Cloning and Sequence Analysis of a Plasmid-encoded 2-Haloacid Dehalogenase Gene from *Pseudomonas putida* No. 109

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The 2-haloacid dehalogenase of *Pseudomonas putida* No. 109 was mediated by a 74-kb conjugative plasmid, which was transferred by mating into *Pseudomonas* and *Escherichia coli* and there expressed the dehalogenase. A 2.8-kb EcoRI-fragment generated from the plasmid was cloned and sequenced. The dehalogenase gene (*dehH109*) was identified by comparison with the N-terminal amino acid sequence and the molecular weight of the enzyme protein. The gene *dehH109* coded for a 224-amino acid protein of M$_r$ 25,231, which showed significant homology to the other four L-specific 2-haloacid dehalogenases from *Pseudomonas* sp. CBS33, *P. putida* AJ1, and *Xanthobacter autotrophicus* GJ10 and also to the haloacetate dehalogenase H-2 from *Moraxella* sp. strain B, but no homology with another haloacetate dehalogenase H-1 and the D-specific 2-haloacid dehalogenase from *P. putida* AJ1.

Haloalkanoic acid dehalogenase catalyzes hydrolytic cleavage of halogen-carbon bonds of C$_2$-halogenated alkanoic acids to yield 2-hydroxy or 2-oxo acids. Many dehalogenases have been found in a variety of microorganisms that can use haloalkanoates as sole carbon and energy sources. Some genes for the dehalogenases have been cloned and sequenced.1–5)

The haloalkanoate dehalogenases had been classified into two groups. One is termed haloacetate dehalogenase [EC 3.8.1.3], which acts specifically on haloacetic acids, and the other is termed 2-haloacid dehalogenase [EC 3.8.1.2], and it acts on short chain 2-haloalkanoates as well as haloacetates. However, it seems that the latter group of enzymes may be divided into four different types according to the stereo-specificity for substrates such as D- and L-2-monochloropropionate and whether the configuration is inverted or not. Type 1 enzymes act only on the L-isomer, inverting the configuration to produce D-lactate,6–10) and type 2 enzymes are specific for the D-isomer, giving a product with inverted configuration.11) The enzymes of type 3 and type 4 act on both stereo-isomers, the former with inversion of configuration10,12,13) and the latter with retention of configuration.13)

*Pseudomonas putida* No. 109 that has been isolated from soil as a 2-monochloropropionate utilizer produces a L-specific 2-haloacid dehalogenase, named H-109, which has already been purified and characterized by Motosugi et al.14) This enzyme acts on 2-halogenated acetates, propionates, and butyrates, but not on fluorinated compounds. Its molecular weight has been estimated to be 25,000. To elucidate the molecular relationships with other dehalogenases, the gene for H-109 has been cloned and analyzed. In this paper we describe the finding of a plasmid specifying H-109 and the cloning and sequencing of the H-109 gene, and also we discuss the relationship with other haloalkanoic acid dehalogenase genes through the comparison of the nucleotide and amino acid sequences.

**Materials and Methods**

*Bacterial strains, plasmids, phages, and culture conditions*. The bacterial strains, plasmids, and phages used in this study are listed in Table. All strains of *P. putida* and *Escherichia coli* were usually grown in LB medium consisting of 1% peptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.3. The seed culture of *P. putida* No. 109 and the selection for *Pseudomonas* transformants were done using chloroacetate- or 2-chloropropionate-minimum medium that consisted of 0.45% Na$_2$HPO$_4$, 0.1% KH$_2$PO$_4$, 0.1% (NH$_4$)$_2$SO$_4$, 0.02% MgSO$_4$, 0.001% CaCl$_2$, traces of Na$_2$MoO$_4$, ZnSO$_4$, and MnSO$_4$, 0.005% methionine if necessary, and 0.2% chloroacetate or 2-chloropropionate. To select *E. coli* transconjugants and transformants, was used M9 medium15) supplemented with 0.2% chloroacetate or 2-chloropropionate instead of glucose as the sole carbon source.

<table>
<thead>
<tr>
<th>Table</th>
<th>Bacterial Strains, Plasmids, and Phages</th>
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<tr>
<td>Strain, plasmid, or phage</td>
<td>Relevant genotype or phenotype</td>
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<tr>
<td><em>P. putida</em> No. 109</td>
<td>A wild-type strain, pUOH109</td>
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<tr>
<td>AC10</td>
<td>Met$^-$</td>
</tr>
<tr>
<td><em>E. coli C600</em></td>
<td>supF hsdR leu thi lacY tonA</td>
</tr>
<tr>
<td>(Nal$^+$)</td>
<td>Nal-resistant strain C</td>
</tr>
<tr>
<td>JM107</td>
<td>supE endA hsdR gyrA relA thiA (lacproAB)$^+$/F$^+$/traD proAB$^+$ lacP$+$ lacZAM15</td>
</tr>
<tr>
<td>MV1184</td>
<td>araA1lac-proABproL (p801lacZAM15) thiA (sr-rc-A)306::Tn10(ter)/F$^+$/traD proAB$^+$ lacP$+$ lacZAM15</td>
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<tr>
<td>Plasmids</td>
<td>pBR322</td>
</tr>
<tr>
<td>pUC119</td>
<td>Amp$^+$ lacZ</td>
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<tr>
<td>pPH109</td>
<td>Found in <em>P. putida</em> No. 109, dehH109</td>
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<tr>
<td>pBR-EJ</td>
<td>2.8-kb EcoRI-fragment of pUOH109 in pBR322</td>
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<tr>
<td>Phages</td>
<td>M13mp18</td>
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<tr>
<td>M13K07</td>
<td>Helper phage</td>
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† Oral presentations of this paper were made at the Annual Meeting of the Society of Fermentation Technology, Japan (October, 1989) and the Annual Meeting of the Agricultural Chemical Society of Japan (April, 1990).

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and if required 0.005% leucine and threonine and a trace of thiamine. *E. coli* JM107 was maintained in M9 glucose medium and grown in YT medium\(^1\) for M13 phage propagation.

**Assay of dehalogenase activity.** The reaction mixture (0.2 ml) containing 50 mM Tris-H\(_2\)SO\(_4\) buffer (pH 9.5), 7.5 mM monochloroacetate and an appropriate amount of the enzyme was incubated at 30°C for 20 min. and the amount of glycolate produced was measured by the colorimetric method of Day and Rodgers.\(^1\) One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of glycolate per min under these conditions. To determine enzyme activities towards 2-chloropropionate, dichloroacetate, and 2,2-dichloropropionate, the respective reaction products, lactate, glyoxylate, and pyruvate, were measured by the Barker and Summerson method\(^2\) and the nitrophenylhydrazone method.\(^3\)

**DNA manipulations.** Plasmid DNA and M13-replicative form DNA were isolated by the alkaline SDS method\(^4\) and purified, if necessary, by CsCl-ethidium bromide gradient centrifugation. Restriction enzymes and DNA-modifying enzymes purchased from Takara Shuzo Co. (Kyoto) and used according to the manufacturer’s manuals. Transformation of *E. coli* with plasmid DNA was done by the CaCl\(_2\) procedure.\(^5\) Agarose gel electrophoresis and other general procedures for DNA manipulations were done as described by Maniatis et al.\(^6\)

**Curing of plasmid with mitomycin C.** Cells of *P. putida* No. 109 were grown overnight in LB medium containing various amounts of mitomycin C, and a culture that showed a temperatately inhibited growth was diluted and spread on LB plates. All colonies grown were replicated onto chloroacetate or 2-chloropropionate minimum plates, on which the cured cells were unable to grow. The selected cells were examined on the absence of the plasmid and the dehalogenase activity.

**Conjugal transfer of plasmid pUOH109 from *P. putida* to *E. coli*.** Matings were done by the centrifugation method of Stuy.\(^7\) *P. putida* No. 109 was cultured with shaking in 5 ml of 2-chloropropionate-minimum medium, and after 24 h, 5 ml of fresh LB medium was added and the cultivation was continued for 6 h without shaking. Nalidixic acid-resistant *E. coli* C that was used as a recipient was grown in LB medium overnight and successively in fresh LB medium for 6 h more with shaking. Equal volumes of the donor and recipient cultures were mixed and centrifuged at 3000 rpm for 5 min. The cell pellet was rinsed with fresh LB medium and incubated at 30°C for 2 h. The cells were suspended and diluted in M9 mineral solution and spread on 2-chloropropionate-M9 plates containing 50 μg/ml nalidixic acid that prevented the growth of *P. putida* No. 109.

**Transformation with plasmid pUOH109.** Transformation of *P. putida* AC10 was done as described by Chakraborty et al.\(^8\) and that of *E. coli* C600 was done using the standard CaCl\(_2\) method.\(^9\) Transformants were selected on chloroacetate- or 2-chloropropionate-minimum plates.

**Cloning of dehalogenase gene.** The plasmid pUOH109 isolated from *P. putida* No. 109 was digested with EcoRI, and the fragments generated were ligated with the vector pBR322 that was cleaved with EcoRI and treated with alkaline phosphatase. CaCl\(_2\)-treated cells of *E. coli* C600 were transformed with the ligated DNA, and dehalogenase-positive clones were selected on LB plates containing 50 μg/ml ampicillin and 0.2% chloroacetate, because 0.2% chloroacetate was toxic enough to prevent the growth of the dehalogenase-negative cells of *E. coli* C600.

**DNA sequencing.** A 2.8-kb EcoRI fragment cloned in pBR322 was cleaved with Xmal to generate 1.35-kb and 1.45-kb fragments, which were subcloned into M13mp18 vector. Additionally, a 0.8-kb fragment generated from *NruI*-SphI digestion of the 2.8-kb EcoRI-fragment was also subcloned into pUC119. A series of deletion mutants of each subclone was constructed using the Kilo Sequence Deletion Kit (Takara Shuzo) according to the supplier’s manual. The derivatives of M13 RF DNA and pUC119 were transformed into *E. coli* JM107 and MV1184, respectively, and single-strand DNA were isolated from the propagated phages.

DNA was sequenced by the dideoxy chain termination method of Sanger et al.\(^10\) by use of the 7-DEAZA Sequencing Kit (Takara Shuzo) and [α-^35^]dCTP as recommended by the supplier. The nucleotide sequence was analyzed by using the GENETYX programs (SDC, Tokyo).

**Purification of dehalogenase from *E. coli*.** The dehalogenase H-109 produced by *E. coli* C600 carrying the recombinant pBR-EJ was purified to homogeneity as follows. The bacterial cells grown for 30 h in 5-liter of LB medium containing 0.1% chloroacetate were ruptured by ultrasonic oscillation to yield the cell-free extract. The enzyme was purified by ammonium sulfate fractionation and three kinds of column chromatography using DEAE-cellulofine, hydroxyapatite, and Toyopearl HW55, resulting in about 800-fold purification. The purified enzyme showed a single band on SDS-polyacrylamide gel electrophoresis.\(^11\)

**Analysis of N-terminal amino acid sequence.** Using 500 pmol of the purified protein, the amino acid sequence was analyzed with an automatic peptide sequencer PSQ-1 (Shimadzu Co., Kyoto).

**Nucleotide sequence accession number.** The nucleotide sequence presented here has been submitted to the DDBJ, EMBL, and GenBank under accession number D17523.

**Results**

**A plasmid encoding 2-haloacid dehalogenase H-109**

That *P. putida* No. 109 harbors a large plasmid has been demonstrated by plasmid extraction with the alkaline-SDS method\(^12\) and agarose gel electrophoresis. The plasmid, designated as pUOH109 tentatively, was purified by CsCl-ethidium bromide gradient centrifugation and analyzed. The plasmid was estimated to be 74 kb by analyses of restriction fragments generated from the plasmid. When the cells of No. 109 were treated with mitomycin C (30 μg/ml), 79% of viable cells tested were no longer able to grow on 2-chloropropionate, and they coincidently lost the plasmid and the dehalogenase activity. Conjugal transfer of the plasmid pUOH109 was observed when the No. 109 cells were mated with the recipient cells of *E. coli* C600. The transconjugants growing on 2-chloropropionate appeared at the frequency of about 10^-2^ per donor cell. When the plasmid pUOH109 was introduced into the cells of *P. putida* AC10 and *E. coli* C600 by transformation, these cells became capable of growing on chloroacetate and 2-chloropropionate. It was ascertained that the transconjugants and the transformants harbored pUOH109 and expressed the 2-haloacid dehalogenase activity. These results indicate that the 2-haloacid dehalogenase H-109 of *P. putida* No. 109 is mediated by the plasmid pUOH109 and also the gene for H-109, designated as dehH109, encoded on the plasmid is expressed in *E. coli* C600.

**Cloning of the gene dehH109**

The plasmid pUOH109 was digested with EcoRI to generate 14 fragments. The digests were ligated with vector pBR322 and transformed into *E. coli* C600. From many transformants that grew on LB plates containing ampicillin (50 μg/ml) and chloroacetate (0.2%), one clone was selected that had definite activity of 2-haloacid dehalogenase. A plasmid isolated from this clone was found to contain an insert of 2.8-kb DNA, which corresponded to the 10th band from the top of the EcoRI-digests of pUOH109 when the digests were electrophoresed on an agarose gel. This recombinant plasmid was designated pBR-EJ.

The dehalogenase produced by this *E. coli* clone was purified and compared with H-109 from *P. putida* No. 109. Both enzymes had identical mobility (M, 25,500) on SDS-polyacrylamide gel electrophoresis, identical patterns of substrate specificity, and identical pH-activity curves (data not shown), suggesting that the dehalogenase produced by the clone was H-109. These facts evidently sup-
Fig. 1. Restriction Map of the 2.8-kb EcoRI-Fragment Encoding the dehH109 Gene and the Ability to Express H-109 of Derivatives of pBR-EJ Constructed by Deletion with Restriction Enzymes.

(1), pBR-EJ; (2)-(8), deletion mutants of pBR-EJ. The open boxes show the segment inserted into pBR322 and the broken line shows a deleted region. Restriction sites: E, EcoRI; H, HinclII; S, SphI; C, ClaI; A, Aval; X, Xmal; N, NruI; B, BseI; Nt, NruI. The right column shows the presence or absence of H-109 activity in each E.coli clone.

Fig. 2. Sequencing Strategy for the 2.8-kb Fragment.

Arrows indicate the direction and extent of sequencing. Restriction sites: E, EcoRI; C, ClaI; S, SphI; X, Xmal; N, NruI. The open box indicates the location of a 675-bp ORF in the 2231-bp sequence analyzed.

ported the idea that the 2.8-kb fragment cloned contained the dehH109 gene.

A restriction map of the 2.8-kb fragment and deletion analysis to locate the dehH109 gene

A physical map of the 2.8-kb fragment was constructed by using the restriction enzymes EcoRI, NruI, NcoI, BseI, Aval, SphI, Xmal, ClaI, HinclII, and SalI (Fig. 1). To locate the dehH109-coding region, the plasmid pBR-EJ was cleaved with the restriction enzymes to yield various deletion derivatives (Fig. 1) and their abilities to express H-109 were examined in E. coli C600. The results showed that the dehH109 gene was within the 1-kb region between the ClaI site and the NruI site shown in Fig. 1.

Nucleotide sequence of the gene dehH109

The 2.8-kb fragment was cleaved with Xmal to yield two segments of 1.35 kb and 1.45 kb and these DNAs were sequenced. Supplementally, the 0.8-kb segment between the SphI site and the NruI site present in the 2.8-kb fragment, which should contain the dehH109-coding region, was also sequenced. The sequencing strategy is shown in Fig. 2.

A contiguous 2231-bp sequence was analyzed, which was a large part of the 2.8-kb fragment. Figure 3 shows only a 999-bp sequence from the ClaI site to the NruI site, in which a single open reading frame (ORF) of 675 bp is found. This ORF, starting at position 183 and ending at position 857, codes for a 224-amino-acid protein with molecular weight 25,231, which is in close agreement with the molecular weight of the enzyme H-109. The deduced amino acid sequence is identical to the N-terminal amino acid sequence (36 residues) found in the H-109 protein. These agreements support the identification of the ORF as the structural gene of H-109 (dehH109). The gene has a G+C content of 63.4%, which is similar to that of the P. putida genomic DNA.

The gene dehH109 is preceded by the sequence GAGGT-G, a putative ribosome-binding sequence, present 10 bp upstream from the ATG initiation codon. No sequence corresponding to the known promoters of Pseudomonas genes has been found in a region of 700 bp upstream from dehH109. A sequence, TGGCCG-5 bp-TCACGCTT, present 290 bp upstream from the initiation codon, is similar to some extent with the Pseudomonas promoters. Although dehH109 carried on pUOH109 was expressed in E. coli, we could not find a sequence corresponding to a consensus sequence of the E. coli promoter. The low expression of dehH109 in E. coli (only 4% of the activity level in P. putida) may be due to the inadequacy of a promoter sequence. A 15-bp inverted repeat with a 14-bp interval, a putative stem-loop structure, is present 9 bp downstream from the stop codon of dehH109.

Discussion

Although many haloalkanoic acid dehalogenases have been found and investigated, only seven enzymes have so far been characterized by cloning and sequencing their genes, to the best of my knowledge. The dehH109 gene was compared with these seven genes.

Four genes, namely dehC1 and dehC2 from Pseudomonas sp. CBS3,11 dhlB from Xanthobacter autotrophicus,21 and
It was definitely shown that t-specific 2-haloacid dehalogenases are not evolutionarily related to t-specific enzymes and should be divided in a separate family. In future, comparison with the genes for DL-nonspecific dehalogenases if obtained may give more evidence for the diversity of haloalkanoate dehalogenases.

By the way, dehH109 has been compared with two haloalkane dehalogenase genes, namely, 1,2-dichloroethane dehalogenase gene (dhIA) from *X. autotrophicus* GJ105 and dichloromethane dehalogenase gene (dcmA) from *Methyllobacterium* sp. DM4, but there was no homology.

The alignment of the amino acid sequences of the six homologous dehalogenases is given in Fig. 4. There are five regions that are highly conserved among these six sequences.

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References