The Role of Spontaneous Cap Domain Mutations in Haloalkane Dehalogenase Specificity and Evolution*

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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 (C1-C4) 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-chlorohexane. 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 mutations 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, chloroaacet-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 indicate that the enzyme catalyzes cleavage of halogenated compounds by nucleophilic displacement of the halogen by Asp126 (8). It was shown that during incubations of DhlA with substrate in H218O incorporation of 18O occurred both in Asp126 and product (9). This indicates that the covalent alkyl-enzyme intermediate, formed by nucleophilic displacement of the halogen by Asp126, is hydrolyzed by nucleophilic attack of a water molecule on the carbonyl carbon atom. His289 probably activates this water molecule to be protonated. Two active site residues, His289 and Asp260, 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 hardly any activity with 1-chlorohexane or chloroalkanes of longer chain length (1). The enzyme has some activity with long-chain bromoalkanes (C6-C10), 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 pJF20 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-chlorobutane, 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-chlorobutane. After three serial transfers in medium containing 2 mM 1-chlorobutane as the sole carbon source, mutants were purified on nutrient broth agar plates containing tetracycline (12 µg/ml).

**Sequencing—**The *dhlA* gene from mutant V152–153 was determined by cloning *Ali-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 AGTTGCG (c), 1928 TTCTGTCGCTACGCGG (c), 1590 CTGCCGAGCATGGCCC (c), 1556 ACCGCACTCTTCTGCCC (c), 1628 AAAAGCCGACAGCTGCG (n), 1322 TGGCAAGCAGGAGTTGGC (n).

Purification of Haloalkane Dehalogenase—Mutants of Pseudomonas GJ31(pPJ20) 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, and stored at -20 °C until use.

Determinaton 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 scans of the gels was taken with a desktop scanner. Accuracy was better than 10%.

For the determination of kcat/Km, Vmax, and kcat/Km, 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 milliunits of 1,2-dibromoethane dehalogenase or 100 milliunits 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 4895 gas chromatograph with a CPWax 52 CB column, using an ECD detector for 2-bromoethanol and a FID detector for 2-chloroethanol and 1-hexanol. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 mM alcohol per minute. 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. Kcat and Vmax 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(pPJ20) was found to be capable of growth on 1-chlorobutane, but not on 1-chlorohexane. The specific activity of purified haloalkane dehalogenase with 1-chlorohexane was 0.017 unit/mg protein, which apparently was not sufficient for growth, although the enzyme was expressed at a high level (4). Mutants of strain GJ31(pPJ20) that utilized 1-chlorohexane as sole carbon source were easily obtained in batch incubations 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. A 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 Ile146–Asp148, 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).

Activities of in Vivo-Mutants—All mutants produced a dehalogenase with increased activity toward 1-chlorohexane and other C2 and C3 1-chloro-α-alkanes (Table II). With all mutant enzymes, both the Kcat and Vmax values for 1-chlorohexane had improved, whereas these values were worse for 1,2-dichloroethane. The Kcat 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 Vmax for 1-chlorohexane and 1,2-dibromoethane whereas the Kcat 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 kcat/Km 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 Vmax and Kcat values were improved. They had a broad activity with long-chain (C4-C6) chloroalkanes. Summarizing, the general substrate range of the mutant enzymes thus had shifted from short chain substrates to 1-chloro-α-alkanes of longer chain length. This modification of specificity was not

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**TABLE I**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mutation</th>
<th>No. of mutants found</th>
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<tr>
<td>D170H</td>
<td>G → C substitution (Asp170 → His)</td>
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</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 (Ile146–Asp148)</td>
<td>1</td>
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</table>

**FIG. 1. Selection of mutants of haloalkane dehalogenase.** Plasmid pPJ20 is a broad host range vector, expressing the dhLA gene product. It was introduced in Pseudomonas GJ31 by conjugation (4). Strains were cultivated as described under "Experimental Procedures."
Spontaneous Cap Domain Mutations in DhIA

**TABLE II**

Activities of the mutant enzymes

Halide production in incubations containing purified dehalogenase (wild type (WT), mutants P168S, Δ164–174 and V152–153) or cell extract (the other mutants). Specific activities represent the amount of halide liberated (micromoles) per min per mg of dehalogenase protein added.

<table>
<thead>
<tr>
<th></th>
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<td>Dichloromethane</td>
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<td>0.15</td>
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<td>&lt;0.1</td>
<td>0.06</td>
<td>&lt;0.1</td>
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<td>1,2-Dichloroethane</td>
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<td>1.11</td>
<td>1.03</td>
<td>0.15</td>
<td>0.43</td>
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<td>1-Chlorobutane</td>
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<td>0.71</td>
<td>1.21</td>
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<td>1-Chloropentane</td>
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<td>0.36</td>
<td>0.48</td>
<td>0.48</td>
<td>0.63</td>
<td>1.80</td>
<td>1.00</td>
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<td>1-Chlorohexane</td>
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<td>0.46</td>
<td>0.70</td>
<td>0.64</td>
<td>0.40</td>
<td>1.16</td>
<td>1.00</td>
</tr>
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<td>1,6-Dichlorohexane</td>
<td>0.28</td>
<td>0.38</td>
<td>0.57</td>
<td>0.66</td>
<td>0.44</td>
<td>0.95</td>
<td>1.57</td>
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<tr>
<td>1-Chloroacetate</td>
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<td>0.17</td>
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<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>0.39</td>
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<td>1,2-Dibromoethane</td>
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<td>3.96</td>
<td>4.30</td>
<td>5.82</td>
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<td>1-Bromopropane</td>
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<td>0.51</td>
<td>1.25</td>
<td>1.12</td>
<td>0.87</td>
<td>0.56</td>
<td>1.22</td>
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<tr>
<td>1-Bromobutane</td>
<td>1.53</td>
<td>0.35</td>
<td>1.53</td>
<td>1.92</td>
<td>1.23</td>
<td>0.77</td>
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<tr>
<td>1-Bromopentane</td>
<td>1.94</td>
<td>0.64</td>
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<td>1.50</td>
<td>0.72</td>
<td>1.75</td>
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<tr>
<td>1-Bromohexane</td>
<td>1.08</td>
<td>0.88</td>
<td>2.50</td>
<td>1.47</td>
<td>1.82</td>
<td>0.97</td>
<td>1.28</td>
</tr>
<tr>
<td>1-Bromoheptane</td>
<td>1.21</td>
<td>0.37</td>
<td>1.37</td>
<td>0.84</td>
<td>1.23</td>
<td>0.72</td>
<td>1.22</td>
</tr>
</tbody>
</table>

**FIG. 2. Repeats and mutations in the dhIA gene.** 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 arrow). 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 bases 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 DhIA molecule at Gln167 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 dhIA 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 all affect the structurally important salt bridge Asp70–Lys61 (Fig. 4). Although the Asp70 O61 is 15.6 Å away from the active site Asp158 O61, the loss of the Asp158–Lys261 salt bridge appears to change the active site cavity in such a way that improved 1-chlorohexane conversion is achieved. It is known that two tryptophans are involved in binding of the halide 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 Phe138, Phe134, Phe172, Phe239, Pro223, Val226, 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 Trp172. This part is mainly stabilized by a surface located salt bridge between Asp179 and Lys261 of the main domain (Fig. 4) and hydrophobic interactions of 4 buried residues (Phe164, Phe154, Phe172, and Gly171). Since buried resi-
dudes serve as structural anchors and strongly resist translac-

tion (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 influ-

ence the Asp<sup>199</sup>-Lys<sup>202</sup> salt bridge, as indicated by examina-

tion of the position and role of the residues influenced by the mu-

tations, then this part of the cap domain could have undergone sig-

nificant 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 \( V_{\text{max}} \) for

1-chloroalkane shows that the reaction of bound substrate is

faster in the mutants. Since this effect is observed with chlori-

nated but not with brominated substrates, formation of the co-

valent 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 CO of the

bound substrate for nucleophilic attack by Asp<sup>234</sup> and stabil-

ization of the reaction intermediate. The lower \( K_m \) 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 sub-

strate 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 ob-

served in the mobile surface loops of lipase of Rhizomucor mie-

hei (26), human pancreatic lipase (27), and lipoprotein lipase

(28, 29), which are also \( \alpha/\beta \)-hydrolyase fold excursions (7).

Mechanism of in Vivo Mutagenesis—Of the six mutations

found, only two appeared to be substitutions. The high fre-

quency with which the 11-amino 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 A164–

174 mutant. This can be followed by excision during mismatch

repair or replication (Figs. 2 and 5). Such a process has been

observed in \( \beta \)-galactosidase of Lactobacillus bulgaricus (15) and

bacteriophage T4 (30) and was also proposed for the evolution-

ary divergence of benzoate dioxygenases (31). A role for repli-

cation was suggested (32, 33). Plasmid pPJ20 is derived from

plasmid RK2, which has an unidirectional mode of replication

(34). The orientation of the dhaA gene in pPJ20 is such that the

direction of transcription is the same as the direction of repli-

cation (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 syn-

thesis (Fig. 5A), excision and mismatch repair occurred only in

the template strand, but if strand slippage occurred during lag-

ging 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 ori-

gin. 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 dhaA genes are not flanked by

repeated sequences, however, indicating that specific se-

quences are not required for their generation. The results sug-

gest that an aspecific stuttering type of process can lead to

the formation of short repeats, irrespective of existing repeti-

tions.

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 hydro-

carbon existed until its industrial production and emission

started in 1922. DhaA 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 evi-

dence that the cap domain, which is an excursion on the \( \alpha/\beta \)

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 cod-

ing 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-

Ala and a 9-bp repeat with one mismatch encoding Phe-Ser-Ala

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

<table>
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<th>Strain</th>
<th>( V_{\text{max}} )</th>
<th>( K_m )</th>
<th>( k_{\text{cat}}/K_m )</th>
<th>( V_{\text{max}} )</th>
<th>( K_m )</th>
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<td>Wild type</td>
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<td>0.25</td>
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<td>V145–154</td>
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FIG. 3. SDS-polyacrylamide gel electrophoresis of crude ex-

tracts from Pseudomonas GJ31(pPJ20) and its 1-chloro-
process similar to the selection of the mutations that arose during cultivation on 1-chloroethylene, with a few additional fine tuning modifications leading to the present wild type DhlA.

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 adaptation of an older dehalogenating enzyme to industrially produced 1,2-dichloroethane.

Acknowledgments—We thank Bauke Dijkstra and Koen Verschueren for many stimulating discussions. We also thank Arjen Smal for experimental help.

REFERENCES