Cloning and expression of DL-2-haloacid dehalogenase gene from *Burkholderia cepacia*

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Abstract *Burkholderia cepacia* strain KY, which can utilize a herbicide 2,4-D as a sole carbon and energy source, catalyzes the hydrolytic dehalogenation of both D- and L-2-haloalkanoic acids. We have cloned the gene encoding DL-2-haloacid dehalogenase, and obtained a recombinant plasmid (pUCDEXL) containing approximately 4.5 kbp insert. In both of *B. cepacia* strain KY and this clone *E. coli* JM109/pUCDEXL, DL-2-haloacid dehalogenase was induced significantly with monohalogenated acetic acids, such as chloroacetate, bromoacetate and iodoacetate. This dehalogenase was also overexpressed in *E. coli* using three different promoters. In pET vector systems with T7 *lac* promoter, a large amount of dehalogenase was selectively expressed, but some parts of the protein were accumulated in the form of inclusion bodies. This problem was overcome to carry on growth and induction at 22°C, and at the same time, the maximum specific activity of dehalogenase was reached at 12.6 U/mg, 500-fold higher activity than in wild strain, *B. cepacia* strain KY grown with 2,4-D.

Keywords Activity; cloning; 2,4-D; 2-haloacid dehalogenase; monohalogenated acetate; overexpression

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Introduction

Modern industrial and agricultural processes require the consumption of many synthetic halogenated compounds, and release them continuously as pollutants into the environment. In many cases, these xenobiotic compounds show high toxicity and persistence, and cause the contamination of soils and groundwaters. Haloaromatics such as chlorinated phenols and PCBs, and haloaliphatics such as trichloroethylene (TCE) are major pollutants. In both cases, dehalogenation step plays an important role in detoxification of these halogenated compounds. Several enzymatic dehalogenations involved in microbial degradation of halogenated compounds have been reported, and their dehalogenation mechanisms are further being investigated (Janssen *et al.*, 1994). Especially, halidehydrolases which catalyze hydrolytic dehalogenation from 2-halocarboxylic acids or haloalkane, monooxygenases which catalyze oxidative dehalogenation from halogenated phenols and TCE (Orser *et al.*, 1993; Schenk *et al.*, 1989; Wackett, 1995), and reductive dehalogenation from haloaromatics which were predominantly proceeding under anaerobic conditions (Neumann *et al.*, 1994) have been expected for application in the field of environmental engineering, cleanup and bioremediation technology. These useful enzymes are, however, generally unstable and show low productivity and activity. Therefore, it is very important to increase production of these enzymes through genetic engineering (Liu *et al.*, 1995; Widersten, 1998) for modification of catalytic function by protein engineering techniques, chemical modification (Jiang *et al.*, 1996), immobilization (Diez *et al.*, 1996), mutagenesis and so on (Asmara *et al.*, 1993; Kurihara *et al.*, 1995).

Burkholderia cepacia, named strain KY, was isolated from soil with herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) as a sole carbon and energy source, in our laboratory. This strain KY can release chloride ion from 2,4-D in the degradation process (Koshikawa *et al.*, 1995). The enzyme extracted from *B. cepacia* strain KY catalyzed the hydrolytic dehalogenation of both D- and L-2- haloalkanoic acids (Reaction 1), however, not for

2,4-D directly. This dehalogenating enzyme was purified and characterized, and was classified into DL-2-haloacid dehalogenase on its reaction characteristics (Ohkouchi *et al.*, 1996).

$$RCHXCOOH+OH^{-}\rightarrow RCHOHCOOH+X^{-},$$

R=H or methyl group, *X*=halogen atom: (Reaction 1)

In this investigation, we have isolated and cloned the structural gene encoding this DL-2-haloacid dehalogenase from *B. cepacia* strain KY, and identified the induction factors necessary to its expression in both *B. cepacia* strain KY and *E. coli*. Furthermore, to obtain more efficient expression, the overexpression systems of this DL-2-haloacid dehalogenase in *E. coli* have been constructed using three different promoters, and conditions of dehalogenase expression in these systems have been examined and optimized. We also have discussed and demonstrated the availability of these expression systems.

Materials and methods

Growth and culture conditions

Burkholderia cepacia strain KY was grown at 30ºC in basic medium containing 0.1% Yeast extract, 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.1% $Na_2HPO_4 \cdot 12H_2O$ and 0.01% $MgSO_4$ and appropriate halogenated compounds. Recombinant *Escherichia coli* JM109 was grown at 37ºC in LB medium, if necessary, ampicillin (final concentration=100 mg/L) and isopropyl-1-thio-β–D-galactoside (IPTG, final concentration=0.5 mM) was added. For *E. coli* BL21 (DE3)pLysS, LB medium was used in small scale culture (5 mL), and SB medium which is more rich than LB medium containing 0.1% Tryptone, 0.2% Yeast extract, 0.5 v/v% Glycerol, 0.13% KH_2PO_4 and 0.04% KH_2PO_4 in large scale culture (3 L) were also used to enhance cell growth.

Dehalogenase activity assay

The harvested cells were disrupted with ultrasonic homogenizer (UltraS Homogenizer VP-5S, TITEC or Vibra cell, Sonics and Materials), and the supernatant after centrifugation served as an enzyme solution. The reaction (total volume 90 μL) was carried out with 100 mM Tris-sulphate buffer (pH 9.5), 25 mM DL-2-chloropropionic acid (DL-2-CPA) as a substrate, and the enzyme solution. Enzyme reaction was stopped by adding of 10 μL of 3 M H_2SO_4. The released chloride ions were analyzed spectrophotometrically (UV-2500, Shimadzu) (Iwasaki *et al.*, 1956). One unit of dehalogenase activity was defined as the amount of enzyme that catalyzes the dehalogenation of 1-μmol of a substrate per minute. The specific activity was also defined as dehalogenase activity per 1 mg-protein. Protein was assayed by Bio-Rad Protein Asay (Bio-Rad Laboratories).

DNA manipulations

Chromosomal DNA extracted and purified from *B. cepacia* strain KY was digested with EcoRI completely. The *Eco*RI fragments were ligated with a vector pUC118 (TAKARA SHUZO, 3.2 kbp) at *Eco*RI site, and transformed into *E. coli* JM109. Transformant, which showed dehalogenase activity, was screened on LB plate containing 0.2% bromoacetate (Liu *et al.*, 1995). Recombinant plasmids were checked by 0.4% agarose gel electrophoresis. Plasmids were extracted using GFX Micro Plasmid Prep Kit (Amersham Pharmacia Biotech).

Dehalogenase expression

B. cepacia strain KY and obtained transformant were cultivated in basic medium and LB medium described above respectively, with bromoacetate (0 to 200 mg/L), and the

dehalogenase activities were determined. Also in transformed *E. coli*, the effect on dehalogenase induction was examined by addition of ten halogenated compounds (1 mM) instead of bromoacetate. Chloroacetate, iodoacetate, dichloroacetate, trichloroacetate, 2-chloropropionate (2-CPA), 3-chloroacetate, 2,2-dichloroacetate, 2,3-dichloroacetate as haloaliphatic acids, and 2,4-dichlorophenol and 2,4-D as haloaromatics were used. The amount of induced dehalogenase was checked visually by SDS-PAGE.

Overexpression in *E. coli*

Preparation of insert fragments by PCR amplification. Oligonucleotide primers were designed based on the open reading frame region of known DL-2-haloacid dehalogenase from *Pseudomonas* sp. Strain 113 (Nardi-Dei *et al.*, 1997). Oligonucleotide primers were ordered and synthesized by Biologica co. The nucleotide sequences of primers used in PCR reaction are as follows. (The italic sequences indicate the recognition site of restriction enzymes added for ligation):

Forward primers: 5'-*GAATT*CGGAGGAGACACAAATGTC-3' (24mer, Italic; *Eco*RI site)

5'-*CATAT*GTCCCATCGACCGATAC-3' (22mer, Italic; *Nde*I site)

Reverse primer: 5'-*GAATTC*TCAGCCCTTCTCTGTTGG-3' (24mer, Italic; *Eco*RI site)

PCR reaction was performed on Programmable Thermal Controller with Hot Bonnet (PTC-100-60-HB, MJ Research) under mineral oil free conditions. A 50 μL reaction mixture contained 0.5μM of each designated primer, 50 ng chromosomal DNA extracted from *B. cepacia* strain KY as templates and 25 μl Premix Taq (Ex Taq version, TAKARA SHUZO). The DNA templates were first denatured at 95ºC for 4 minutes. The subsequent 25 cycles consisted of a 1-min denaturation step at 94ºC, a 1-min annealing step at 62ºC and a 2-min primer extention step at 72ºC. The amplified fragments were checked by 1.2% agarose gel electrophoresis. These fragments were digested with restriction enzymes described as below.

Construction of plasmids for dehalogenase overexpression. Three plasmids, pUC118, pKK223-3 (Amersham Pharmacia Biotech., 4.6 kbp) and pET-21a (Novagen, 5.4 kbp) were used as vectors. The PCR fragments prepared previously were digested with EcoRI for ligation to pUC118 and pKK223-3. For ligation to pET-21a, the fragments were digested with two restriction enzymes, *Nde*I and *Eco*RI. These *Eco*RI-*Eco*RI fragments were ligated with vectors, pUC118 and pKK223-3 at the *Eco*RI site, and also *Nde*I-*Eco*RI fragments were ligated with pET-21a vector at the corresponding restriction site.

Conditions for dehalogenase overexpression. For pUC and pKK vectors, *E. coli* JM109 was used as host strain for expression as well as for subcloning. Only a pET vector was transformed into *E. coli* BL21 (DE3)pLysS which carries the gene for T7 RNA polymerase for target enzyme expression. Dehalogenase was induced by addition of IPTG to a final concentration 0.5 mM. IPTG was added at culture start when dehalogenase was expressed in *E. coli* JM109, and cells were incubated at 37ºC for 15 hours. On the other hand, *E. coli* BL21(DE3)pLysS was incubated until OD_{660} reached approximately 0.6 at 37, 30, 25, 22ºC. Then, IPTG was added and the incubation and expression were continued. The time for the expression at each temperature was optimized experimentally. The amount of induced dehalogenase was checked visually by SDS-PAGE.

Results and discussion

Cloning of 2-haloacid dehalogenase gene

In many transformant grown on LB plates containing 0.2% bromoacetate, one clone which showed the highest activity was selected. The recombinant plasmid isolated from this clone was found to contain an insert of approximately 4.5 kb-DNA. This recombinant plasmid was designated as pUCDEXL, and was shown in Figure 1. This clone showed approximately 3.5 U/mg-dehalogenase activity in the presence of 0.2% bromoacetate added for selection of dehalogenase clones. In the absence of bromoacetate, however, the dehalogenase activity was decreased to a level of 0.3 U/mg. This suggests that bromoacetate can play a role as an inducer for this DL-2-haloacid dehalogenase.

Dehalogenase expression

In both *B. cepacia* strain KY and JM109/pUCDEXL, by addition of bromoacetate to medium, dehalogenase activities were raised dramatically as shown in Figure 2. In the range of 0 to 1.0 mM, the activities were increased depending on concentration of bromoacetate. This result suggests that DL-2-haloacid dehalogenase from *B. cepacia* strain KY was inducible enzyme, which has been observed in some other dehalogenases, for example, fluoroacetate dehalogenase H-I from *Moraxella* sp. Strain B (Motosugi *et al.*, 1982), haloacid dehalogenase BhlB from *Xanthobacter autotrophicus* GJ10 (van der Ploeg *et al.*, 1995). These dehalogenases were reported as inducible enzymes with fluoroacetate and bromoacetate, respectively.

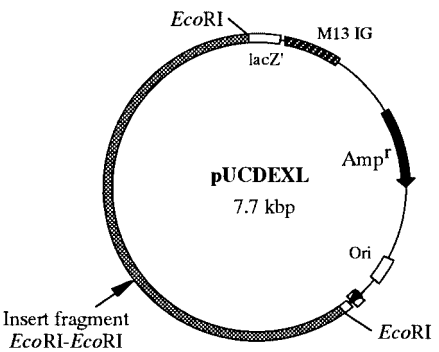

Figure 1 Structure of the recombinant plasmid, pUCDEXL for the dehalogenase expression. a pUCDEXL contained approximately 4.5 kbp insert fragment encoding DL-2-haloacid dehalogenase at *Eco*RI restriction site of pUC118

Table 1 Effect of various halogenated compounds on the dehalogenase activity in *E. coli* JM109/pUCDEXL

Halogenated compounds	Specific activity (U/mg)	Relative activity (%)
Control	0.30	100
Chloroacetate	0.65	217
Bromoacetate	1.79	597
Iodoacetate	3.10	1033
Dichloroacetate	0.35	117
Trichloroacetate	0.34	113
DL-2-Chloropropionate (2-CPA)	0.38	127
3-Chloropriopionate	0.34	113
2,2-Dichloropropionate	0.36	120
2,3-Dichloropropionate	0.25	83.3
2.,4-Dichlorophenol	N.D.	–
2,4-Dichlorophenoxy acetate (2,4-D)	0.42	140

Control: No halogenated compounds were added to LB medium.
N.D.: The cell growth was completely inhibited

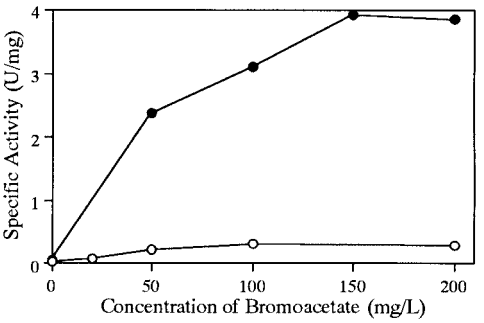

Figure 2 Effect of bromoacetate concentration on the dehalogenase activity in *B. cepacia* and *E. coli* JM109/pUCDEXL. ○: The specific activity of *B. cepacia* cultivated in basic medium. ●: The specific activity of *E. coli* JM109/pUCDEXL in LB medium containing ampicillin and IPTG. (bromoacetate 1 mM=139.0 mg/L)

The effect of various halogenated compounds on dehalogenase activity in JM109/pUCDEXL was also shown in Table 1. Only monohalogenated acetates, chloroacetate, bromoacetate and iodoacetate showed significant increase in the dehalogenase activity. The wild strain *B. cepacia* strain KY was also grown with 2-CPA very well, and the dehalogenase activity was higher than that with 2,4-D (with 2,4-D, 0.025 U/mg; with 2-CPA, 0.28 U/mg). Against our expectation, however, 2-CPA could not act as dehalogenase inducer. This suggests that *B. cepacia* strain KY can catalyze the transformation of 2-CPA to chloroacetate, and dehalogenase was induced by chloroacetate accumulated in culture medium. On the other hand, *E. coli* JM109/pUCDEXL could not grow with 2-CPA as a sole carbon and energy source. So, it could not catalyze this transformation; the dehalogenase induction with 2-CPA had not occurred consequently. In the presence of 1 mM 2,4-dichlorophenol, the cell growth of JM109/pUCDEXLwas completely inhibited.

Each crude extract (15 μg-protein/each lane) was applied to SDS-PAGE, and it was found that the amount of dehalogenase increased with monohalogenated acetates (data not shown). These results demonstrate the effect of monohalogenated acetates as inducers on this dehalogenase expression. In JM109/pUCDEXL, IPTG did not function as inducer of the dehalogenase. These results also suggest that intrinsic promoter on this dehalogenase gene from *B. cepacia* strain KY regulates the dehalogenase expression instead of *lac* promoter on pUC vector.

Overexpression of dehalogenase in *E. coli*

Construction of plasmids for dehalogenase overexpression. DL-2-haloacid dehalogenase from *B. cepacia* strain KY showed a high similarity to DL-DEX 113 from *Pseudomonas* sp. Strain 113 in its enzyme characteristics and molecular weight (Motosugi *et al.*, 1982; Ohkouchi *et al.*, 1996). The PCR amplification with designed primer pairs and chromosomal DNA from *B. cepacia* strain KY as template DNA was successful. The amplified PCR fragments had length approximately 1.0 kbp. The constructed plasmids for overexpression were shown in Figure 3, which were designated pUCDEXs, pKKDEX and pETDEXa, respectively. The *lac* promoter in pUCDEXs, the *tac* promoter in pKKDEX, and the T7 *lac* promoter in pETDEXa regulate the expression of the dehalogenase, respectively. Only in pETDEXa, the ribosome-binding site on pET-21a vector was used for expression, and the initiation codon (ATG; Met) was located at *Nde*I recognition site. In pUCDEXs and pKKDEX, the sequences of ribosome-binding site and initiation codon from *B. cepacia* strain KY were contained in the insert *Eco*-RI-*Eco*RI fragments amplified by PCR. The termination codon on this dehalogenase gene, which was located on the insert fragments was used in all vector systems.

Table 2 Effect of incubation and induction temperature on the dehalogenase activity in *E. coli* BL21(DE3)pLysS/pETDEXa

Temperature (°C)	Induction times (hours)	Specific activity in soluble fraction (U/mg)
37	3.0	2.64
30	5.0	4.49
25	10.0	7.47
22	12.0	12.5

Table 3 The dehalogenase expression in overexpression systems with three promoters

Vector	Promoter	Host *E. coli* strain	Inducer	Induction conditions	Specific activity (U/mg)
pUC118	*lac*	JM109	IPTG	37°C, 15 hours	3.98
pKK223-3	*tac*	JM109	IPTG	37°C, 15 hours	4.27
pET-21a	T7 *lac*	BL21(DE3)pLysS	IPTG	22°C, 12 hours	12.6

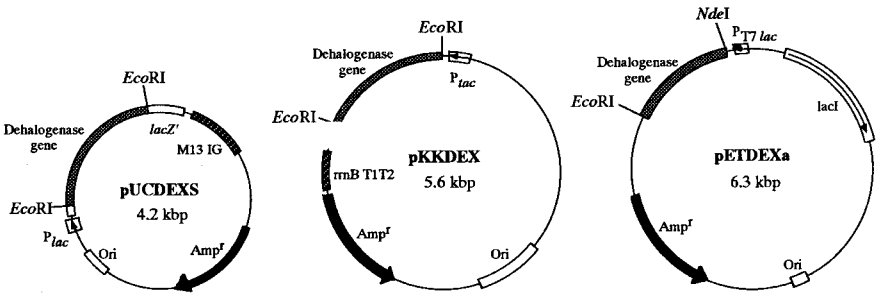

Figure 3 Physical maps of plasmids pUCDEXS, PKKDEX and pETDEXa constructed for overexpression of the dehalogenase in *E. coli*. Each plasmid contains the ampicillin resistance gene (Amp^r) and origins of replicon for *E. coli* (Ori). The dehalogenase gene was located understream of each promoter, P_{lac}, P_{tac} and $P_{T7\ lac}$

Overexpression of DL-2-haloacid dehalogenase. The dehalogenase activity in soluble fraction extracted from BL21(DE3)pLysS/pETDEXa grown at each temperature was compared in Table 2. The growth and induction at 37°C showed poor cell growth and caused decrease of dehalogenase activity. Because the amounts of dehalogenase in both soluble and insoluble fractions had not diminished dramatically in SDS-PAGE (data not shown), so many part of the producd dehalogenase seemed to be accumulated in the form of an inclusion body (Noguchi *et al.*, 1998).

The inclusion body has a merit of protein stability against proteases, but it is insoluble and biologically inactive. The reactivation of protein in the form of the inclusion body is generally difficult because it is very experimental and varies for each protein. Therefore, the dehalogenase may be transferred to the soluble fraction from the insoluble fraction. Incubation of the cells at lower temperature improved the dehalogenase specific activity in soluble fraction. Finally, when the growth and induction were carried out slowly at 22°C, the dehalogenase activity was up to 12.5 U/mg. An explanation for this could be that the speed of dehalogenase synthesis was slower and the folding of protein could proceed more completely (Ishii *et al.*, 1995).

The maximum expressions of pUCDEXs, pKKDEX and pTDEXa were compared in Table 3. The expression of pETDEXa at 22°C had reached the highest specific activity,

12.6 U/mg. The measured tendency of dehalogenase activity corresponded well for the transcription ability of each promoter. In pUCDEXs and pKKDEX, the cell growth was very good. Therefore, it was expected that various protein syntheses following the cell growth were proceeding at the same time, which causes the decrease of dehalogenase specific activity.

But in pETDEXa, the cell growth was slow and poor, and the many other proteins co-existing in cells could be inhibited slightly of their synthesis. These results demonstrate that the target protein of dehalogenase was expressed selectively in *E. coli* cells. This over-expression system using a pETDEXa will become very useful for enzyme purification and further studies. In addition, it has the advantage that the product, DL-2-haloacid dehalogenase can retain natural characteristics and the structure of a wild type enzyme, because expressed dehalogenase in this pETDEXa has no tag sequences, but specific affinity purification using tag sequences cannot be applied for its purification.

Conclusions

The following conclusions were obtained in this investigation.

1. The structural gene encoding DL-2-haloacid dehalogenase from 2,4-D degrading microorganism, *B. cepacia* strain KY, has been cloned, and cloned *E. coli* showed the specific activity, approximately 3.5 U/mg.
2. This dehalogenase was induced with monohalogenated acetate in *E. coli* significantly. Bromoacetate induced the dehalogenase successfully also in *B. cepacia* strain KY.
3. Overexpression systems of DL-2-haloacid dehalogenase with three different promoters, *lac*, *tac* and T7 *lac* promoter were constructed. When the dehalogenase was expressed in *E. coli* using pETDEXa at 37ºC, some parts of the dehalogenase were taken in the form of inclusion body, and as a result, the dehalogenase activity in the soluble fraction was decreased. But this phenomenon was improved to carry out growth and induction slowly at 22ºC, and the maximum activity was reached at 12.6 U/mg in the pETDEXa system.

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