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Cloning and expression of a haloacid dehalogenase from *Pseudomonas* sp. strain 19 S

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Abstract

A dehalogenase gene specifying the utilization of a variety of haloacids by *Pseudomonas* sp. Strain 19S has been cloned and expressed in *E. coli*. Our cloning strategy employed specific amplification of a fragment homologous to *Pseudomonas* dehalogenase gene by Polymerase Chain Reaction (PCR). The PCR amplicon successfully acted as a probe to detect the dehalogenase gene in the Southern Blot of the digested *Pseudomonas* total DNA. Corresponding fragments were cloned into pUC 18 vector and amplified in *E. coli* MV 1190. One clone with a substantial dehalogenation activity carried a recombinant plasmid containing a 5.5 kb insert.

Abbreviations: 2-CPA – 2-chloropropionate, MCA – monochloro acetate, IPTG – isopropyl-1-thio-β-D-galactoside, NBT – nitroblue tetrazolium salt, PCR – polymerase chain reaction, X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactoside, X-phosphate – 5-bromo-4-chloro-3-indolyl phosphate

Introduction

Various soil microorganisms are capable of metabolizing aliphatic and aromatic halogenated hydrocarbons which are highly toxic in the environment. Those enzymes which catalyze hydrolytic release of halogen ions from halogenated hydrocarbons are named 'dehalogenases'. Haloalkanoate dehalogenases from different soil-borne bacteria with considerable variation in their substrate and stereospecificities have been isolated and characterized (Motosugi et al. 1982; Weightman et al. 1982; Allison et al. 1983; Beeching et al. 1983; Kloges et al. 1983; Leigh et al. 1988; Tsang et al. 1988; Smith et al. 1990). Despite several reports on the enzymology of the dehalogenation of the haloalkanoic acids, the molecular genetics of these activities has been investigated only to a limited extent. Recently, Schneider et al. (1991) described the cloning and complete nucleotide sequences of two haloalkanoate dehalogenases from *Pseudomonas* sp. Strain CBS3. An operon encoding two stereospecific haloalkanoic acid dehalogeneses from an another *Pseudomonas* strain, *P. putida* AJ1, has been cloned and sequenced (Barth et al. 1992; Jones et al. 1992). Fine genetic characterizations and detailed structure-function analyses of the isofunctional dehalogenases would no doubt provide new insights concerning their origin and evolution, as well as the molecular mechanism of the dehalogenation reaction.

In a previous report, we described isolation and the preliminary characterization of a haloalkanoate dehalogenase from *Pseudomonas* sp. Strain 19S (Kocabiyik & Turkoglu 1989). This study presents molecular cloning and expression of the gene encoding this enzyme in *E. coli*.

Materials and methods

Bacterial strains and plasmids

High copy number plasmids pUC18 and Bluescript SK+ were used as vectors in cloning. *E. coli* MV 1190 [*Δ(lac – pro AB) thi, supE, Δ(serl-recA) 306:: Tn10*

Fig 1. PCR amplification primers were designed from homologous amino acid sequences of *deh* C I, *deh* C II and *Had* L (Schneider et al. 1991; Johns et al. 1992). Mixed primer A was based on the codons for amino acids 45 to 49, and mixed primer B was based on the codons for amino acids 176–179.

WRQRQ	L	E	Y	S	W	TRTLMG (40–55)
	leu	glu	tyr	ser	trp	
Primer A 5′	CTX	GAA	TAT	ACX	TGG	3′
	T	G	C	TG		
	ser	asn	ala	trp		
Primer B 3′	AGX	TTA	CGT	ACC	CT	5′
	TC	G	A			
			C			
			G			
FVSSN	S	N	A	W	D	..GA..FGF (173–189)
X: Inosine						

(tet^r): traD36:proAB, lacIq Z M15] and *E. coli* JM 101 [*(lac-proAB) lacI^q ZM15*] were the recipient strains in the transformation and expression studies. Cloned *deh CI* and *deh CII* genes were kindly supplied by Prof. Dr. R. Müller. *Pseudomonas* sp. 19S was isolated from soil as described elsewhere (Kocabiyik & Turkoglu 1989).

Media and growth conditions

E. coli clones were grown at 37° C in LB broth or on LB agar plates supplemented with ampicillin (100 μg/ml). For protein expression, *E. coli* (at 37° C) and *Pseudomonas* sp. (at 30° C) were grown in the Minimal Medium (MM) (Slater et al. 1979) plus pyruvate (1%) and 10 mM 2-dichloropropionate (2-CPA), or 30 mM monochloroacetate (MCA).

Measurement of dehalogenase activity

Crude cell lysates were prepared by sonication (Kocabiyik & Turkoglu 1989). Dehalogenase activity was measured with pure MCA by determining the free chloride release spectrophotometrically (Iwasaki et al. 1959).

DNA isolation and manipulation techniques

Chromosomal DNA was isolated from cultures of *Pseudomonas* sp. 19S in LB, by a modified alkaline lysis method (Sambrook et al. 1989). After lysis of the cells, 5 M freshly prepared $NaClO_4$ was added to obtain a final concentration of 1 M. Then, 1 volume Chloroform: Isoamylalcohol (24:1, v/v) added and the solution was incubated in ice, by swirling, for 30 min. The mixture was centrifuged at 12,000 rpm for 10 min, at 4° C. Then, the aqueous phase was transferred to a beaker, and overlaid by 2 volume cold ethanol. DNA in the interphase was collected by using a thin glass stick, dissolved in 1.5 ml of TE buffer, and purified by Cesium Chloride-Ethidium Bromide gradient centrifugation (Sambrook et al. 1989). Isolation of plasmid DNA for restriction analysis and cloning was accomplished by use of Circle Prep Kit (BIO 101, Inc., La Jolla, CA). Digestion of DNA with restriction endonucleases and electrophoresis of agarose gels were performed according to the standard protocols (Sambrook et al. 1989). Linear DNA fragments were recovered from gel by using a GeneClean Kit as described by the manufacturer (BIO 101, Inc., La Jolla, CA).

Primers for polymerase chain reaction (PCR)

Primers for the PCR were designed from the published amino acid sequences of *deh CI deh CII* and *Had L* (Schneider et al. 1991; Jones et al. 1992). Comparison of their deduced amino acid sequences showed three regions containing highly conserved motifs. Among these regions extending from 40 to 55, and 173 to 189 were considered for the preparation of a 15 mer forward (Primer A) and a 14 mer reverse (Primer B) mixed primers, respectively (Fig. 1). These oligonucleotides were synthesized by National Biosciences, Hamel, MN.

PCR amplification

PCR reaction mixture, in a final volume of 100 μl contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM MgCl, 0.2 mM each deoxynucleoside triphosphates (dNTP), 1 μM each primers (A and B) and 500 ng template DNA. The total DNA from *Pseudomonas* sp. 19S was the template in PCR. The mixtures were overlaid with mineral oil, placed in thermal cycler (Perkin Elmer Cetus, Norwalk, CT) and held at 94° C for 5 min before addition of 0.5 μl aliquote containing 2U of AmpliTaq DNA polymerase (Perkin Elmer Cetus). The reaction mixture was then subjected to 30 cycles of: denaturation at 94° C for 1 min, primer annealing at 50° C for 1 min, and primer extension at 72° C for 1 min. An additional 5 min was allowed for the completion of primer extension after the last cycle. Amplicons were analyzed by electrophoresing 3 μl of PCR product besides a sample of 123 bp ladder DNA molecular size marker (no. 5613 SA/SB GIBCO-BRL/Life Technologies, Gaithersburg, MD) in each gel for size determination.

PCR amplification yielded a unique 1.4 kb fragment. Both ends of the fragment were blunt ended with T4 DNA polymerase (United States Biochemical, Cleveland, OH) and ligated into Eco V site of Bluescript SK+ vector. Recombinant plasmids thus constructed, were transferred to (Chung et al. 1989) and amplified in *E. coli* JM101.

Southern blot hybridization

DNA from gels was transferred to Zeta Probe membranes (BioRad Laboratories, Richmond, CA) with 10 × SSC buffer as the transfer solution, as described in the manufacturer's protocol.

For hybridization, nucleic acid molecules were non-radioactively labelled by incorporation of biotinylated dUTP by nick-translation method (Bio Nick Labelling System, GIBCO BRL). Hybridization and washings were carried out as described in the manufacturer's protocol (Zeta Probe, Blotting Membranes Instruction Menual., BioRad). Biotin labelled probes were detected, after hybridization to target nucleic acids, by enzyme-linked immunoassay using streptavidin-alkaline phosphatase conjugate. A subsequent enzyme-catalyzed color reaction with X-phosphate and NBT was used to visualize hybrid molecules.

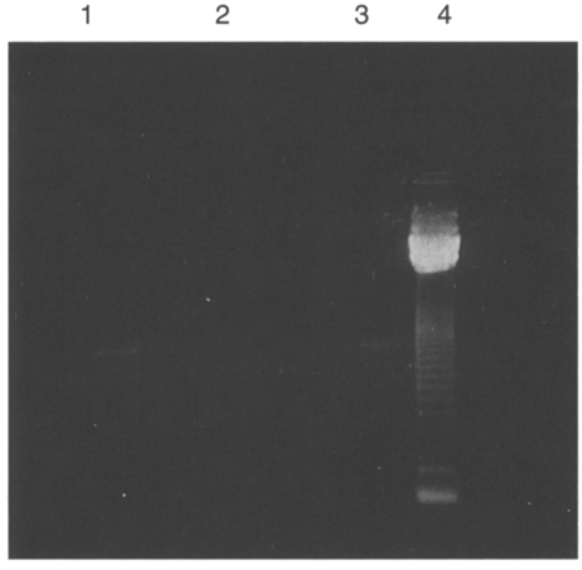

Fig. 2. Analysis of PCR products by gel electrophoresis in 1% agarose gel. The amplification reaction was carried out as described in the text. Template DNA was used at 500 ng (Lane 1), 100 ng (Lane 2) and 300 ng (Lane 3) in 100 μl of reaction mixture. Lane 4 is the 123-bp ladder marker.

Cloning and expression of dehalogenase gene in E. coli

Chromosomal DNA from *Pseudomonas* sp. Strain 19S was digested with BamH I and in the presence of Hind III cut DNA (United States Biochemical, Cleveland, OH) as size marker, was electrophoresed on the 0.65% agarose gel. Using the biotin labelled 1.4 kb PCR fragment as probe, a single unique band (Fig. 4a) at a position of about 5.5 kb was detected on the lane containing BamH I digested chromosomal DNA. Then, 5–6 kb BamH I fragments of the *Pseudomonas* total DNA were isolated from the gel using GeneClean Kit and inserted into pUC18 at BamH I site. Aliquotes of the ligation reactions were used to transform competent *E. coli* MV1190 cells using a one-step transformation procedure (Chung et al. 1989). Recombinant colonies were selected on the X-Gal, IPTG and ampicillin containing LB agar medium. Positive colonies were then, transferred into wells of a microtiter dish containing MM supplemented with 2-CPA and pyruvate. After incubation at 37° C for 5 days, chlorine release was detected according to the white precipitate formation upon $AgNO_3$ addition. Putative recombi-

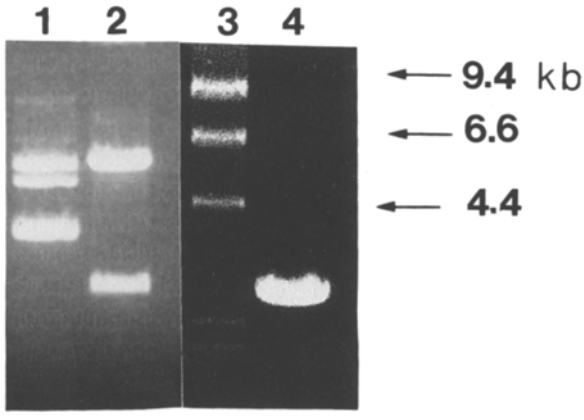

Fig. 3. Cloning of *Pseudomonas* 19S dehalogenase into pUC 18 vector. pUC 18.5SB recombinant plasmid carrying a 5.5 kb insert has contained the dehalogenase gene. Cloning strategy employed was as described in the Materials and methods. Lane 1: uncut pUC 18.5SB; Lane 2: Bam HI digested pUC 18.5SB; Lane 3: Hind III digested λ DNA as size marker; Lane 4: linearized pUC 18.

nant clones were tested for dehalogenase activity in their crude lysates. One clone dechlorinating 2-CPA and MCA was identified which carried a recombinant pUC18 with a 5.5 kb insert.

Results

PCR amplification

Primers based on the conserved amino acid sequences of three haloalkanoic acid dehalogenases (*deh* CI, deh CII and *Had L*) were used for amplification of homologous sequences from total chromosomal DNA of *Pseudomonas* sp. 19S. Figure 2 shows that this amplification produced a single unique band which migrated at an apparent molecular size of 1.4 kb without any contamination. Therefore, under the conditions described in the Materials and methods PCR amplification was specific.

Hybridization

Biotin labelled PCR amplicon and *deh* CI and *deh* CII genes themselves were used as probes in a Southern Blot analysis of BamH I digested total DNA isolated from *Pseudomonas* sp. 19S. Hybridizations with each probe were carried out separately at different temperatures ranging from 25° C to 68° C. BamH I fragments, about 5.5 kb in size gave hybridization signals with 1.4 kb PCR probe. Neither *deh* CI nor *deh* CII probes hybridized with *Pseudomonas* DNA even under the least stringent hybridization and washing conditions.

Cloning of Pseudomonas sp. 19S dehalogenase gene

BamH I fragments of 5–6 kb in size, from *Pseudomonas* total DNA were ligated to pUC 18 at BamH I site and cloned in *E. coli* MV1190. About 200 Amp^r white colonies (putative recombinants) were collected from selective LB agar medium (containing X-gal, IPTG and ampicillin). Growth of these clones tested on MM containing MCA or 2-CPA according to CL^- release. Selected 10 positive clones were further analyzed for dehalogenase activity in their cell free extracts. One clone (designated *E. coli* pUC 18.5SB) dechlorinating 2-CPA and MCA was identified which carried a recombinant pUC 18 with a 5.5 kb insert (Fig. 3). Cell extracts prepared from *E. coli* pUC18.5SB dehalogenated MCA at a rate comparable to that of the cell extracts from induced *Pseudomonas* sp. 19S. Similar dehalogenase expression was observed when the recombinant plasmid was subcloned in *E. coli* JM 101 (data not shown). In *E. coli* pUC18.5SB expression of the dehalogenase was constitutive and no longer induced by MCA. Significant dehalogenase activity was detected in the crude extract when this clone was grown with or without MCA.

Hybridization with a labelled 1.4 kb PCR amplicon showed that 5.5 kb insert isolated from pUC 18.5 SB was homologous (Fig. 4b).

Discussion

We have cloned and expressed an aliphatic dehalogenase from *Pseudomonas* sp.19S in *E. coli*. Our cloning strategy made use of specific amplification of a part of *Pseudomonas* dehalogenase gene by PCR, for which the primers were designed considering the highly retained amino acid motifs of *deh* CI, *deh* CII and *Had L*. The PCR amplicon successfully acted as probe to detect the dehalogenase gene in the Southern Blot of the digested total DNA of *Pseudomonas* 19S. Failure of the cloned deh CI and deh CII genes as probes to detect the dehalogenase in the *Pseudomonas* total DNA is of particular interest. This might imply a high degree of homology on the amino acid level rather than

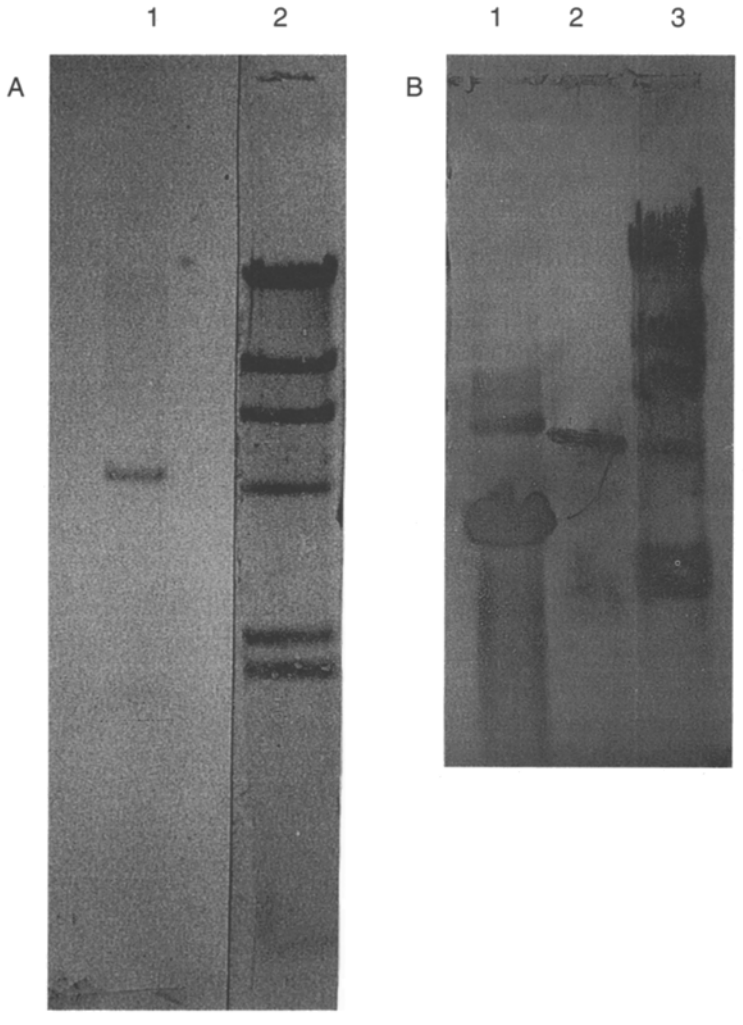

Fig. 4. DNA hybridization. The DNA hybridization was done with biotin labelled 1.4 kb PCR amplicon. A. Total DNA (Lane 1) from *Pseudomonas* sp. 19S was isolated as described in the Materials and methods, digested with BamH I and blotted onto nylon membrane together with Hind III digested λ DNA (Lane 2). B. lane 1: uncut pUC 18.5SB; lane 2: 5.5 kb fragment isolated from pUC 18.5SB; lane 3: Hind III cut λ DNA.

DNA level in dehalogenases, which is consistent with a 38% identity of the deduced amino acids of *deh* CI, *deh* CII and *Had L.* A haloacid dehalogenase from *Xanthobacter autotrophicus* GJ 10 also, has a high degree of homology to above proteins (Van der Ploeg et al. 1991). Functional and structural significance of these conservations are of interest and being investigated by site-specific mutagenesis (Schneider et al. 1993) and X-ray crystallography (Verschueren et al. 1993).

On the other hand, constitutive production of the cloned dehalogenase in *E. coli* pUC 18.5SB might be due to absence of regulatory sequences that control the induction. Structure analysis and the nucleic acid

sequencing of the cloned gene are still in progress in our laboratory.

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