

Cloning and Expression in *Escherichia coli* of the Haloacetate Dehalogenase Genes from *Moraxella* Plasmid pUO1

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Plasmid pUO1, which is present in a fluoroacetate-assimilating strain of *Moraxella* sp., specifies two kinds of haloacetate dehalogenases, H-1 and H-2, and mercury resistance. The H-1 and H-2 genes were cloned onto plasmid pBR322 in *Escherichia coli*. The analysis of the cloned DNA fragments revealed the loci of both genes on the pUO1 map and also the direction of transcription. Two different hybrid pBR322s containing the H-1 gene were maintained stably in *E. coli*, while three hybrids containing the H-2 gene were so unstable that they readily disappeared or changed into various deletion mutants. It was suggested that the instability of the H-2 hybrid plasmids might be caused by a certain DNA structure adjacent to the H-2 gene which might be concerned in the deletion of the H-2 gene from pUO1.

In recent years, the large amounts of halogenated organic compounds released into the environment have caused serious pollution problems. We have been working on the biodegradation of halogenated compounds, especially dehalogenation of haloaliphatics, to aid in the removal of such pollutants.

We have previously reported the isolation¹⁾ and characterization^{2~4)} of plasmid pUO1 which specifies the dehalogenation of haloacetates and mercury resistance in a fluoroacetate-assimilating strain of *Moraxella* species. The plasmid (43 megadaltons, Mdal) is transmissible to several strains of *Pseudomonas* spp. and *Escherichia coli*. Haloacetate dehalogenase [EC 3.8.1.3] catalyzes the reaction, $XCH_2COOH + H_2O \rightarrow HOCH_2COOH + HX$ (X: halogen). pUO1 encodes two kinds of the dehalogenase, H-1 and H-2; H-1 acts preferentially on monofluoroacetate, and H-2 acts on monochloro-, monobromo- and monoiodo-acetate, but not on monofluoroacetate.⁵⁾ The H-1 and H-2 enzymes are similar in molecular weight (42,000 and 43,000, respectively).^{5,6)} It is strange that two analogous enzymes are encoded together on one plasmid. Since enzyme evolution is known to be

initiated by duplication of a gene and followed by mutation of either gene,⁷⁾ it is interesting to compare the H-1 and H-2 genes. It is also considered possibly that the H-2 gene has transferred from another replicon into pUO1 as a transposable element. In fact, pUO1 frequently loses the H-2 function coincidentally with the loss of a specific 3 Mdal segment, generating a deletion mutant, pUO11.⁴⁾

A few studies on plasmids concerned in the biodegradation of halogenated compounds have been done,^{8~15)} but genes of dehalogenases have been neither cloned nor analyzed in detail. Recently, Slater *et al.* have isolated an R68.44-prime plasmid carrying a dehalogenase gene from *Pseudomonas putida* capable of degrading chloropropionates.¹⁶⁾ For studies on genetic relationships among various dehalogenases, it is necessary to clone the enzyme genes.

In this paper, we describe the cloning of the H-1 and H-2 genes onto plasmid pBR322 in *E. coli*, and refer to the instability of the H-2 clones. We also estimate the loci of the H-1 and H-2 genes on the pUO1 map and the direction of transcription.

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmids pUO1 and pUO11, and *Moraxella* sp. B have been described elsewhere.²⁾ *E. coli* C600 (r_K^- , m_K^- , *leu thr trp thi*) and plamid pBR322 were used as the cloning host-vector. Eight plasmids from *E. coli* V517 were used as size references (pVA517A to H; 35.8, 4.82, 3.67, 3.39, 2.63, 2.03, 1.79, and 1.36 Mdal).¹⁷⁾

Culture conditions. Strains of *E. coli* were grown at 37°C in L-broth (1% Polypepton, 0.5% yeast extract, 0.5% NaCl, pH 7.0) or L-agar plates (L-broth containing 1.5% agar). Antibiotic selections were performed on L-agar plates containing 50 µg of ampicillin (Ap) or tetracycline (Tc) per milliliter. *Moraxella* sp. B was grown as described previously.¹⁾

Preparation and analysis of plasmid DNA. Plasmid pUO1 was isolated from *Moraxella* sp. B according to the method of Hansen and Olsen.¹⁸⁾ Isolation of pBR322 and its derivatives from *E. coli* C600 was performed by the alkaline extraction method described by Birnboim and Doly.¹⁹⁾ cc-DNAs were purified by CsCl-ethidium-bromide equilibrium centrifugation as described before.¹⁾

DNA digestion with various restriction endonucleases were carried out in the buffers prescribed by the supplier. Analysis of the restriction fragments was done by agarose gel electrophoresis as previously described.¹⁾

Ligation and transformation. pUO1 and pBR322 were separately digested to completion with the appropriate restriction enzyme and exposed at 65°C for 10 min. Both digests were mixed, precipitated with ethanol, and dissolved in the ligation buffer (66 mM Tris-HCl, pH 7.6, 6.6 mM MgCl₂, 10 mM dithiothreitol, and 0.5 mM ATP). T4 DNA ligase-reaction was carried out at 15°C overnight. Transformation of CaCl₂-treated cells of *E. coli* was done according to the method of Cohen *et al.*²⁰⁾ Transformants with hybrid pBR322 carrying an insert at the restriction site of *Sal*I, *Hind*III or *Bam*HI were selected by ampicillin resistance (Ap^r) and tetracycline susceptibility (Tc^s), and then examined for H-1 and H-2 activities by a convenient method described below. Transformants with hybrids carrying *Eco*RI inserts were selected first on ampicillin plates and then examined for dehalogenase activities. On occasion, H-2⁺ transformants were selected by forming yellow colonies on L-agar plates containing 0.2% monochloroacetate and bromothymol blue (50 µg/ml), because the dehalogenation of monochloroacetate lowers pH of the medium.

Assay of H-1 and H-2 activities. The dehalogenase activities were assayed by measuring glycolate produced from monofluoro- or monochloroacetate by the colorime-

tric method of Dagley and Rodgers.²¹⁾ Cells grown overnight in 5 ml of L-broth or on an L-agar plate were harvested, washed and suspended in 2 ml of 0.05 M phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol. Adding one drop of toluene, the suspension was incubated at 30°C for 30 min with shaking. A 50 µl aliquot of the toluenized cell suspension was added to an assay mixture containing 100 µl of 0.1 M Tris-HCl, pH 9.0, and 50 µl of 0.03 M monofluoro- or monochloroacetate. After 20 min incubation at 30°C, the reaction was stopped by the addition of 0.5 ml of 2% chromotropic acid and 5 ml of conc H₂SO₄, and then the mixture was heated in boiling water for 30 min. The blue color developed was measured by the optical density at 570 nm.

Chemicals and reagents. The restriction endonucleases *Sal*I, *Eco*RI, *Hind*III, *Sma*I, and *Bam*HI, and T4 DNA ligase were purchased from the Takara Shuzo Co., Ltd., Kyoto, Japan. Ampicillin was obtained from Meiji Seika Kaisha, Ltd., Tokyo, Japan, and tetracycline from the Sigma Chemical Co., St. Louis, U.S.A. Other chemicals were commercial products.

RESULTS AND DISCUSSION

Transformation of E. coli with pUO1

The CaCl₂-treated cells of *E. coli* C600 were transformed with pUO1 DNA prepared from *Moraxella* sp. B. Transformants selected on L-agar plates containing HgCl₂ (30 µg/ml) exhibited dehalogenase activities. However, they could not grow on monofluoro- or monochloroacetate as a source of carbon and energy, though they could utilize glycolate. This is probably due to the toxic action of haloacetate, such as the "lethal synthesis" of fluorocitrate, an inhibitor of the Krebs cycle.

Plasmid DNA isolated from the transformants was proved to include both pUO1 and its deletion mutant pUO11. To examine the stability of pUO1 in *E. coli*, pUO1-harboring cells were cultured in L-broth for 40 hr, then spread on L-agar, and colonies were analyzed for the plasmid. It was revealed that approximately half of the colonies tested consisted of cells harboring either pUO1 or pUO11 and cells of the remaining colonies had no plasmids. The pUO11-harboring cells were about half the population of plasmid-inheritors. This suggested that pUO1 was liable to disappear or to be deleted in *E. coli* cells. A supplement of monochloroacetate in the

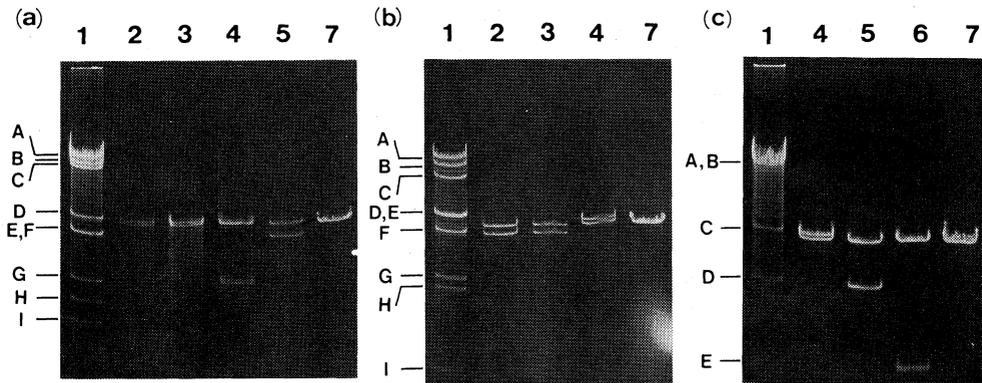


FIG. 1. Restriction Analysis of Hybrid Plasmids Containing (a) *Sal*I-Digests, (b) *Eco*RI-Digests, and (c) *Hind*III-Digests.

pUO1, pBR322 and hybrid plasmids were cleaved by (a) *Sal*I, (b) *Eco*RI, and (c) *Hind*III, and subjected to gel electrophoresis. 1, pUO1; 2 and 3, H-1⁺ hybrids; 4, H-2⁺ hybrid; 5 and 6, other hybrids; 7, pBR322.

growth medium allowed an increase in the population of pUO1-carrying cells.

Cloning of H-1 and H-2 genes of pUO1

After digesting the pUO1 DNA with restriction endonucleases *Sal*I, *Eco*RI, *Hind*III and *Bam*HI, the resulting fragments were ligated to pBR322 DNA, and provided for transformation of *E. coli* C600.

1) *Cloning of Sal*I digests. Four Ap^r Tc^s transformants were obtained. Two of them produced the H-1 enzyme, one, the H-2 enzyme, and the other, neither. Analysis of their plasmids (Fig. 1a) clarified that the two H-1⁺ clones (lanes 2 and 3) had an identical 5.4 Mdal plasmid, which was cleaved by *Sal*I into two fragments of 2.6 and 2.8 Mdal, corresponding to pBR322 DNA and the *Sal*I-D fragment of pUO1. The names and loci of the restriction fragments of pUO1 are given in Fig. 2.³⁾ The H-2⁺ clone (lane 4) carried a 3.8 Mdal hybrid plasmid which contained a 1.2 Mdal insert corresponding to the *Sal*I-G fragment. Another clone (lane 5) had a 4.8 Mdal hybrid plasmid containing a 2.2 Mdal insert corresponding to the *Sal*I-E fragment. The inserted fragments were identified by further restriction analysis. The H-1 and H-2 hybrid plasmids containing the *Sal*I-D and -G were tentatively called pBRSD1 and pBRSG2.

2) *Cloning of Eco*RI digests. Two H-1⁺

clones and one H-2⁺ clone were obtained. The H-1⁺ clones had an identical hybrid plasmid containing an insert of the *Eco*RI-F fragment (2.4 Mdal) (Fig. 1b). The H-2⁺ clone had a hybrid plasmid with the *Eco*RI-E fragment (3.0 Mdal). The H-1 and H-2 hybrid plasmids were tentatively named pBREF1 and pBREE2.

3) *Cloning of Hind*III digests. Clones of the *Hind*III-D (1.48 Mdal) and -E (0.60 Mdal) fragments were obtained, but they did not exhibit the dehalogenase function. Then the *Hind*III-C fragment (3.0 Mdal), which was expected to carry the H-2 gene, was purified by agarose gel electrophoresis and cloned onto pBR322 (Fig. 1c, lane 4). It was confirmed that the clone expressed H-2 enzyme. This plasmid was tentatively named pBRHC2.

4) *Cloning of Bam*HI digests. Fifteen Ap^r Tc^s transformants were obtained, but they did not express the dehalogenase. It was surprising that plasmids of these transformants were deletion mutants of pBR322, lacking the various lengths of DNA around the insertion site (*Bam*HI site); the range of plasmid size was from 1.4 to 2.5 Mdal. Purified fragments *Bam*HI-C (2.2 Mdal) and -D (1.22 Mdal) were cloned into pBR322, but they did not express the dehalogenase.

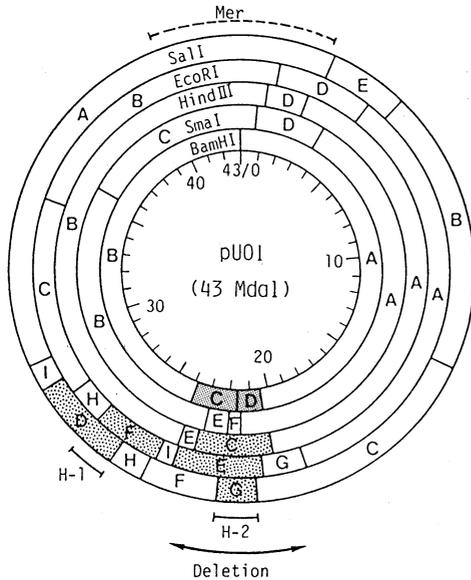


FIG. 2. Locations of the H-1 and H-2 Genes on a Restriction Map of pUO1.

Cloned fragments are indicated by stippling; coarsely-stippled ones are capable of expressing the dehalogenase and finely-stippled ones are not.

Loci of H-1 and H-2 genes on the pUO1 map

Since both clones of the fragments *SalI*-D and *EcoRI*-F expressed the H-1 enzyme, the H-1 gene should be located in the overlap of the two fragments (Fig. 2); the overlap region has a span of 1.6 kb. The H-2 enzyme was expressed by the clones of the *SalI*-G, *EcoRI*-E, and *HindIII*-C fragments. Therefore, the location of the H-2 gene was within the region of *SalI*-G (1.8 kb). The H-2 gene was wholly included in the 3 Mdal region which was liable to be deleted.

The structure genes of the H-1 and H-2 enzymes both have a length of about 1.3 kb, calculated from the molecular weights of the enzymes. Therefore, the limits we have set for both genes still include an extra length of 0.3 and 0.5 kb, respectively.

Orientation and expression of the inserted genes

The insertion of a restriction fragment into a vector DNA occurs in either of two possible orientations, which can be judged by the cleavage with another restriction enzyme; in

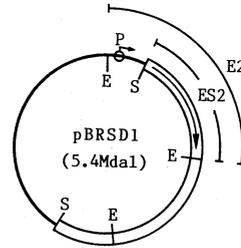


FIG. 3. Subcloning of the H-1 Structure Gene from Hybrid pBRSD1 Containing the *SalI*-D Fragment.

The thick line indicates pBR322 DNA and the open box, the inserted *SalI*-D fragment. P indicates the promoter of the Tc gene, and arrows indicate the direction of transcription. Restriction sites of *SalI* and *EcoRI* are expressed by S and E, respectively. ES2 and E2 are the fragment subcloned.

practice the hybrids with *EcoRI*-F and -E by a *SalI* cleavage, the hybrids with *SalI*-D and *HindIII*-C by an *EcoRI* cleavage, and the hybrid with *SalI*-G by *SmaI*-*BamHI* double cleavages. When each hybrid plasmid was cleaved into the inserted DNA and the vector DNA, and then ligated again, two hybrid plasmids with the dehalogenase gene inserted in opposite orientations were obtained. The *EcoRI*-F-inserted hybrid expressed the H-1 gene regardless of the orientation of the insert, while the hybrid with *SalI*-D inserted in the opposite orientation to the parent hybrid pBRSD1 did not express the H-1 gene. This suggests the possibility that the H-1 promoter is contained in the *EcoRI*-F fragment but not in the *SalI*-D, that is, the promoter should be located at the right of the H-1 gene limit indicated in Fig. 2, and the direction of transcription of the H-1 gene should be clockwise.

Why did the parent *SalI*-D hybrid express the H-1 gene without its own promoter? This hybrid carried the H-1 structure gene downstream from the promoter of Tc gene. When the fragment E2 (Fig. 3) which contained the H-1 structure gene and the Tc promoter was cleaved out and newly inserted into an *EcoRI*-cleaved pBR322 DNA, two hybrid plasmids with the fragment in opposite orientations expressed the H-1 gene equally. On the other hand, when the fragment ES2,

TABLE I. STABILITY OF HYBRID PLASMIDS

After each clone was grown in L-broth for 2 days, the populations of Ap^r and Ap^s cells were determined by the replica method. It was confirmed that Ap^s cells carried no plasmid.

Hybrid plasmid	Fragment inserted (Mdal)	Phenotype	Retention of plasmid (%)
pBRSD1	<i>Sal</i> I-D (2.8)	H-1 Ap ^r	99
pBREF1	<i>Eco</i> RI-F (2.4)	H-1 Ap ^r Tc ^r	97
pBRSG2	<i>Sal</i> I-G (1.2)	H-2 Ap ^r	5
pBREE2	<i>Eco</i> RI-E (3.0)	H-2 Ap ^r Tc ^r	1
pBRHC2	<i>Hind</i> III-C (3.0)	H-2 Ap ^r	4
pBR322	—	Ap ^r Tc ^r	100

which contained the H-1 structure gene alone, was subcloned into an *Eco*RI-*Sal*I-cleaved pBR322 DNA which lacked the Tc promoter, the resulting hybrid plasmids did not express the H-1 gene. Therefore, the H-1 expression of the *Sal*I-D hybrid and the E2 hybrid can be explained as being caused by the readthrough transcription initiated at the Tc promoter of pBR322. Quantitative expression of the H-1 gene of these hybrid plasmids was about three times that of pBREF1, which suggests that the Tc promoter is more efficient than the H-1 one in *E. coli*.

The *Sal*I-G hybrid plasmids expressed the H-2 gene equally without regard to the orientation of the insertion. Therefore, it seems that the promoter of the H-2 gene resides in the region of *Sal*I-G.

Stability of the H-1 and H-2 hybrid plasmids

After the H-1 and H-2 clones were cultured serially twice in L-broth, the preservation of the plasmid in the cell was examined using the criterion of Ap resistance of the cell. The results (Table I) indicate that the H-1 hybrid plasmids, pBRSD1 and pBREF1, were maintained stably in *E. coli*, while the H-2 hybrids, pBRSG2, pBREE2, and pBRHC2, were very unstable. It was confirmed that the Ap^r cells maintained the plasmid and expressed the dehalogenase, and that the Ap^s cells did not have the plasmid. As an exceptional case, Ap^r H-2⁻ mutants arising from the clones of

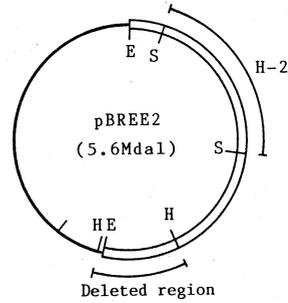


FIG. 4. Map of Unstable Hybrid pBREE2 Containing *Eco*RI-E Fragment.

The thick line and open box indicate pBR322 DNA and the inserted *Eco*RI-E fragment, respectively. Restriction sites of *Sal*I, *Eco*RI, and *Hind*III are expressed by S, E, and H, respectively. The 0.6 Mdal segment cut off by *Hind*III is indicated as a deleted region.

pBREE2 and pBRHC2 contained the deletion plasmids which were lacking not only the inserted DNA but also various lengths of the pBR322 DNA around the insertion site. Similar deletions were observed when cloning the *Bam*HI-C fragment.

The plasmid pBREE2 was a very unstable hybrid pBR322 containing *Eco*RI-E fragment. Enzymatic deletion of a small specific segment (0.6 Mdal) from the inserted fragment of pBREE2 (Fig. 4) resulted in stabilizing the plasmid. The region deleted from *Eco*RI-E was nearly equivalent to *Hind*III-E, in which resided an end of the specific 3 Mdal deletion region described above. The *Hind*III-E region is included too in the *Bam*HI-C fragment whose clone was also unstable. Another end of the 3 Mdal deletion region was included in the *Hind*III-C fragment, whose clone pBRHC2 was unstable too. These suggested that the instability of the hybrid plasmids containing *Eco*RI-E, *Bam*HI-C and *Hind*III-C might be caused by a particular DNA structure participating in the deletion of the 3 Mdal segment from pUO1.

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