Lack of homology between two haloacetate dehalogenase genes encoded on a plasmid from *Moraxella* sp. strain B

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Two genes encoding haloacetate dehalogenases, H-1 and H-2, are closely linked on a plasmid from *Moraxella* sp. strain B. H-1 predominantly acts on fluoroacetate, but H-2 does not. To elucidate the molecular relationship between the two enzymes, we compared their structural genes. Two restriction fragments of the plasmid DNA were subcloned on M13 phages and their nucleotide sequences were determined. The sequence of each fragment contained an open reading frame that was identified as the structural gene for each of the two dehalogenases on the basis of the following criteria; N-terminal amino acid sequence, amino acid composition, and molecular mass. The genes for H-1 and H-2, designated *dehH1* and *dehH2*, respectively, had different sizes (885 bp and 675 bp) and G + C contents (58.3% and 53.4%). Sequence analysis revealed no homology between the two genes. We concluded that the dehalogenases H-1 and H-2 have no enzyme-evolutionary relationship. The deduced amino acid sequence of the *dehH1* gene showed significant similarity to those of three hydrolases of *Pseudomonas putida* and a haloalkane dehalogenase of *Xanthobacter autotrophicus*. The *dehH2* coding region was sandwiched between two repeated sequences about 1.8 kb long, which might play a part in the frequent spontaneous deletion of *dehH2* from the plasmid.

Introduction

A number of soil micro-organisms have been isolated that can utilize short-chain haloalkanoic acids such as chloroacetate and 2-chloropropionate as their sole carbon source (Jensen, 1960; Kearney & Kaufman, 1972). These micro-organisms have enzymes, known as 2-haloalkanoic acid dehalogenases, which catalyse the hydrolytic cleavage of halogen-carbon bonds by the following reaction (Goldman, 1965; Little & Williams, 1971):

The dehalogenases are roughly classified into two groups on the basis of their substrate specificities. One group, termed haloacetate dehalogenase (EC 3.8.1.3),

acts specifically on halogenated acetates to yield glycolate. The other group, termed 2-haloacid dehalogenase (EC 3.8.1.2), acts broadly on short-chain 2-haloalkanoic acids to produce 2-hydroxy acids. However, individual enzymes in each group exhibit different substrate-specificities for carbon chain length, the halogen species, and the configuration of the substitute (Kawasaki, 1988). The diversity of the dehalogenases may result from selection for micro-organisms able to degrade a variety of novel halogenated compounds.

Enzyme evolution may be initiated by tandem duplication of a gene, followed by the accumulation of multiple mutations on either gene copy, which results in the creation of a modified enzyme (Rigby *et al.*, 1974). If gene duplication is important in evolution, we would expect to find two similar enzymes in a cell at some stage of the evolutionary process. The nylon-oligomer-degrading enzymes found encoded on a plasmid by Okada *et al.* (1983) are an example of this. Although several bacteria have been isolated that contain two or more similar dehalogenases (Goldman *et al.*, 1968; Weightman *et al.*, 1979; Klages *et al.*, 1983; Allison *et al.*, 1983), the relationships between these enzymes have not been elucidated.

Moraxella sp. strain B, isolated from industrial wastewater, has two haloacetate dehalogenases, H-1 and H-2.

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The nucleotide sequence data reported in this paper has been submitted to GenBank and has been assigned the accession numbers D90422 for *dehH1* and D90423 for *dehH2*.

These enzymes differ in halogen specificity; H-1 acts better on monofluoroacetate than on monochloro- or monobromoacetate, but has little activity against monoiodoacetate. H-2 acts on monochloro-, monobromo-, and monoiodoacetate, but not on monofluoroacetate. The two enzymes have been purified and characterized (Kawasaki *et al.*, 1981a). The molecular masses estimated by SDS-PAGE were 33 kDa for H-1 and 26 kDa for H-2. Both enzymes were very sensitive to inhibition with thiol-blocking reagents such as *p*-chloromercuribenzoate. The other properties also indicated that H-1 and H-2 differed in molecular features, but not in catalytic features except for halogen specificity.

The genes for H-1 and H-2, designated *dehH1* and *dehH2*, respectively, are borne on a conjugative plasmid, pUO1 (65 kb) (Kawasaki *et al.*, 1981b), and have previously been cloned onto pBR322 in *Escherichia coli* (Kawasaki *et al.*, 1984). The *dehH1*- and *dehH2*-coding restriction fragments that were cloned were located very close to each other on the restriction map of pUO1. Additionally, the two fragments formed a hybrid in a DNA-DNA hybridization test, implying that the fragments contained homologous sequences (Kawasaki, 1985). These findings suggested an enzyme-evolutionary relationship between H-1 and H-2.

Results presented in this paper show that *dehH1* and *dehH2*, and the encoded enzymes H-1 and H-2, show no sequence conservation, indicating that the two genes have not evolved from a common ancestral gene.

Methods

Bacteria, plasmids, phages, and growth conditions. Recombinant plasmids pBREF1 and pBRSG2 were used as sources for cloned *dehH1* and *dehH2*, respectively. These plasmids were constructed previously by inserting a 3.9 kb *EcoRI* restriction fragment of pUO1 (termed *EcoRI*-F fragment) and a 2.1 kb *SalI* restriction fragment of pUO1 (termed *SalI*-G fragment) into pBR322 to generate pBREF1 and pBRSG2, respectively (Kawasaki *et al.*, 1984). These plasmids were maintained and propagated in *E. coli* C600 (*leu*, *thr*, *thi*, *hsdR*, *hsdM*, *supE*), which was grown in LB medium consisting of 10 g tryptone, 5 g yeast extract, and 5 g NaCl per litre, pH 7.3.

Phages M13mp18 and mp19 and *E. coli* JM107 (Δ *lac-pro*, *endA*, *gyrA*, *thi*, *hsdR*, *supE*, *relA/F'* *traD*, *proAB*, *lacZ* Δ M15) were used as the vectors and host, respectively, for subcloning and sequencing. JM107 was maintained in M9 minimal medium (Maniatis *et al.*, 1982) and grown for phage propagation in YT medium consisting of 8 g tryptone, 5 g yeast extract, and 5 g NaCl per litre, pH 7.3.

DNA manipulations. Plasmid DNA and M13 replicative form (RF) DNA were isolated by the alkaline SDS method (Birnbom & Doly, 1979). Isolation of M13 single-stranded DNA and subcloning into M13 vectors were performed as described by the M13 Cloning and Sequencing Handbook provided by Amersham. General procedures for DNA manipulations were according to Maniatis *et al.* (1982).

Construction of deletion subclones. The *dehH1* coding region is located within the overlap (1.75 kb) between the *EcoRI*-F fragment and the *SalI*-D fragment of pUO1, which is shown in the restriction map of

Fig. 1. Restriction map of the region encoding *dehH1* and *dehH2* in the plasmid pUO1. Fragments yielded by *EcoRI* and *SalI* are shown by boxes with alphabetical letters. The *SalI*-*KpnI* fragment and the *SalI*-G fragment that were subcloned and sequenced are shown by boxes under the map. Arrows indicate locations and the direction of transcription of *dehH1* and *dehH2*. Broken lines indicate locations of homologous sequences.

pUO1 in Fig. 1 (Kawasaki *et al.*, 1984). However, to facilitate subsequent deletion with exonuclease III, a 1.5 kb segment from a *SalI* site to a *KpnI* site that contains a large portion of the overlap (Fig. 1) was subcloned for sequencing. The *SalI*-*KpnI* fragment was cut out from the pBREF1 DNA and inserted into two M13 vectors, mp18 and mp19. For the *dehH2* gene, the 2.1 kb *SalI*-G fragment from pBRSG2 was subcloned in different orientations in M13mp19 vector.

A series of deletion subclones was constructed from each of the four subclones by use of the Kilo Sequence Deletion Kit (Takara Shuzo) according to the supplier's manual, essentially as described by Henikoff (1984). RF DNA was cleaved with two different restriction enzymes and digested with exonuclease III and mung bean nuclease, followed by ligation and transfection of JM107. Before transfection, the ligated DNA was treated with one of the restriction enzymes to linearize undigested DNA.

Nucleotide sequencing. DNA was sequenced by the dideoxy chain termination method of Sanger *et al.* (1977) by use of the 7-DEAZA Sequencing Kit (Takara Shuzo) and [α - 35 S]dCTP (500 Ci mmol $^{-1}$, 18.5 TBq mmol $^{-1}$, New England Nuclear Corp.) as recommended by the supplier. Gel electrophoresis and autoradiography were done as described by the Amersham sequencing handbook. Sequence data were analysed and organized into a contiguous sequence with the aid of a sequence alignment program of the Genetic Data Processing Software, SDC-GENETYX (SDC).

Purification of dehalogenases from *E. coli*. Dehalogenases produced by *E. coli* clones harbouring pBREF1 and pBRSG2 were purified to homogeneity by the procedures described for the purification of H-1 and H-2 from *Moraxella* sp. B (Kawasaki *et al.*, 1981a). Cells grown in LB broth containing 30 μ g ampicillin ml $^{-1}$ were ruptured with an ultrasonic disintegrator. The enzymes were purified by ammonium sulphate fractionation, followed by three column chromatography steps using DEAE-Toyopearl 650M, Toyopearl HW-50 gel (Tosoh) and hydroxyapatite. The assays for the dehalogenases were as described previously (Kawasaki *et al.*, 1981a).

Determination of the N-terminal amino acid sequence. The purified enzymes were desalted by dialysis, lyophilized, and dissolved in 1% (w/v) SDS solution at about 100 pmol protein per 100 μ l. Amino acid sequence analysis was carried out with an automatic peptide sequencer (Applied Biosystems Model 470A). Phenylthiohydantoin amino acid derivatives were identified by HPLC essentially as described by Hunkapiller & Hood (1983).

Results and Discussion

Characteristics of dehalogenases produced from pBREF1 and pBRSG2 in *E. coli*

Plasmids pBREF1 and pBRSG2 are known to express haloacetate dehalogenases in *E. coli* (Kawasaki *et al.*, 1984). The dehalogenases purified from *E. coli* were shown to have the same mobility on SDS-PAGE and the same substrate specificity and catalytic properties as the corresponding enzymes from *Moraxella* (data not shown). These results confirmed that the dehalogenases produced from pBREF1 and pBRSG2 in *E. coli* were identical to H-1 and H-2, respectively.

The N-terminal amino acid sequences determined with the enzymes from *E. coli* were Met-X-Phe-Pro-Gly-Phe-Lys-Asn-Ser-Thr for H-1 and Met-Lys-Lys-Ile-Glu-Ala-Ile-Ala-Phe-Asp for H-2. The second amino acid (X) of H-1 was unclear.

Nucleotide sequence of the *dehH1* gene

A total of 26 deletion subclones derived from the recombinants of M13mp18 and 19 containing the 1.5 kb

SalI-*KpnI* restriction fragment were subjected to DNA sequencing, and gave the fully overlapped sequence of both strands. A contiguous 1536 bp sequence determined for the *SalI*-*KpnI* fragment is shown in Fig. 2. Computer analysis of the sequence revealed an open reading frame (ORF) of 885 bp, beginning at the 413th nucleotide from the 5' *SalI* end of the fragment. This ORF could be assigned to the H-1 structural gene (*dehH1*) based on the following arguments: The first ten amino acid residues deduced from the nucleotide sequence of the ORF were identical to the N-terminal amino acid sequence determined for the H-1 enzyme. The amino acid composition and the molecular mass (33 307 Da) for the deduced protein closely resembled those of the H-1 protein (Table 1).

A sequence, AGGAGA, located 6 bp upstream from the ATG initiation codon of the *dehH1* gene constituted a potential ribosome-binding sequence. Plasmid pBREF1 containing the *EcoRI*-F fragment expressed the *dehH1* gene regardless of the orientation of the insert, while the recombinant containing the *SalI*-*KpnI* fragment did not express the *dehH1* without the aid of a promoter belonging to the vector. Such a case was a

GTCTGACGATTCACTCACCGTGCCTGCCGTGTCGGCCGCCTTACCGGCTTTTCCGACATGCCGCCGTGTTGCCACGGCTTACATGGTCGGCTTCGTCGAATGGCGTGCATTGAGGCA	120
CTCCGGCCATACCTTTGCCCCCTCGCAACGCACGGTGGGCACGCACGTCAATCTGAGCCACAGCCGCCGACCCCGTGGGCATGCAGGTTACGGCAGAGGTTGAGCTGATCGAGGTCGAA	240
GGCAAGCGCTTGACATTCAAGGCTTGTGCCGCGACGAGGTGGACGTGATCTCGAGGGCCGGCAGCAGCGCTTATCGTTGAAGCGCAGCAGTTTCATCAGACGAGTCACGTCCAAGGGT	360
GAACGTGGCTGAGCTTTAGGGCTAAAGAAGTTGATCAACCCAGGAGCAAAA ATG GAC TTT CCA GGA TTC AAG AAC AGC ACC GTT ACC GTG GAT GGT GTG GAC	463
Met Asp Phe Pro Gly Phe Lys Asn Ser Thr Val Thr Val Asp Gly Val Asp	
ATC GCC TAC ACC GTA AGC GGC GAA GGC CCT CCG GTG CTG ATG CTG CAT GGG TTC CCG CAG AAC CGG GCC ATG TGG GCG GCG GTG GCT CCC	553
Ile Ala Tyr Thr Val Ser Gly Glu Gly Pro Pro Val Leu Met Leu His Gly Phe Pro Gln Asn Arg Ala Met Trp Ala Arg Val Ala Pro	
CAA CTC GCC GAG CAC CAT ACC GTG GTG TGT GCC GAC CTG CGA GGC TAT GGC GAT TCG GAC AAG CCC AAG TGC CTG CCG GAC CGG TCA AAC	643
Gln Leu Ala Glu His His Thr Val Val Cys Ala Asp Leu Arg Gly Tyr Gly Asp Ser Asp Lys Pro Lys Cys Leu Pro Asp Arg Ser Asn	
TAC TCA TTC CGC ACG TTT GCC CAT GAC CAA CTC TGT GTG ATG CGC CAC CTG GGG TTC GAG CGC TTC CAC CTC GTC GGA CAT GAT CGC GGC	733
Tyr Ser Phe Arg Thr Phe Ala His Asp Gln Leu Cys Val Met Arg His Leu Gly Phe Glu Arg Phe His Leu Val Gly His Asp Arg Gly	
GGG CGT ACC GGT CAC CGC ATG GCG CTG GAT CAT CCC GAA GCG GTG CTG TCG CTG ACC GTC ATG GAC ATC GTG CCG ACG TAT GCG ATG TTC	823
Gly Arg Thr Gly His Arg Met Ala Leu Asp His Pro Glu Ala Val Leu Ser Leu Thr Val Met Asp Ile Val Pro Thr Tyr Ala Met Phe	
ATG AAC ACC AAC CGT CTG GTT GCC GCT TCC TAC TGG CAT TGG TAT TTC CTG CAG CAG CCT GAG CGG TTC CCC GAG CAC ATG ATC GGT CAG	913
Met Asn Thr Asn Arg Leu Val Ala Ala Ser Tyr Trp His Trp Tyr Phe Leu Gln Gln Pro Glu Pro Phe Pro Glu His Met Ile Gly Gln	
GAC CCG GAC TTC TTC TAT GAG ACC TGT TTG TTC GGG TGG GGC GCA ACC AAG GTG TCG GAC TTT GAC CAA CAA ATG CTG AAC GCA TAT CGG	1003
Asp Pro Asp Phe Phe Tyr Glu Thr Cys Leu Phe Gly Trp Gly Ala Thr Lys Val Ser Asp Phe Asp Gln Gln Met Leu Asn Ala Tyr Arg	
GAG TCT TGG CGC AAC CCA GCC ATG ATT CAC GGC TCA TGC TCG GAC TAC CGC GCC GCC GCA ACA ATT GAC CTT GAA CAC GAT AGC GCG GAC	1093
Glu Ser Trp Arg Asn Pro Ala Met Ile His Gly Ser Cys Ser Asp Tyr Arg Ala Ala Ala Thr Ile Asp Leu Glu His Asp Ser Ala Asp	
ATC CAA CGG AAG GTG GAA TGC CCC ACC TTG GTT TTC TAC GGC TCA AAG GGC CAG ATG GGC CAG CTA TTC GAC ATA CCA GCC GAG TGG GCA	1183
Ile Gln Arg Lys Val Glu Cys Pro Thr Leu Val Phe Tyr Gly Ser Lys Gly Gln Met Gly Gln Leu Phe Asp Ile Pro Ala Glu Trp Ala	
AAG CGC TGC AAC AAC ACT ACA AAC GCA TCT CTG CCA GGA GGC CAT TTC TTC GTG GAT CAG TTC CCC GCG GAA ACA TCA GAG ATT CTT TTG	1273
Lys Arg Cys Asn Asn Thr Thr Asn Ala Ser Leu Pro Glu His Phe Phe Val Asp Gln Phe Pro Ala Glu Thr Ser Glu Ile Leu Leu	
AAG TTT CTT GCT CGA AAC GGC TGA TACAGCAGCGCTGCTTCGTTCTTGTGCCAGCTAAAAATAACGCTAGAGAGAGGGCGAAGCAGTGTGAAACCTGTTCTTCCGATTG	1385
Lys Phe Leu Ala Arg Asn Gly * TACAGCAGCGCTGCTTCGTTCTTGTGCCAGCTAAAAATAACGCTAGAGAGAGGGCGAAGCAGTGTGAAACCTGTTCTTCCGATTG	
CCTNTGGTTCGGTACTTGGCGGCTGCTGCGATGGGCTTGGGCTCAAATTCAACAATCTGCTTCCCATATTCCACCGGGAACATTAACTGTGCGATCCCGTACGATCATATGGTCGC	1505
TTGTGAAACAGGTGCGGGTGGGTCTGGTACC	1536

Fig. 2. Nucleotide sequence of the *SalI*-*KpnI* fragment containing *dehH1* and its deduced amino acid sequence. The potential ribosomal binding site is underlined. The termination codon is indicated by an asterisk. Numbers under the amino acid sequence refer to the distances from the N-terminal. Arrows represent a palindromic sequence located distal to the *dehH1* gene.

Table 1. Comparison of the amino acid compositions and molecular masses deduced from the two ORFs with those of the H-1 and H-2 enzymes

Amino acid	Number of residues per mol of enzyme			
	H-1		H-2	
	DNA analysis*	Protein analysis†	DNA analysis*	Protein analysis†
Gly	22	24	16	17
Ala	24	24	20	21
Val	18	23	16	23
Leu	22	22	20	21
Ile	8	8	12	12
Ser	15	15	13	12
Thr	16	15	9	9
Asp	21	31	12	19
Asn	11		6	
Glu	13	26	18	25
Gln	12		8	
Lys	8	10	11	12
Arg	17	17	12	11
Cys	7	5	5	5
Met	12	6	7	8
Tyr	10	12	11	10
Phe	20	19	10	10
His	14	13	6	5
Trp	6	8	4	5
Pro	18	19	8	8
Molecular mass (Da)	33307	33000	25345	26000

* The total number of residues deduced from the ORF was 294 for H-1 and 224 for H-2.

† The number of residues was recalculated from previous data (Kawasaki, 1985) based on molecular masses of 33 kDa for H-1 and 26 kDa for H-2.

M13mp18 recombinant with the *SalI*-*KpnI* fragment, in which the direction of the *dehH1* gene was opposite to that of transcription by the vector's *lac* promoter. This suggested that a promoter capable of operating *dehH1* in *E. coli* was included in the *EcoRI*-F fragment but not in the *SalI*-*KpnI* fragment. No sequence similar to the -10 and -35 consensus sequences of *E. coli* promoters was found upstream from the *dehH1* gene.

Nucleotide sequence of the *dehH2* gene

The *dehH2*-containing 2.1 kb *SalI*-G fragment was sequenced in both strands by use of 30 deletion subclones, and a contiguous 2105 bp sequence was determined for the fragment (Fig. 3), in which an ORF of 675 bp was found and identified as the H-2 structural gene (*dehH2*). The N-terminal amino acid sequence deduced from the DNA sequence of the ORF corresponded to that of the H-2 enzyme, and the predicted

amino acid composition and molecular mass of the *dehH2* gene product corresponded closely to those of the H-2 enzyme (Table 1).

The *dehH2* gene was preceded by the sequence GGAGA, the putative Shine-Dalgarno sequence, present 6 bp upstream from the ATG initiation codon. A promoter that initiates transcription of *dehH2* in *E. coli* must be present in the *SalI*-G fragment, because pBRSG2 and its derivative containing the *SalI*-G fragment, inserted in different orientations, expressed similar levels of H-2. The location of the promoter was analysed by progressively deleting the 5'-terminal region of the *SalI*-G fragment (Fig. 3). Deletion of 720 bp from the 5' terminus to a *SmaI* site did not obstruct the expression of *dehH2*, but further deletion of 275 bp up to a *BamHI* site resulted in failure to express the gene. When the remaining portion of the fragment was inserted downstream of one promoter carried by a vector, it could express the *dehH2* gene. Therefore, the *dehH2* promoter working in *E. coli* should be located in the 275 bp *SmaI*-to-*BamHI* region. A sequence TTGACA-(17 bases)-TAACCT present about 240 bp upstream from the *dehH2* coding region was presumed to be the -35 and -10 sequences of the *dehH2* promoter.

Plasmid pUO1 expressed *dehH1* and *dehH2* in several *Pseudomonas* hosts as well as in *E. coli* and *Moraxella*. However, no sequence similar to the known *Pseudomonas* promoters (Inouye *et al.*, 1986) was found in either of the sequenced fragments. The *dehH1* and *dehH2* genes were coded on the plasmid pUO1 in the same transcription direction, that is, from right to left in the map given in Fig. 1.

Comparison of *dehH1* and *dehH2*

The G+C content of *dehH1* was 58.3% and that of *dehH2* was 53.4%. Both values were higher than that of the genomic DNA of genus *Moraxella* (40-47%) (Bovre, 1984). As regards G+C content alone, it seemed that the DNA of *dehH1* was rather similar to *Pseudomonas* DNA and the DNA of *dehH2* was analogous to *E. coli* DNA. The two genes differed somewhat in their codon usage. Base preference for G and C in the wobble position was apparent in the *dehH1* gene (72%), a phenomenon observed in a variety of organisms with high G+C levels.

Comparison of the nucleotide sequences of *dehH1* and *dehH2* with the aid of a computer program (SDC-GENETYX) revealed no homology; the two genes showed no sequences 30 bases long with more than 50% identity. The amino acid sequences deduced from the genes also showed no significant similarity. Harr plot analysis (Staden, 1982) reconfirmed no homology between the two genes on the nucleotide sequence level and the amino

<u>CTCGACGTCGCTTTGATCGAAAGCATTCGGATAGTCTGGTGACCTGGCAGCCTCGGTCATGAGCGGACATGCCAGTCGGGTGGCAGCTCTTGCGCGGTTGGCTTCGCGCCAGGGC</u>	120
<u>GATCCAAATCTATGAGGCTGGCGTCGCAATGGGGCGGTTGGCTGCGTACGGCGTTTGTGGCTGACTACTTTGTCAAGGACGCTTTCAGGAACGAGTTGCGCCGGGTGCTCAATCGGGCGGAG</u>	240
<u>GTTGTTAAAGCCCTCAAGCGCGCATTTATACCGCGGATCAGCCCGGCGCAGGCCAAACGCTGATGAAATGCAGGCTGTGGCCGATGCGTTGAGCCTGATGGCCAACATCGTGATG</u>	360
<u>CGGTGGAATACCTCACAGATGCAGGCGGTCCTGGATCGCTGGTGAACCGCGCCAGGTCATTCACCGGAATGATCGGGAAGATTGCGCCACACAGGCTGGAGAGCATCAACTTGGCG</u>	480
<u>GCTGTGTTTCGCTTCCCGGTTGACCGCTATGCTGACCAATCTTGCCTTCGCGGGCAAATGCATCGATAAATGGCACCAATGGATGAAACCGACCAACGGTTTGACGCCACGAATCGCAGA</u>	600
<u>TTTGAAGTGAACAGGAAAGTCAATGAAATCAACGATCTACCAACACCACCTCCGCGCCAGTGTAGCTTTTCGTACCGTCACTTATTGCACTGAAACGAGGAGACCCGAGTCGGGCC</u>	720
<u>GGGCGCCCTGATGGAATTTAAAAATCGAGCGCAGCAATCCGCGACGAACATCGCCTTGCTTCGTCCTGGTGTGTCATCTTCAGCAAATCGCGAATCGTTGTCTGGGCTGATGAACAT</u>	840
<u>AGTCTCGTCTAACAATTTGAGTAAACAGTATAAAAGCTATTGACAAGTTACGCTCCGCTGCTTAACTCCCGGTGCTGCTGATAGGTTACTTCATTGGGTACGACAGCATTCAGGATCT</u>	960
<u>AGGCGAGCGGCCCGCGTGGGGGGCGGTTTTCGCGGATCCGAGAGGCTAGATGTGACGGGTACAACGAAGATCGATTGCTGGCAGCGCAGCGAGCGGTGCGGAAACGCTACAAGCAGC</u>	1080
<u>GAGCCATCGACCGACGCATATCTTAGAAAAATGAGACATGA</u>	1178
ATG AAG AAG ATC GAA GCC ATT GCA TTC GAC ATG TAC GGC ACC CTC TAC GAT GTG CAT Met Lys Lys Ile Glu Ala Ile Ala Phe Asp Met Tyr Gly Thr Leu Tyr Asp Val His	
TCG GTA CTG GAC GCA TGT GAG AAG CAG TAT CCA GGG AAG GGA AAA GAC ATC AGC GTC CTG TGG CGC CAA AAG CAA CTC GAA TAC GCT TGG Ser Val Val Asp Ala Cys Glu Lys Gln Tyr Pro Gly Lys Gly Lys Asp Ile Ser Val Leu Trp Arg Gln Lys Gln Leu Glu Tyr Ala Trp	1268
TTG CGG TGC CTC ATG GGG CAG TAC ATC AAG TTC GAG GAG GCG ACA AAT GCG TTG ACC TAC ACG TGC AAC CAG ATG AAG TTG GAT TGC Leu Arg Cys Leu Met Gly Gln Tyr Ile Lys Phe Glu Glu Ala Thr Ala Asn Ala Leu Thr Tyr Thr Cys Asn Gln Met Lys Leu Asp Cys	1358
GAC GAG GGT TCG GCC ATG CGG CTC ACC GAG GAA TAT TTA CGC CTA AAA CCT TTT CCG GAG GTT CGA GGC GCA CTT CGA GCG CTG CGG CAG Asp Glu Gly Ser Ala Met Arg Leu Thr Glu Glu Tyr Leu Arg Leu Lys Pro Phe Pro Glu Val Arg Gly Ala Leu Arg Ala Leu Arg Gln	1448
CGA GGA ATG CGG CTT GCG ATC CTG TCC AAC GGA TCG ACA GAA ACG ATT CAT GAC GTT GTT CAT AAC TCC GGC GTG GAG GGC GAG TTC GAG Arg Gly Met Arg Leu Ala Ile Leu Ser Asn Gly Ser Thr Glu Thr Ile His Asp Val Val His Asn Ser Gly Val Glu Gly Glu Phe Glu	1538
CAT TTG ATC AGC GTG GAT TCC GCC CGG GCT TAC AAG CCC CAC CCT CTT GCC TAC GAA CTC GGA GAG GAA GCG TTC GGA ATA TCG CGC GAA His Leu Ile Ser Val Asp Ser Ala Arg Ala Tyr Lys Pro His Pro Leu Ala Tyr Glu Leu Gly Glu Glu Ala Phe Gly Ile Ser Arg Glu	1628
TCC ATT CTC TTT GTA TCG TCG AAT CCA TGG GAT GTA TCG GGA GCA AAA GCG TTC GGC TAT CAA GTC TGT TGG ATC AAT CGC TAT GGC TTT Ser Ile Leu Phe Val Ser Ser Asn Pro Trp Asp Val Ser Gly Ala Lys Ala Phe Gly Tyr Gln Val Cys Trp Ile Asn Arg Tyr Gly Phe	1718
GCG TTT GAC GAA CTG GGG CAG ACT CCT GAC TTC ACG GTT CCC GTG ATG GAT GCG ATT GTG CAT TTG ATC GCT GTA TGA GCCGATTACGGCAGGA Ala Phe Asp Glu Leu Gly Gln Thr Pro Asp Phe Thr Val Pro Val Met Asp Ala Ile Val His Leu Ile Ala Val * 224	1812
GGACAGAAGGAGAGGGGGCTGGTCCCAAAACCGAGGCGCCTGCTCTCAAGGATCGTCAGACGCGCTTGTGTAAAGCCTGCTGCGGAAACAAAGAGGATAAATGCTTCCAGGCTCGTCT	1932
GACCAGCAGCGTAGCGTTTCGCGCGCCAGCCAGGNTGCTTCGACCTCGTTCGCTGGCCTCGTAAGGCTGCGTCTAGTTGCGTTCCAGTAGCCCGCGGAAACGGCCAGCCCGGTCGCGCC	2052
AGCAGGCGCAGCGCTCGTTCGTAGAGCGAAACGCTGCGGTAGGCCGCGCTCGAC	2105

Fig. 3. Nucleotide sequence of the *SalI*-G fragment containing *dehH2* and its deduced amino acid sequence. The termination codon is marked by an asterisk. The putative Shine-Dalgarno sequence and the sequences similar to the -35 and -10 consensus of *E. coli* promoters are underlined. The region showing homology to that in the *EcoRI*-F fragment is overlined. Arrows represent palindromic sequences located distal to the *dehH2* gene.

DehH1	23	SGEGPPVLMHLHG	94	LGFERFHLVGHDRGG	(294 a.a.)
DmpD	26	SGAGFPLMMIHG	96	LEIEQADLVGNSFGG	(283 a.a.)
BphD	30	AGQGERVIMLHG	101	LGIEKAHLVGNMGG	(277 a.a.)
Tpes	33	NPSGDPVLLLHG	99	MGLHNTTVIGHSMGS	(272 a.a.)
Dh1A	44	SDAEDVFLCLHG	113	LDLRNITLVWQDWGG	(310 a.a.)

Fig. 4. Comparison of the predicted amino acid sequence of the N-terminal region of H-1 with those of three *Pseudomonas* hydrolases and a *Xanthobacter* dehalogenase. The sequence comparison was carried out using the program GENETYX. Well-conserved amino acids are emboldened. The number preceding each sequence refers to the amino acid number within each protein. Whole amino acid sequence length is shown in parentheses. DehH1, haloacetate dehalogenase H-1 (this work); DmpD, 2-hydroxymuconic semialdehyde hydrolase of *P. putida* (Nordlund & Shingler, 1990); BphD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienate hydrolase of *Pseudomonas* sp. strain KKS102 (Kimbara *et al.*, 1989); Tpes, tropinesterase of *P. putida* (Hessing, 1983); Dh1A, haloalkane dehalogenase of *X. autotrophicus* GJ10 (Janssen *et al.*, 1989).

acid sequence level. The results suggest that the dehalogenases H-1 and H-2 have not descended from a common ancestral enzyme.

A computer-assisted search for nucleotide sequences

homologous to *dehH1* and *dehH2* in the nucleotide sequence data bases available to the GENETYX program gave no significant results. However, a search for amino acid sequences homologous to the deduced amino acid

sequences of *dehH1* and *dehH2* in the protein data base SWISS-PROT yielded haloalkane dehalogenase (the gene product of *dhlA*, Janssen *et al.*, 1989) of *Xanthobacter autotrophicus* and three hydrolases of *Pseudomonas putida*, i.e. 2-hydroxymuconic semialdehyde hydrolase (the gene product of *dmpD*, Nordlund & Shingler, 1990), 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (the gene product of *bphD*, Kimbara *et al.*, 1989) and tropinesterase (Hessing, 1983). The alignment of the amino acid sequences of these five proteins showed significant similarity in the N-terminal region of each protein. Two highly conserved regions in all proteins are shown in Fig. 4. These similar N-terminal regions (about 120–130 amino acids) might be common domains, which were derived from an ancestral *Pseudomonas* hydrolase.

The homology with other haloacetate dehalogenases of different bacterial strains isolated was previously examined by dot hybridization to the whole DNA extracted from the bacteria using the *dehH1* DNA and *dehH2* DNA as probes (Kawasaki *et al.*, 1985). All 20 strains tested had DNA that hybridized with either of *dehH1* or *dehH2* DNA; the 9 strains whose DNA hybridized with the *dehH1* DNA had H-1-like dehalogenases acting preferentially on fluoroacetate, and the 11 strains containing DNA that hybridized with the *dehH2* DNA had H-2-like dehalogenases that did not act on fluoroacetate. This suggests that bacterial haloacetate dehalogenases may be classified into two enzyme families, an H-1 family and an H-2 family.

Repeated sequences preceding *dehH1* and *dehH2*

We wondered why the *dehH1*-containing *EcoRI*-F fragment had hybridized with the *dehH2*-containing *SaII*-G fragment (Kawasaki, 1985). This question was answered by sequencing and analysing the complete 3.9 kb *EcoRI*-F fragment (data not shown). Two fragments of *EcoRI*-F and *SaII*-G contained identical sequences of about 700 bp upstream of the *dehH1* and *dehH2* coding regions (Fig. 3). Further sequencing of pUO1 DNA adjacent to these fragments revealed that the homologous sequence extended another 1100 bp up to a *HindIII* site located in each flanking fragment as shown in Fig. 1.

The function of the homologous sequences and their relation to the dehalogenase genes are not clear. The two sequences were located on pUO1 repeatedly in the same orientation with the space of about 4 kb and held the *dehH2* coding region between. This region is deleted spontaneously from pUO1 to form the deletion mutant pUO11 as reported previously (Kawasaki *et al.*, 1983). The repeated sequences might possibly play a part in the deletion.

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