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Genetic Organization of the *dhlA* Gene Encoding 1,2-Dichloroethane Dechlorinase from *Xanthobacter flavus* UE15

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***Xanthobacter flavus* strain UE15 was isolated in wastewater obtained from the Ulsan industrial complex, Korea. This strain functions as a 1,2-dichloroethane (1,2-DCA) degrader, via a mechanism of hydrolytic dechlorination, under aerobic conditions. The UE15 strain was also capable of dechlorinating other chloroaliphatics, such as 2-chloroacetic acid and 2-chloropropionic acid. The *dhlA* gene encoding 1,2-DCA dechlorinase was cloned from the genomic DNA of the UE15 strain, and its nucleotide sequence was determined to consist of 933 base pairs. The deduced amino acid sequence of the DhlA dechlorinase exhibited 100% homology with the corresponding enzyme from *X. autotrophicus* GJ10, but only 27 to 29% homology with the corresponding enzymes from *Rhodococcus rhodochrous*, *Pseudomonas pavonaceae*, and *Mycobacterium* sp. strain GP1, which all dechlorinate haloalkane compounds. The UE15 strain has an ORF1 (1,356 bp) downstream from the *dhlA* gene. The OFR1 shows 99% amino acid sequence homology with the transposase reported from *X. autotrophicus* GJ10. The transposase gene was not found in the vicinity of the *dhlA* in the GJ10 strain, but rather beside the *dhlB* gene coding for haloacid dechlorinase. The *dhlA* and *dhlB* genes were confirmed to be located at separate chromosomal loci in the *Xanthobacter flavus* UE15 strain as well as in *X. autotrophicus* GJ10. The *dhlA* and transposase genes of the UE15 strain were found to be parenthesized by a pair of insertion sequences, IS1247, which were also found on both sides of the transposase gene in the GJ10 strain. This unique structure of the *dhlA* gene organization in *X. flavus* strain UE15 suggested that the dechlorinase gene, *dhlA*, is transferred with the help of the transposase gene.**

Key words: *dhlA*, 1,2-dichloroethane, dehalogenase, *Xanthobacter flavus* UE15

Haloaliphatic hydrocarbons are currently in wide industrial usage, as solvents and ingredients in pesticides and herbicides. In particular, trichloroethylene (TCE) and perchloroethylene (PCE), and their catabolic intermediate, 1,2-dichloroethane (1,2-DCA), have been used in the organic synthesis of detergents and degreasing agents. However, these compounds can cause very harmful effects in animals and humans, due to their potential toxicity and carcinogenicity, when they contaminate the environment.

The halogenated aliphatic hydrocarbons can be degraded by a variety of microorganisms. Some of the microorganisms capable of degrading these chemicals have been isolated from the environment, and the genes and enzymes involved in their degradative pathways were intensively studied (Fetzner *et al.*, 1994; Janssen *et al.*, 2001). The most critical reaction for microbial degradation of the haloaliphatics is known to be dehalogenation. This reaction can be conducted by dehalogenases, which break the carbon-halogen covalent bonds of the haloaliphatics under aerobic or anaerobic conditions (Fetzner *et al.*, 1994).

1,2-DCA was reported to be degraded by *Xanthobacter autotrophicus* GJ10 via hydrolytic dehalogenation, as shown in Fig. 1. The dehalogenation of 1,2-DCA (A) is initiated by haloalkane dehalogenase (DhlA) and other enzymes, producing mono-chloro acetic acid (D). Monochloro acetic acid is further dehalogenated by haloacetate dehalogenase (DhlB) to produce glycolic acid (E). Two chlorine atoms can be released from 1,2-DCA via reactions catalyzed by two types of dehalogenases. These dehalogenases are encoded by *dhlA* and *dhlB* genes (Hill *et al.*, 1999; Janssen *et al.*, 1985; 1989). The hydrolytic dehalogenation of haloalkane compounds has also been reported in *Rhodococcus rhodochrous*, which degrades 1-chlorobutane via a dehalogenase encoded by the *dhlA* gene (Newmam *et al.*, 1999).

Some halogenated hydrocarbons were reportedly degraded via reductive dehalogenation under anaerobic conditions. *Dehalococcoides ethenogenes* transformed trichloroethene via reductive dehalogenase, mediated by

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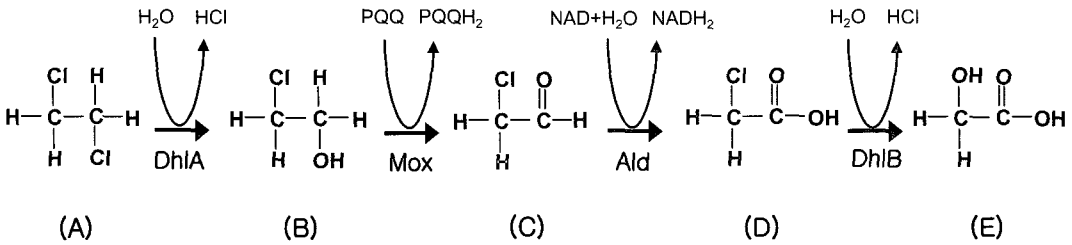

Fig. 1. Proposed catabolic pathway of 1,2-DCA. (A), 1,2-Dichloroethane (1,2-DCA); (B), 2-Chloroethanol; (C), 2-Chloroacetaldehyde; (D), Monochloroacetic acid (MCA); (E), Glycolic acid; DhlA, haloalkane dehalogenase; Mox, alcohol dehydrogenase; Ald, aldehyde dehydrogenase; DhlB, haloacetate dehalogenase.

the *tceA* gene (Magnuson *et al.*, 2000; Maymo-Gatoll *et al.*, 1999). Tetrachloroethene was reportedly dehalogenated via reductive dehalogenase, mediated by the *pceA* gene (Neumann *et al.*, 1998). The *pceA* gene was also reported in *Desulfitobacterium dehalogenans,* which has the ability to dehalogenate *ortho*-chlorophenol.

Xanthobacter flavus UE-15 was previously isolated as an aerobic dehalogenater of 1,2-DCA (Song *et al.*, 2003). This bacterial strain is able to degrade monohaloacids, such as 2-chloroacetic acid and 2-chloropropionic acid. In this study, the *dhlA* gene encoding for hydrolytic dechlorinase, a degrader of 1,2-DCA, was cloned from *X. flavus* UE-15, and its genetic organization was analyzed.

Materials and Methods

Cultivation of Bacterial Strains

Xanthobacter flavus UE15 is a bacterial isolate capable of degrading and utilizing 1,2-dichloroethane as a carbon source under aerobic conditions (Song *et al.*, 2003). *X. flavus* UE15 was cultivated in nutrient broth and MM2 minimal medium ($FeSO_4 \cdot 7H_2O$, 1 μM; $(NH_4)_2SO_4 \cdot 7H_2O$, 1 mM; $CaCl_2 \cdot 2H_2O$, 100 μM; NaCl, 8.5 mM; 10 mM phosphate buffer, pH 7.0) containing 0.5 mM 1,2-dichloroethane at 30°C, as described by Song (2004). *Escherichia coli* XL1-Blue was cultivated in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg/ml) at 37°C.

Dechlorination Assay

After 16 h cultivation under aerobic conditions, isolated strains were washed twice with a 10 mM potassium phosphate buffer and incubated at 30°C, for 24 h in a 50 mM potassium phosphate buffer, supplemented with 0.5 mM of each substrate, including 1,2-DCA. One ml of culture supernatant was treated with $Hg(SCN)_2$ (0.69 g/l, 0.5 ml) and incubated for 15 min at room temperature, and then mixed with 0.5 ml of 0.25 M ferric ammonium sulfate dissolved in 4.86 N HNO_3, as described by Bergman and Sanik (1957). The concentration of released chloride ions was measured with a spectrophotometer (LKB4046, Pharmacia, UK) at the wavelength of 453 nm, and the results were evaluated by comparison with blanks having no bacterial cells, as described by Chae and Kim (2000).

Primer Design and PCR

The *dhlA* and *dhlB* genes from *X. flavus* were amplified by PCR, using gene-specific primers. All primers were designed based on *Xanthobacter autotrophicus* GJ10, using the Primer 3 and Netprimer software. Two sets of primers were designed from the *dhlA* gene. The 1024F (ACT AAC GCT TCA GCA ATC TC) and 1906R (TGT CTC GGC AAA GTG TTT CA) primers were designed to amplify partial *dhlA*, and the 562F (GAT TGC CCA GTA CTC CCT GA) and 2171R (GCA AAT ACC GAC ATC AAG CA) primers were designed to amplify full *dhlA* and the *dhlA* flanking region. Partial *dhlB* genes were amplified with primers dhlBF (GAT CAA GAT CCC GAG ACA GC) and dhlBR (ATA GGT TTC TTC CCG CAT CC).

PCR mixtures (50 μl) contained 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM concentrations of each deoxynucleotide triphosphate, 50 pM of each primer, 2.5 U of *Taq* DNA polymerase (Posco Chem, Korea), and about 10 ng of genomic DNA, which was used as a PCR template. PCR amplification of the *dhlA* gene was performed using a Programmable Thermal Controller (MJ Research, USA), programmed as follows: 2 min of pre-denaturation at 95°C, followed by 30 cycles (95°C for 30 sec, 55°C for 45 sec, and 72°C for 90 sec), and a final extension at 72°C for 10 min.

Cloning and Southern Hybridization

Genomic DNA preparation from *X. flavus* UE15 was performed by the alkaline lysis method (Sambrook *et al.*, 1989). The amplified 3.7-kb PCR products from the genomic DNA were ligated with pGEM-T vector (Promega, USA) and were transformed into the *E. coli* XL1-blue. This *dhlA* transformant is hereafter referred to as pUE1516. PCR products which were amplified with 1024F and 1096R, and dhlBF and dhlBR designed from *X. autotrophicus* GJ10, were used as the *dhlA* and *dhlB* gene probes for Southern hybridization, respectively. Genomic DNA isolated from bacteria was digested with restriction enzymes and resolved by electrophoresis on 0.7% agarose gel. The gel was incubated in 0.25 mM HCl for 10~20 min and then treated with denaturation solution (1.5 M NaCl; 0.5 N NaOH) for 20 min, as described by

Sambrook *et al.* (1989). The gel was treated with neutralizing buffer (1 M Tris-HCl [pH 7.4]; 1.5 M NaCl) for 5 min. DNA fragments in the gel were transferred onto a Hybond-N^+ nylon membrane (Amersham, Little Chalfont, Buckinghamshire, UK) by using 1 M NaOH for 2 h (Koetsier *et al.*, 1993), and the membrane was backed at 80°C for 2 h. The DNA fragments in the membrane were hybridized with DNA probes, which were labeled with peroxidase by using the ECL system (Amersham, Little Chalfont, Buckinghamshire, UK), according to the manufacturer's instructions.

Sequencing and Analysis of the dhlA Gene

The plasmids for sequencing were isolated with the Wizard DNA purification system (Promega, USA). The DNA sequences obtained using 562F and 2171R were determined from the purified plasmid with T7 and SP6 PCR primers. The nucleotide sequence was determined by the dideoxy-chain termination method (Sanger *et al.*, 1977). Sequencing was performed using a ThermoSequenase kit (Part number: US78500, Amersham Life Science, USA) and LongRealR 4200 sequencer. The nucleotide sequences obtained were analyzed with DNASIS, PROSIS, and Clustal W software. All other bacterial DNA information was obtained from the NCBI database for the analysis of bacterial dehalogenases.

Nucleotide sequence accession numbers

The nucleotide sequences of the 16S rDNA and *dhlA* gene for *X. flavus* UE15 have been deposited in the GenBank database, under accession numbers AY561847 and AY561848, respectively.

Results and Discussion

Cloning of dhlA Gene

X. flavus UE15 was isolated in wastewater from the Ulsan industrial complex [20]. UE15 can degrade not only 1,2-dichloroethane (1,2-DCA), but also several haloacids, such as 2-chloroacetic acid and 2-chloropropionic acid, as seen in Table 1. The degradation activities of the strain indicate that the hydrolytic dehalogenases encoded for by *dhlA* and *dhlB* genes are involved in degradation. Similar

Table 1. Hydrolytic dehalogenation of several haloaliphatic compounds by *Xanthobacter flavus* UE15

Substrate	Hydrolytic dechlorination
1,2-dichloroethane (1,2-DCA)	+++
2-chloroacetic acid (2-CA)	+++
2-chloropropionic acid (2-CPA)	+++
2,3-dichloropropionic acid (2,3-DCPA)	–
2-bromoacetic acid (MBA)	+++
2-bomopropionic acid (2-BPA)	+++

+++, good; –, none

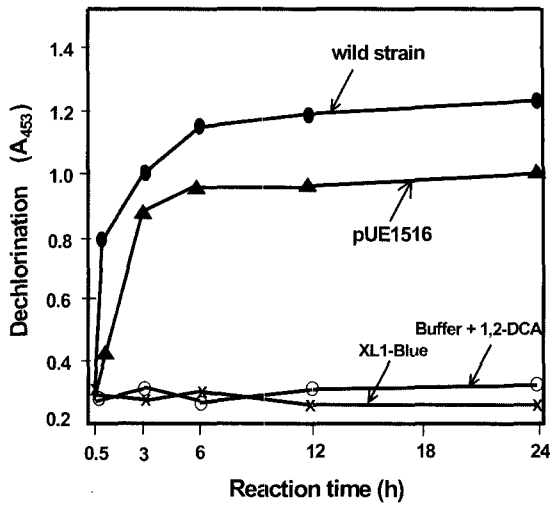

Fig. 2. 1,2-dichloroethane dechlorination activity exhibited by *Xanthobacter flavus* UE15, and transformant with pUE1516.

ability to degrade 1,2-DCA was also reported in *X. autotrophicus* GJ10 (Janssen *et al.*, 1985; 1989). The *dhlA* gene used in this study was cloned from the genomic DNA of *X. flavus* UE15 by the PCR method.

The PCR product was obtained using the 562F and 2171R primers, which were designed from the nucleotide sequences flanking the *dhlA* gene of *X. autotrophicus* GJ10 (Janssen *et al.*, 1989). The *dhlA*-carrying PCR products amplified from *X. autotrophicus* GJ10 and *X. flavus* UE15 were measured to be 1.6-kb and 3.7-kb in size, respectively.

The recombinant plasmid of pUE1516 was transformed into *E. coli* XL1-Blue, and then the transformant was examined for dechlorination activity against 1,2-DCA, as seen in Fig. 2. The transformant cells carrying pUE1516 showed dechlorination activity at levels as high as that of the wild strain of *X. flavus* UE15. This clearly indicates that the 3.7-kb PCR product obtained from *X. flavus* UE15 contains the *dhlA* gene, and that the gene conferred the ability to hydrolytically degrade 1,2-DCA, just as in the wild-type strain.

Sequence and Genetic Organization of dhlA

The 3.7 kb PCR product obtained from *X. flavus* UE15 was sequenced and analyzed, in order to ascertain the genetic organization of the *dhlA* gene. The *dhlA* gene of *X. flavus* UE15 consists of 993 nucleotides and 310 amino acids, as is shown in Fig. 3A. Its G+C content was 55.2%, the terminal codon was TAG, and the putative ribosomal binding site was GAGG (-5 to -8) inside the double promoters. The 504-bp region downstream of the *dhlA* gene, one 1356-bp open reading frame (ORF1) was found. The *dhlA* and ORF1 were parenthesized by pairs of insertion sequences (IS) which are located at the TTCA (-106 to -109) and TGAA (2888 to 2891) regions.

The DhlA protein of the UE15 strain showed 100%

homology with the corresponding haloalkane dehalogenases from *X. autotrophicus* GJ10, but only 27 to 29% homology with the corresponding haloalkane dehalogenases from *Mycobacterium* sp. GP1 (Poelarends *et al.*, 2000), *Pseudomonas pavonaceae* (Poelarends *et al.*, 2000) and *Rhodococcus rhodochrous* (Kulakova *et al.*, 1997), as is shown in Table 2. The ORF1 from strain UE15 consists of 1356 nucleotides and 451 amino acids. The GC composition of the ORF1 was 63.7%, the terminal codon was TGA, and the sequence of the putative ribosomal binding site was CGCG (2798~2801). The amino acid sequences of the ORF1 in *X. flavus* UE15

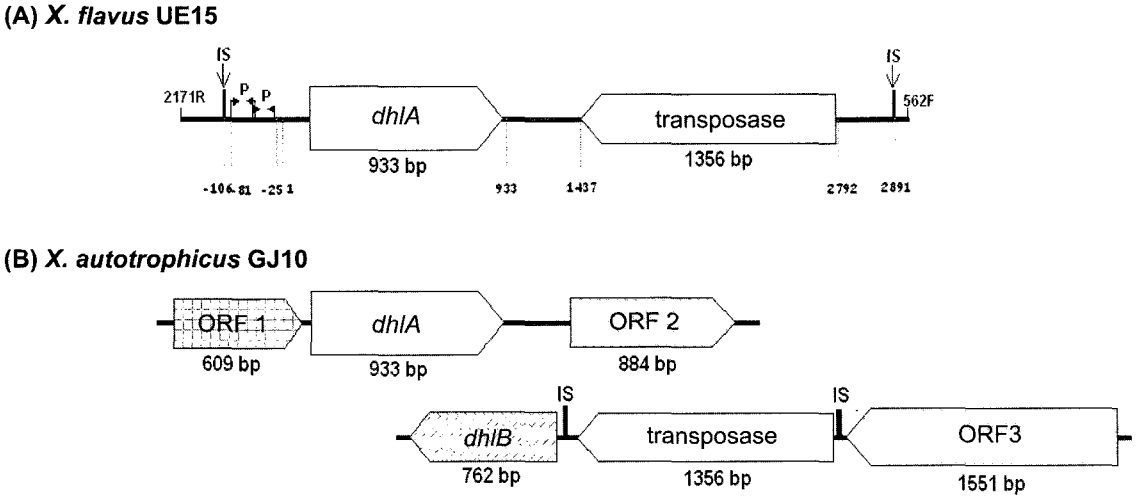

Fig. 3. Genetic organization of *dhlA* and transposase genes from *Xanthobacter flavus* UE15 (A) and *Xanthobacter autotrophicus* GJ10 (B). P, promoter; IS, target site for the insertion element IS1247; 562F and 2171R, primer target sites

Table 2. Similarity (%) of nucleotide and amino acid sequences of *dhlA* from *Xanthobacter flavus* UE15 with those of several bacteria

Strains/Gene	Genebank Accession No.	Similarity (%)	
		Nucleotide	Amino acid
Xanthobacter autotrophicus GJ10 / *dhlA*	M26950	100.0	100.0
Mycobacterium sp. GP1 / $dhaA_f$	AJ250372	49.4	28.6
Rhodococcus rhodochrous / *dhaA*	AF060871	48.2	27.0
Pseudomonas pavonaceae / *dhaA*	AJ250371	48.6	27.0

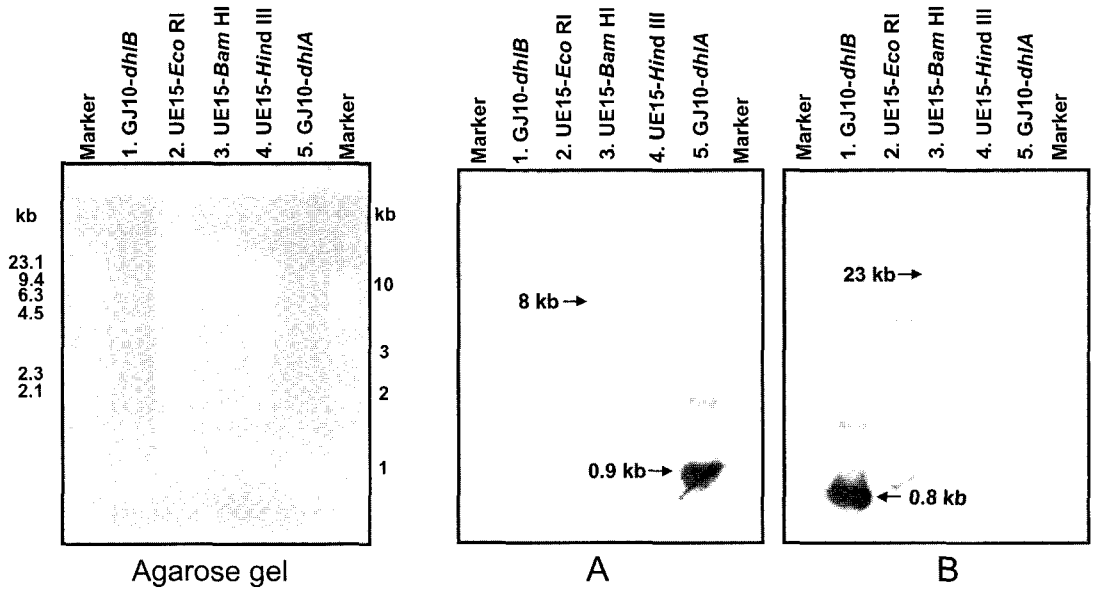

Fig. 4. Genomic DNA fragments of *X. flavus* UE15, hybridized with *dhlA* (A) and *dhlB* (B) as probes. The *dhlA* and *dhlB* probes were isolated from *X. autotrophicus* GJ10, by PCR amplification.

showed 99% similarity with the transposase gene reported to be located upstream of the *dhlB* gene in *X. autotrophicus* GJ10 (Janssen *et al.*, 1989). The sequences of the transposase gene and insertion target site are identical to the sequences of the insertion element, IS1247, which was reported in *Xanthobacter autotrophicus* GJ10 (Van der Ploeg *et al.*, 1995).

Location of dhlA and dhlB Genes

The genomic DNA of *X. flavus* UE15 was digested with several different restriction enzymes, and then electrophoresed onto an agarose gel. The DNA fragments of the gel were hybridized separately with *dhlA* and *dhlB* genes from *X. autotrophicus* GJ10, as reported previously by Song (2004). The 8 kb *Bam* HI fragment was hybridized with the *dhlA* gene probe, as seen in lane 3 of Fig. 4A. The 23 kb fragment was hybridized with the *dhlB* probe, as can be seen in lane 3 of Fig. 4B. These results indicate that the *dhlA* and *dhlB* genes are located at different sites on the genomic DNA of *X. flavus* UE15.

The *dhlA* gene coding for haloalkane dehalogenase, and *dhlB* coding for haloacid dehalogenase, were reported to be located at different chromosomal loci in *X. autotrophicus* GJ10 (Janssen *et al.*, 1989; Van der Ploeg *et al.*, 1991), as shown in Fig. 3B. The transposase gene which was located beside the *dhlB* gene in *X. autotrophicus* GJ10 was observed, strangely, in the flanking region of the *dhlA* gene in *X. flavus* UE15, as seen in Fig. 3A. The insertion element IS1247, including the transposase gene and the insertion target site, was reported in the *X. autotrophicus* GJ10 strain by Van der Ploeg *et al.* (1995). IS1247 was also reported to be identical to the insertion element IS2112 in *Rhodococcus rhodochrous* (Kulakov *et al.*, 1999).

This configuration of *dhlA* and transposase genes in *X. flavus* UE15 suggests that the hydrolytic dechlorinase gene, *dhlA*, has been transferred, with the help of the transposase gene.

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