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Use of haloacetate dehalogenase genes as selection markers for *Escherichia coli* and *Pseudomonas vectors*

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Abstract

The haloacetate dehalogenase gene, *dehH2*, cloned from *Moraxella* sp. strain B could be used as a selection marker gene for vectors in *Escherichia coli* and *Pseudomonas putida*. Haloacetates, especially iodoacetate, inhibit the growth of some microorganisms. The *dehH2* gene introduced into the cells conferred iodoacetate resistance on them. Therefore, *E. coli* and *P. putida* transformed with vectors marked with *dehH2* could be easily selected on plates containing iodoacetate.

Introduction

Haloacetate dehalogenase catalyzes the hydrolytic cleavage of a halogen-carbon bond of haloacetate to yield glycolate. *Moraxella* sp. strain B was isolated as a fluoroacetate utilizer from industrial waste-water and has two haloacetate dehalogenases, H-1 and H-2 (Kawasaki et al. 1981a). H-1 acts preferentially on fluoroacetate rather than on chloro- and bromoacetate and slightly on iodoacetate. H-2 shows good activity on chloro-, bromo- and iodoacetate, but not on fluoroacetate. The genes for H-1 and H-2, called *dehH1* and *dehH2* respectively, are encoded on a conjugative plasmid, pU01 (Kawasaki et al. 1981b). They have been cloned in pBR322 (Kawasaki et al. 1984) and sequenced (Kawasaki et al. 1992). The cloned genes express H-1 and H-2 in *E. coli* and *Pseudomonas* spp.

Fluoroacetate is known to inhibit the tricarboxylic acid cycle and iodo- and bromoacetate inhibit many enzymes, especially those containing essential thiol groups. Therefore, haloacetates are generally toxic to many organisms and inhibit their growth. Haloacetate dehalogenase is assumed to play two main roles in microorganisms; the detoxification of harmful haloacetates and the utilization of them as carbon sources.

This study demonstrates that *E. coli* and *Pseudomonas* acquiring the haloacetate dehalogenase genes, *dehH1* and *dehH2*, became resistant to haloacetates and therefore these genes could be used as genetic markers of vectors to be easily selected.

Methods

Bacteria, plasmids, and growth conditions

Plasmids pBREF1 (8.3 kb, *dehH1*, Ap^r, Tc^r) and pBRSG2 (6.5 kb, *dehH2*, Ap^r) were constructed by inserting a 3.9-kb DNA fragment containing the *dehH1* gene (885 bp) and a 2.1-kb fragment containing the *dehH2* gene (657 bp), respectively, into pBR322 (Kawasaki et al. 1984). A 1.3-kb segment including a *dehH2* promoter-coding region was removed from pBRSG2 to generate plasmid pBRSG25 (5.2 kb). The *dehH2* gene on pBRSG25 should be expressed using *tet*-promoter of pBR322. Plasmid pMFYSG2 was constructed by inserting the 2.1-kb fragment encoding *dehH2* into the broad host-range plasmid pMFY40 (11.6 kb, Ap^r, Tc^r), derived from RSF1010 (Fukuda & Yano 1985). Helper plasmid pRK2013 (Ditta et al. 1980) was a kind gift from Prof. K. Yano, Tokyo University. These plasmids were maintained and propagated in *E. coli* C600 (*leu, thr, thi, lacY, hsdR, hsdM, supE, tonA*) and in *E. coli* HB101 (*ara, proA, lacY, galK, rpsL, xyl, mtl, hsdS, recA, supE*).

Moraxella sp. strain B harboring pUO1 and its plasmid-cured strain were described previously (Kawasaki et al. 1981b). *P. putida* AC10 (met$^-$) was gifted from Dr. Furukawa, Kyushu University, and *P. putida* IFO 12996 and *P. aeruginosa* IFO 3445 were obtained from the culture collection of IFO (Japan).

All strains of *E. coli* and *Pseudomonas* were grown in LB medium (Maniatis et al. 1982) consisting of 10 g tryptone, 5 g yeast extract, 5 g NaCl per litre, pH 7.3 and when required 50 μg/ml of ampicillin (Meiji Seika Co., Ltd.) and various concentrations of sodium-haloacetates (Wako Pure Chemical) were added.

Transformation and tri-parental mating

Transformation of *P. aeruginosa* IFO 3445 with plasmid pMFYSG2 was done as described by Chakrabarty et al. (1975) and transformants were selected on LB plates containing 600 μg ampicillin per ml. *E. coli* C600 and HB101 were transformed with pMFYSG2 and recombinant pBR322 using the standard $CaCl_2$ method (Maniatis et al. 1982) and selected on ampicillin (50 μg/ml)-LB plates. When selected on iodoacetate plates, the cells were incubated in fresh LB medium at 37° C for 1 h prior to plating.

The introduction of pMFYSG2 into *P. putida* AC10 and IFO 12996 were performed by the tri-parental mating method described by Ditta et al. (1980). The culture of *P. putida* was mixed with the cultures of *E. coli* C600 carrying pMFYSG2 and *E. coli* HB101 carrying the helper plasmid pRK2013 and filtered onto a membrane filter. The filter was incubated at 30° C on an LB plate overnight. The cells were suspended in a small volume of physiological saline solution and plated on LB-agar media containing 600 μg/ml of ampicillin and 10 μg/ml of chloramphenicol for AC10 transconjugants and 50 μg/ml of ampicillin and 10 μg/ml of $HgCl_2$ for IFO 12996 transconjugants.

DNA manipulations

Plasmid DNA was isolated by the alkaline SDS method (Birnboin & Doly 1979) and analyzed by agarose-gel electrophoresis. Other general procedures for DNA manipulations were according to Maniatis et al. (1982).

Assay of haloacetate dehalogenase activity of cells

Cells grown overnight in LB medium were ruptured by ultrasonic oscilation and centrifuged to yield a cell-free extract. The haloacetate dehalogenase activity of the extract was assayed as described previously (Kawasaki et al. 1981a). A 50 μl aliquot of the extract 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 monochloroacetate, and after 20 min incubation at 30° C, glycolate produced was measured by the colorimetric method of Dagley and Rodgers (1953). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of glycolate from chloroacetate per min under the assay conditions.

Table 1. Minimum growth-inhibitory concentrations (MIC) of haloacetates towards *E. coli* with or without the genes *dehH1* and *dehH2*. The cells were grown at 30° C on LB plates containing various concentrations of fluoroacetate (FA), chloroacetate (ClA), bromoacetate (BrA) or iodoacetate (IA) and after 24 h, MICs were determined.

Strain of *E. coli*	Plasmid	MIC (mg/ml) of			
		FA	ClA	BrA	IA
C600	–	> 10	3	0.1	0.03
	pBREF1 (*dehH1*)	> 10	3	0.1	0.03
	pBRSG2 (*dehH2*)	> 10	3	1	3
HB101	–	3	3	0.1	0.1
	pBREF1 (*dehH1*)	> 10	3	0.1	0.1
	pBRSG2 (*dehH2*)	3	3	1	3

Determination of minimum growth-inhibitory concentration (MIC)

An overnight culture was inoculated onto LB plates or into LB broth containing haloacetate, and after 24-h cultivation at 30° C, MICs was determined by the absence of growth.

Results and discussion

Changes of haloacetate susceptibilities of E. coli *by acquiring dehalogenase genes dehH1 and dehH2*

E. coli had different susceptibilities to fluoro-, chloro-, bromo-, and iodoacetates. The MICs of these haloacetates were determined for *E. coli* C600 and HB101 (Table 1). The strain C600 was very susceptible to iodoacetate (MIC of 30 μg/ml) but resistant to fluoroacetate (MIC higher than 10 mg/ml), while the strain HB101 was relatively less resistant to fluoroacetate

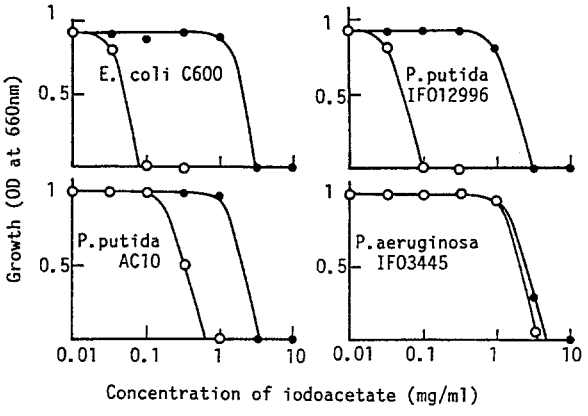

Fig. 1. Growth-inhibitory effects of iodoacetate on *Pseudomonas* strains. The cells were grown at 30° C in LB medium containing iodoacetate and after 24 h the growth was measured by OD at 660 nm. • and o represent the growth of the cells with and without plasmid pMFYSG2 (*dehH2*), respectively. For contrast, the growth of *E. coli* C600 with or without pBRSG2 was indicated.

(MIC of 3 mg/ml). The decreasing order of MICs in *E. coli* was generally fluoro-, chloro-, bromo-, and iodoacetates.

The genes *dehH1* and *dehH2*, carried on the plasmids pBREF1 and pBRSG2 respectively, were introduced into the two strains of *E. coli*. The *dehH2* introduction made both strains very resistant to iodoacetate, their MICs increased 30~100-fold (3 mg/ml). The MICs to bromoacetate also increased 10-fold, but those to chloroacetate and fluoroacetate did not change. The introduction of *dehH1* into the strain HB101 raised the MIC to fluoroacetate from 3 mg/ml to more than 10 mg/ml.

Next, we examined the relationship between the dehalogenase H-2 activity of the cell and the iodoacetate resistance. Four *dehH2*-plasmids, pBRSG2, pBRSG25, pMFYSG2, and pUO1, were used, which expressed H-2 activity in *E. coli* C600 at different levels, 790, 180, 580, and 55 mU/mg, respectively. These strains showed increased but the same MIC to iodoacetate (3 mg/ml) regardless of their different H-2 content. Even if *E. coli* acquired the highest level of dehalogenase activity, the MICs to chloro-, bromo- and iodoacetate never increased over 3 mg/ml. It was probably due to pH-lowering caused by too much hydrogen halide produced.

Increased iodoacetate resistance of Pseudomonas acquiring dehH2

Although the *Pseudomonas* strains tested were rather resistant to haloacetates compared with *E. coli*, two strains of *P. putida*, IFO 12996 and AC10, gave relatively low MICs to iodoacetate (0.1 and 0.5 mg/ml), respectively, Fig. 1). Plasmid pMFYSG2 (*dehH2*) was introduced into these *Pseudomonas* and their MICs to iodoacetate were determined. The two strains of *P. putida* have acquired increased iodoacetate resistance to give a MIC of 3 mg/ml but *P. aeruginosa* IFO 3445 showed no increase in the resistance (MIC 3 mg/ml). *Moraxella* sp. strain B with plasmid pUO1 showed a 10-fold increased MIC to iodoacetate (0.3 mg/ml) compared with its plasmid-cured strain.

Selection of transformants on iodoacetate plates

The fact that *E. coli* with *dehH2* could grow on an iodoacetate (100 μg/ml) plate, while the cells without *dehH2* could not, suggested the usage of the *dehH2* gene as a selection marker of vectors. *E. coli* HB101 cells were transformed with pBRSG2 (*dehH2* and *bla*) and transformants were selected on iodoacetate plates and ampicillin plates. Prior to plating the transformed cells on the selection plates, they were incubated in fresh LB medium at 37° C for 1 h to express the *dehH2* and *bla* genes and to acquire the resistance to iodoacetate and ampicillin. Table 2 shows that the transformation frequency for iodoacetate resistance was equal to that for the ampicillin resistance. Since iodoacetate resistance phenotype conferred by *dehH2* was easily selected, the gene was usable as a genetic marker for vectors.

Use of iodoacetate as a selection pressure to maintain dehH2-containing cells

Plasmid pBRSG2 in *E. coli* is very unstable (Kawasaki et al. 1984). When *E. coli* HB101 (pBRSG2) was cultured in LB medium at 37° C for 24 h approximately 40% of the cells lost the plasmid. The cells cultured in the presence of iodoacetate (100 μg/ml) almost all maintained the plasmid as well as the cells cultured in the presence of ampicillin (50 μg/ml). Iodoacetate could exert a selection pressure to prevent the loss of the *dehH2*-plasmid.

Thus haloacetate dehalogenase gene *dehH2* can be used as a selection marker in *E. coli* and some *Pseudomonas*. Especially, we expect the effective use of it

Table 2. Selection of *E. coli* cells transformed with pBRSG2 on an iodoacetate plate. $CaCl_2$-treated cells of *E. coli* HB101 (3.0×10^8/0.1 ml) were transformed with about 0.1 μg of pBRSG2 DNA and incubated in 1 ml of LB medium at 37° C for 1 h prior to spreading 0.1 ml aliquots on the selection plates. Transformation frequency was given in parentheses.

Competent cell	Plasmid	No. of colonies grown on plates containing		
		Iodoacetate		Ampicillin
		100 μg/ml	300 μg/ml	50 μg/ml
E. coli HB101	–	0	0	0
E. coli HB101	pBRSG2	1.2×10^2 (4×10^{-6})	1.2×10^2 (4×10^{-6})	1.2×10^2 (4×10^{-6})

in multiple antibiotic resistant bacteria where antibiotic resistance genes do not serve as selection markers.

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