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Cloning and nucleotide sequence of a D,L-haloalkanoic acid dehalogenase encoding gene from *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV

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Abstract

We have cloned DNA fragments of plasmid pFL40 from *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV encoding a D,L-2-haloalkanoic acid halidohydrolase (DhlIV). A 6.5-kb *Eco*RI/*Sal*I-fragment with inducible expression of the halidohydrolase was cloned in *Pseudomonas fluorescens* and *Escherichia coli*. A 1.9-kb *Hin*dII-fragment demonstrated expression of the dehalogenase only due to the presence of the promoter from the pUC vector in *Escherichia coli*. The nucleotide sequence of this DNA-fragment was determined. It had an open reading frame coding for 296 amino acid residues (molecular weight of 32783 D). The *dhlIV* gene showed sequence homology to a short segment of a D-specific dehalogenase (*hadD*) from *Pseudomonas putida* AJ1, but not to any other known DNA sequences. Restriction enzyme patterns indicated similarity between *dhlIV* and the D,L- isomer specific *dehI* dehalogenase gene from *Pseudomonas putida* PP3. There are some indications from restriction enzyme patterns and initial sequencing data, that a gene encoding a σ^{54}-dependent activator protein, similar to the $dehR_I$ regulatory gene from *Pseudomonas putida* PP3 is located upstream of *dhlIV*. In contrast to DehI, dehalogenation of D- or L-chloropropionic acid by the DhlIV-protein leads to lactic acid of inverted configuration.

Introduction

Since (Jensen 1957) first successfully isolated haloalkanoic acid degrading bacteria, a large number of various bacteria with different dehalogenases were characterized, reviewed by (Fetzner & Lingens 1994), (Hardman 1991), (Janssen et al. 1994), (Slater 1994) and (Slater et al. 1995). One group of dehalogenases is called halidohydrolases, which hydrolytically replace the halogen atom from haloalkanes or haloalkanoic acids by a hydroxyl group. A subgroup of these enzymes acts on C2-halogenated short chain aliphatic acids. Differences in substrate specifity, stereospecificity and sensitivity to sulfhydryl-blocking reagents led to the postulation of distinct reaction mechanisms by Little & Williams (1971) and Weightman et al. (1982). Based on these data, the enzymes were divided into haloacetate- and haloalkanoic acid- halidohydrolases and subclassified into two, respectively five mechanistic groups (11, 8).

Studies on the origin, evolutionary relationship and dissemination of these enzymes and their genetic basis revealed that halidohydrolase genes frequently are encoded by mobile genetic elements, such as plasmids or transposon like elements (Hardman et al. 1986; Kawasaki et al. 1981 & 1994; Thomas et al. 1992), possibly directing their dissemination via natural horizontal gene transfer, as demonstrated for the dissemination of the *dhlIV* halidohydrolase gene carrying plasmid pFL40 from *A. xylosoxidans* ssp. *denitrificans* strain ABIV to indigenous soil bacteria in soil microcosms (Brokamp & Schmidt 1991).

This strain was isolated from garden soil by repeated batch culture in the presence of dichloroacetic acid. *A. xylosoxidans* ABIV utilized different 2-haloalkanoic acids, such as mono- and dichloroacetic acid and mono- and dichloropropionic acid as sole carbon

source by the action of its inducible D-, L-isomers specific hydrolytic dehalogenase (Brokamp & Schmidt 1991).

Up to now, sequences from nine L-isomer-specific halidohydrolase genes (Cairns et al. 1996; Jones et al. 1992; Kawasaki et al. 1992 & 1994; Murdiyatmo et al. 1992; Nardi-Dei et al. 1994; Schneider et al. 1991; van der Ploeg et al. 1991), two D-isomer-specific- (Barth et al. 1992; Cairns et al. 1996) and one halidohydrolase gene with activity toward monofluoroacetic acid (MFA) (Kawasaki et al. 1992) have been reported. Based on homologies of DNA- and protein-sequences or similarities of dehalogenation mechanisms, the actual classification of dehalogenases has been worked out by Janssen et al. (1994) and Slater et al. (1995) differing in part from former classifications, based on substrate ranges and inhibitor tests. However, in all classification schemes one group of halidohydrolases is representing enzymes acting on both isomers of 2-CPA, either by inversion (class 2I) or by retention (class 2R) of configuration at the chiral C2 atom (Slater et al. 1995).

In this paper we present some biochemical and genetic properties of the DhlIV dehalogenase and to our knowledge the first DNA-sequence of a D,L-halidohydrolase gene.

Materials and methods

Chemicals

The halogenated compounds were obtained from Fluka, Neu-Ulm, with exception of D- and L-chloropropionic acid, purchased from Sigma, Deisenhofen.

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

Growth conditions

Nutrient I (NI) medium (Merck, Darmstadt) or Isosensitest medium (Oxoid, Wesel) were used as rich media. 2-haloalkanoic acid degrading bacteria were detected on indicator medium agar (IM) (Strotmann & Röschenthaler 1987). Cells were grown on minimal medium (MM) (Janssen et al. 1984) supplemented with 20 mM halogenated compound, prior to tests for dehalogenase expression. To achieve growth of recombinant *E. coli* strains, 0.1 x NI was added to IM or MM. AIX medium, composed of Isosensitest agar, ampicillin, 0.2 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) (40 mg l^{-1}) was used to select *E. coli* cells with recombinant pUC18- or pUC19-plasmids. Antibiotics were generally used at the following concentrations: ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), rifampicin (100 μg ml^{-1}).

Dehalogenase activities and protein analysis

Halide ion release from halogenated substrates was followed by a colorimetric method of Bergmann & Sanik (1957). Cell-free extracts were prepared by ultrasonic treatment (Janssen et al. 1984). The standard dehalogenase assay (1 ml) contained 10 μmol of halogenated substrate in a 50 mM Tris-sulphate buffer (pH 9.0) and 25 - 100 μl crude extract. The mixture was incubated at 30°C for 30 min and at different times 50 μl samples were assayed for halide liberation. One unit of enzyme activity was defined as the amount of enzyme that dehalogenated 1 μmol MCA min^{-1}.

Activity-stain polyacrylamide gel electrophoresis (PAGE) (Weightman & Slater 1980), SDS-PAGE (Laemmli 1970) and rapid purification of the halidohydrolase (Brokamp & Schmidt 1991), were carried out as described previously.

Estimation of effect of sulfhydryl-blocking reagents

Freshly prepared crude cell lysates without the addition of DTT were used and the effect of either 10μM $HgCl_2$ or p-chloro-mercuribenzoic acid (pCMB) was tested in the standard dehalogenase assay.

Induction experiments

Cells were grown twice on NI medium, harvested by centrifugation and resuspended to an optical density (OD_{546}) of 1.0 in resting cell medium (Strotmann et al. 1990), facilitating comparison of the in vivo dehalogenation rate of various chlorinated substrates from different cultures by measuring the liberation of chloride. The medium was supplemented with 20 mM halogenated aliphatic acids and liberation of chloride was estimated with or without the addition of 100 μg ml^{-1} chloramphenicol.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Alcaligenes xylosoxidans ssp. denitrificans ABIV	wild type, DhlIV$^+$, pFL40	(Brokamp & Schmidt 1991)
Pseudomonas fluorescens R2f	DhlIV$^-$, Rif^r	(van Elsas et al. 1988)
Escherichia coli RRIΔM15	*leu, pro, thi, strA, hsdr, hsdm*, pro$^+$, lacZΔM15/F' lacI^{Q}ZΔM15	(Rüther 1982)
Plasmids		
pFL40	60 kb, Dhl IV$^+$	(Brokamp & Schmidt 1991)
pME285	Km^r and Hg^r	(Itoh & Haas 1985)
pUC18/19	Ap^r, *lacZ'*	(Yanisch-Perron et al. 1985)
pFL410	pME285 with a 10.5 kb *Eco*RI fragment of pFL40, DhlIV$^+$ and Km^r	this study
pFL420	pME285 with a 9.5 kb *Sal*I fragment of pFL40, DhlIV$^+$ and Km^r	this study
pFL417	pME285 with a 6.5 kb *Eco*RI-*Sal*I fragment of pFL40, DhlIV$^+$ and Km^r	(Brokamp & Schmidt 1991)
pFL45	pUC18 with a 6.5 kb *Eco*RI-*Sal*I fragment of pFL40, DhlIV$^+$, Ap^r	this study
pFL451	4.2 kb *Hin*dIII-*Eco*RI fragment of pFL45 in pUC18, DhlIV$^-$, Ap^r	this study
pFL453	5.7 kb *Hin*dIII-*Eco*RI fragment of pFL45 in pUC18, DhlIV$^-$, Ap^r	this study
pFL458	3.9 kb *Xba*I fragment of pFL45 in pUC18, DhlIV$^-$, Ap^r	this study
pFL48	1.9 kb *Hin*dII fragment of pFL45 in pUC18, DhlIV$^+$, Ap^r	this study
pFL49	1.9 kb *Hin*dII fragment of pFL45 in pUC19, DhlIV$^-$, Ap^r	this study
pFL481	1.55 kb *Hin*dII-*Sfu*I fragment of pFL48 in pUC18, DhlIV$^+$, Ap^r	this study
pFL489	1.3 kb*Sfu*I-*Ssp*I fragment of pFL48 in pUC18, DhlIV$^+$, Ap^r	this study
pFL482-18	1.1 kb *Dra*I-*Ssp*I fragment of pFL48 in pUC18, DhlIV$^+$, Ap^r	this study
pFL482-19	1.1 kb *Dra*I-*Ssp*I fragment of pFL48 in pUC19, DhlIV$^-$, Ap^r	this study
pFL483	0.55 kb *Bst*E2 fragment of pFL48 in pUC18, DhlIV$^-$, Ap^r	this study

Optical configuration of lactate product

The formation of either D- or L- lactate from D- or L-2-chloropropionic acid (CPA), was monitored by NADH production by D- or L-lactate dehydrogenase (Boehringer, Mannheim) respectively, as described by Noll (1984). A dehalogenase assay (1.5 ml) containing 200 μl cell-free extract was started by addition of either 10 μmol D- or L-CPA. The mixture was incubated for 20 to 30 min at 37 °C. The reaction was stopped by boiling the mixture for 1 min. To 600 μl of this assay 600 μl H_2O, 60 μl 1M glycine (pH 9.0) and 40 μl freshly prepared 50mM NAD were added, mixed and transferred into 2 cuvettes. Then 7.5 U D- or L-lactate-dehydrogenase (Boehringer, Mannheim) was pipetted to one cuvette and the absorbance was measured over a period of 5 min at 340 nm against the reference cuvette in a Uvicon 930 spectrophotometer (Kontron, Eching).

DNA manipulations

DNA manipulations were performed with *P. fluorescens* R2f with plasmid pME285 and with *E. coli* RRI with the pUC18 or pUC19 vector system.

Plasmid DNA was isolated by the methods of Panayotatos (1987), Kado & Liu (1981) or by CsCl - ethidium bromide equilibrium centrifugation (Clewell & Helinski 1969). Transformation of plasmid-DNA was performed by electroporation in a Gene Pulser (BioRad, München), using *E. coli* cells as hosts according to Dower et al. (1988) and with *P. fluorescens* cells by the method described by Fiedler & Wirth (1988).

Agarose gel electrophoresis, restriction analysis and molecular cloning were carried out following standard protocols (Sambrook et al. 1989). Restriction enzymes and other DNA-modifying enzymes were obtained from Boehringer, Mannheim.

Conjugation of plasmids

The estimation of conjugative transfer of plasmid carrying strains was performed on nylon filters (Schleicher & Schüll, Dassel) according to Simon et al. (1983).

Curing of plasmids

Cells were grown overnight in NI medium supplemented with 100 μg l^{-1} ethidium bromide and then striked

onto NI-agar plates. Colonies were picked and transferred parallely to a NI-agar plate and to a IM-agar plate. Colonies not able to dehalo- genate were tested for plasmid-loss.

DNA hybridization

Colony hybridization was performed on Hybond-N-membranes (Amersham, Braunschweig) as described (Sambrook et al. 1989). Southern blots were carried out with a VacuGene Blotting system (Pharmacia, Freiburg). Labeling and detection procedures were performed with the Digoxigenin Labeling and Detection Kit (Boehringer, Mannheim).

DNA sequence analysis

DNA sequencing was carried out with the T7-sequencing-kit (Pharmacia, Freiburg) with overlapping double-stranded plasmids by the didesoxynucleotide sequencing method (Sanger et al. 1977), using [α-^{35}]dATP (Amersham, Braunschweig) and pUC/M13 universal and reversed primers (Boehringer, Mannheim).

Additional sequencing was performed with the DIG Taq DNA sequencing kit (Boehringer, Mannheim) with the direct blotting electrophoresis method on a GATC1500 DNA sequencer (GATC, Konstanz).

Computer analysis of the DNA sequence data was performed using the DNASIS/PROSIS programs (Hitachi, Japan). The nucleotide- and deduced amino acid sequence of the halidohydrolase gene was compared with those of other nucleotide sequences of the EMBL data base and those of other proteins in the Swiss-Prot protein sequence data base by using the Husar program package (EMBL, Heidelberg).

N-terminal amino acid sequence

Proteins from a crude extract were separated by SDS-PAGE and then transferred to a PVDF- membrane (Millipore, Eschborn) by electroblotting. The putative dehalogenase carrying band was cut off and the N-terminal sequence was determined by the Edman degradation method on a model 470A sequencer with online PTH identification on a model 120A PTH analyser (Applied Biosystems, Weiterstadt).

Nucleotide sequence accession number

The nucleotide sequence data in this paper has been submitted to EMBL under accession number X77610.

Results

Biochemical characterization of DhlIV-halidohydrolase

The strain *A. xylosoxidans* ssp. *denitrificans* ABIV and basic biochemical features of its single inducible halidohydrolase DhlIV (formerly named DhlC) have been described (Brokamp & Schmidt 1991). The enzyme acts on both isomers of CPA with approximately the same rate of dehalogenation and is completely inhibited by thiol reagents (Table 2). Using the coupled NADH-assay for determination of the dehalogenation products from L- and D-CPA respectively, D- and L-lactic acid of opposite C2-configuration were obtained (Table 3). Thus the enzyme can be identified as a D,L-halidohydrolase, which inverted substrate-product configuration. A comparison to other characterized D,L- halidohydrolases of class 2I and class 2R is shown in Table 2.

Cloning and expression of the structural dehalogenase gene encoded by plasmid pFL40

The *dhlIV* gene is located on the 60 kb sized self transmissible plasmid pFL40. It is able to replicate and express the dehalogenase in *Pseudomonas* sp. and related species, but not in *E. coli*. In crude extracts prepared from DCPA or CPA grown cultures dehalogenase specific activities of 3 U mg^{-1} were obtained. No dehalogenase activity was detectable in crude extracts cultivated in rich medium without addition of haloalkanoic acids. Curing of pFL40 from these strains resulted in loss of dehalogenase activity. We could not detect further interesting features like degradation of aromatic compounds (toluene, 3-chlorobenzoic acid or 2,4-dichlorophenoxyacetic acid) or antibiotic resistance genes on plasmid pFL40. Comparing size and some restriction enzyme patterns of pFL40, no homology to other degradative plasmids, such as the catabolic plasmids from soil bacteria characterized by Hardman et al. (1986) or to plasmid pUO1, encoding two halidohydrolases (Kawasaki et al. 1981), could be detected.

Shotgun cloning with fragments of pFL40 into the pME285 vector yielded DNA inserts with a 10.5 kb

Table 2. Comparison between D,L-halidohydrolases (class 2I and 2R)[a]

Enzyme from (name)	Class[a]	Location	Molecular-weight [kD]	Inhibition by Thiolreagents [%]			Relative Rates of Hydrolysis [%]							Reference
				pCMB p/CMBS	NEM	$HgCl_2$	MCA	MFA	MBA	MJA	DCA	CPA	DCPA	
P. putida PP3 (DehII)	2I	chromo-some	50	0 (10μM)	50 (1mM)	ND	100	ND	ND	ND	150	25 9 (L) 12 (D)	12	(Weightman et al. 1979, 1982; Slater 1994)
P. sp. 113 (D/L-Hal)	2I	ND	35	0 (1 mM)	0 (1 mM)	94 (1 mM)	100	0	848	291	12	208 (L) 150 (D)	42	(Motosugi et al. 1982 a, b)
Rhizobium sp. (Dehal II)	2I	ND	110	2 (10μM)	97 (1mM)	ND	100	ND	866	215	4.5	58 60 (L) 56 (D)	15	(Allison et al. 1983; Leigh et al. 1988)
*P. putida*YL (DL-DEX YL)	2I	ND	ND	2 (10 μM)	11 (1 mM)	ND	100	ND	ND	ND	4.9	97 61 (L) 92 (D)	7.8	(Hasan et al. 1994)
Agrobacterium tumefaciens - RS4 (Dhl-RS4)	2I	chromo-some	35	0 (0.1mM)	ND	0 (0.1mM)	100	0	96	52	32	87 (L) 140 (D)	65	(Schwarze et al. 1996)
A. xylosoxidans ABIV (DhlIV)	2I	pFL40	32.8	100 (10μM)	ND	100 (10μM)	100	0	126	61	14	58 62 (L) 58 (D)	24	(Brokamp & Schmidt 1991)
P. putida PP3 (DehI)	2R	trans-poson	46	100 (10μM)	100 (1mM)	ND	100	ND	ND	ND	17	65 37 (L) 46 (D)	31	(Weightman et al. 1979, 1982; Slater 1994)

a: according to Slater et al. (1995): Class 2I: D-, L-isomers as substrates, inversion of configuration; Type 2R: D-, L- isomers as substrates, retention of configuration.
ND: Not determined, pCMB: p-chloromercuribenzoic acid, pCMBS: p-chloromercuribenzenesulfonic acid, NEM: N-ethylmaleimide, MCA: monochloroacetic acid, MFA: monofluoroacetic acid, MBA: monobromoacetic acid, MJA: monoiodoacetic acid, DCA: dichloroacetic acid, CPA: 2-chloropropionic acid, DCPA: 2,2-dichloropropionic acid.

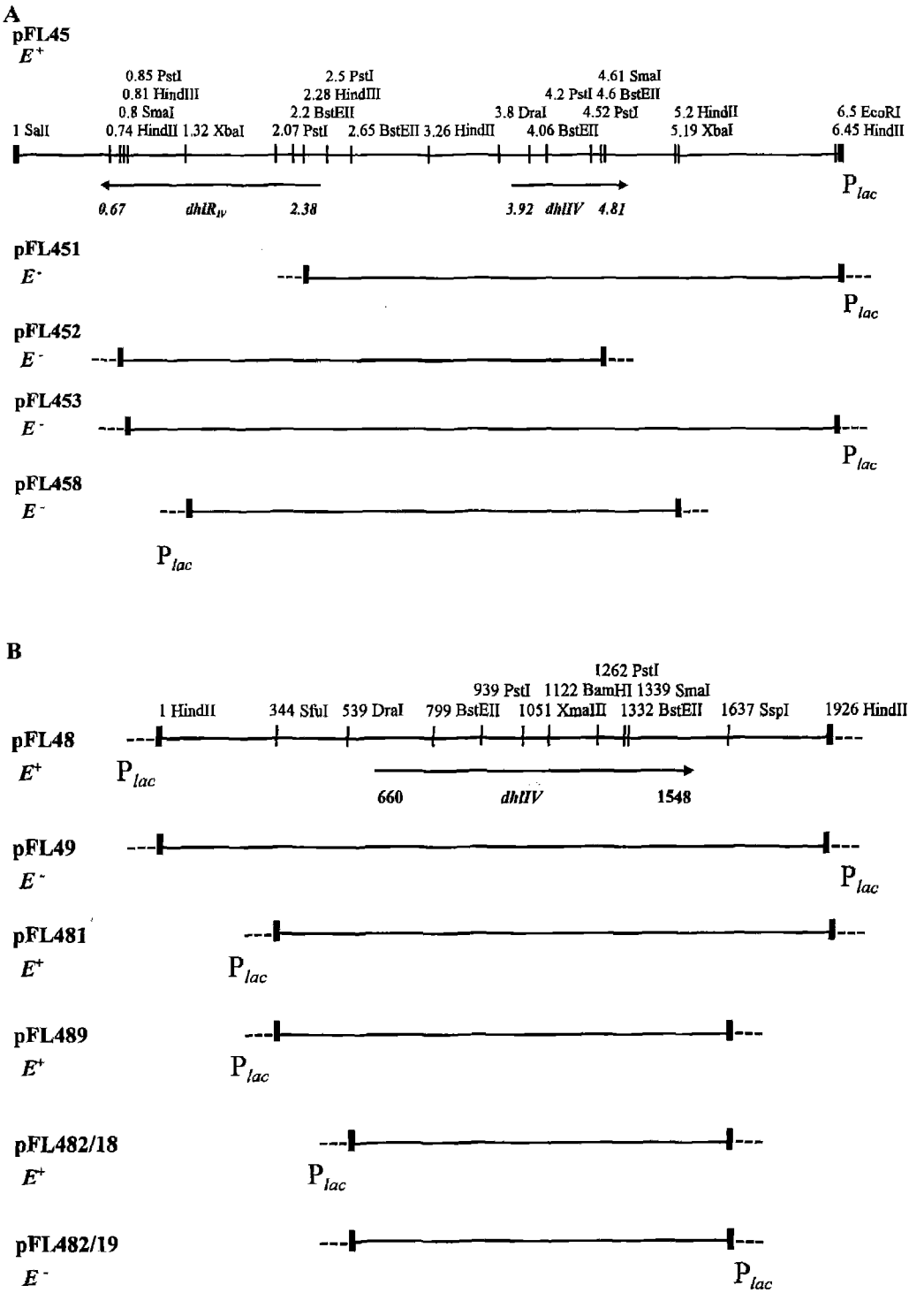

Figure 1. Physical maps of the inserted DNA in pUC18 or pUC19. *(A)* subclones from pFL45; distances between restriction enzyme sites are given in kb. *(B)* subclones from pFL48; distances between restriction enzyme sites are given in bp. E^+: Expression of the halidohydrolase. E^-: No expression of the halidohydrolase. The ORF of *dhlIV* and its corresponding regulatory gene $dhlR_{IV}$ are indicated by arrows. p_{lac}: position of p_{lac} promoter in pUC18 or pUC19.

Table 3. Determination of the optical configuration of the product of the dehalogenation reaction from D- or L-CPA

Substrate[a]	Enzyme	ΔE_{340}*5 min^{-1}
L-CPA	D-LDH	0.286
L-CPA	L-LDH	0.012
D-CPA	D-LDH	0.008
D-CPA	L-LDH	0.366
L-lactate [b]	L-LDH	0.401
D-lactate [b]	D-LDH	0.447

a: Complete dehalogenation of CPA will produce 4.6 mM lactate in the test assay.
b: Controls, addition of 5 mM lactate in the test assay instead of CPA

*Eco*RI fragment (pFL410) and a 9.5 kb *Sal*I fragment (pFL420), respectively, which expressed the halidohydrolase in *P. fluorescens* R2f (Figure 3). In contrast *Bam*HI, *Hin*dIII or *Pst*I restriction fragments of pFL40 failed in expressing the enzyme in appropriate shotgun cloning experiments. Successful subcloning of the dehalogenase gene from pFL420 was carried out by digestion with *Eco*RI, prior to religation. The resulting plasmid pFL417 carried a 6.5 kb *Eco*RI/*Sal*I fragment with *dhlIV*. Cloning this DNA fragment into pUC18 resulted in plasmid pFL45 (Figure 1a). Electroporation of this plasmid into *E. coli* RRI enabled this strain to grow on MCA, D,L-CPA and 2,2-DCPA.

Regulation of the halidohydrolase

Like the parent strain, all these constructs showed inducible expression of the halidohydrolase. No dehalogenase activity was detectable in crude extracts from NI-grown cultures. If a second non halogenated substrate, such as succinic acid, glucose or 0.1 x NI was added to MM-medium, these compounds were degraded first. No release of chloride from these cultures or dehalogenase activity in crude extracts was measurable before the second substrate was degraded. Additionally no liberation of chloride and no dehalogenase activity was detected in cultures, in which protein biosynthesis was interrupted by the addition of chloramphenicol (Figure 2 a,b). It is likely to suggest, that *dhlIV* is positively regulated in the presence of haloalkanoic acids and negatively regulated if additional substrates are present.

Dehalogenation efficiencies of pFL417 and pFL45 were nearly identical. About 0.75 mM DCPA or CPA was dehalogenated within one hour from induced cells

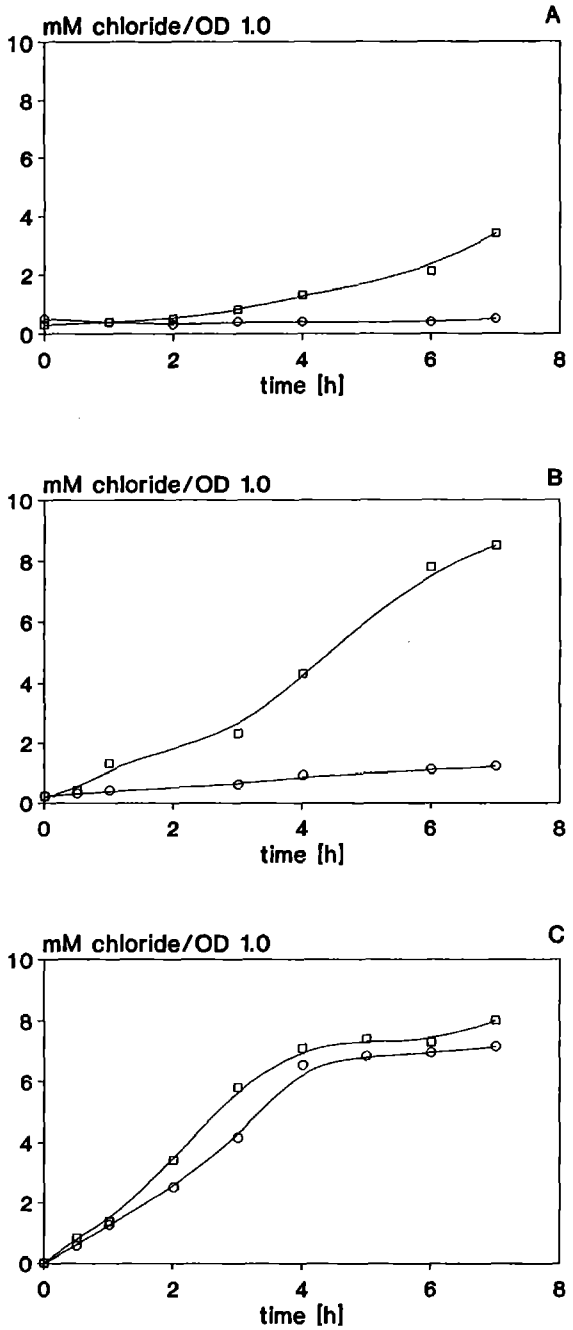

Figure 2. Induction of the DhlIV halidohydrolase. Non induced cells were suspended in resting cell medium with 10 mM DCPA to a optical density (OD_{546}) of 1.0. The de novo protein biosynthesis was inhibited by addition of 100 $\mu g\ ml^{-1}$ chloramphenicol (+ Cam). The release of chloride was measured from: *(A) A. xylosoxidans* ABIV (pFL40); *(B) P. fluorescens* R2f (pFL417); *(C) E.coli* RRI (pFL48). ○ DCPA (+ Cam), □ DCPA (- Cam).

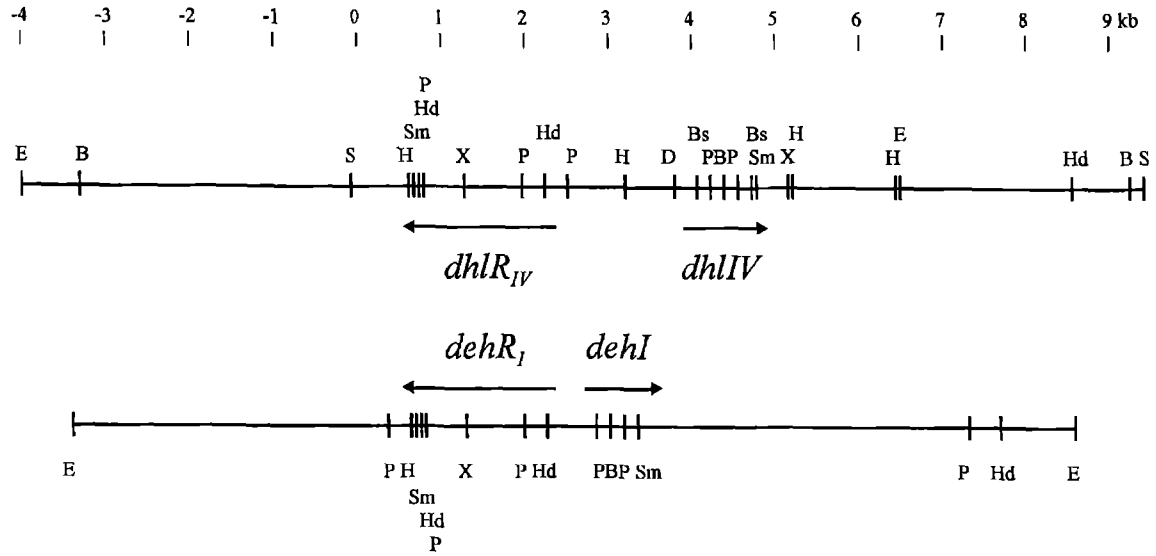

Figure 3. Physical map of a region of pFL40. A detailed restriction map has been constructed, containing the D,L-halidohydrolase *dhlIV* and its regulatory gene, $dhlR_{IV}$. Comparision to the transposable element *DEH* (redrawn from Topping et al. 1995) with the σ^{54}dependent regulatory gene $dehR_I$ and the corresponding dehalogenase *dehI* is shown. Whereas orientation and position of the key restriction sites of both dehalogenase- and regulatory genes are identical, the gap between $dehR_I$ and *dehI* (0.28 kb) is different to that between $dhlR_{IV}$ and *dhlIV* (1.5 kb). Restriction sites: B, *Bam*HI; Bs, *Bst*EI; E, *Eco*RI; H, *Hin*dII; Hd, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; X, *Xba*I.

(OD_{546}= 1.0). Whereas the induction time with DCPA and CPA was in the range of one to three hours, it took more than ten hours to induce cells with MCA and DCA. In crude extracts from these cultures specific activities up to 2 U mg^{-1} were measured. Although there were remarkable differences in growth rates between *P. fluorescens* R2f (pFL417) and *E. coli* RRI (pFL45), the specific activities of their dehalogenases were similar, indicating a neglectable effect of the low copy vector pME285 versus the high copy vector pUC18.

To achieve subsequent cloning of *dhlIV* fragments, plasmid pFL45 was mapped by restriction analysis (Figure 1a). A 1.9 kb *Hin*dII fragment was cloned into pUC18 (pFL48) (Figure 1b) and transferred into *E. coli*, that in contrast to the constructs described above, immediately produced chloride from halogenated substrates (Figure 2c). Dehalogenase activities in crude extracts in the range of 1.5 - 2 U mg^{-1} using MCA as a substrate were obtained from cells either grown on NI medium or on MM medium with MCA, CPA or DCPA. In contrast no activity of the dehalogenase was measured from the same 1.9 kb DNA fragment cloned by *Eco*RI/*Hin*dIII digestion into pUC19 (pFL49) (Figure 1b), where the *lac*-promoter (P_{lac}) is positioned at the opposite end of the polycloning site and of the inserted DNA fragment. Considering these results, we suggest that these clones had lost their regulatory region, thus constitutively expressing the dehalogenase in the presence of P_{lac}.

Several DNA fragments from pFL45 with different upstream regions and the complete *dhlIV* gene were cloned (Figure 1a). *Hin*dIII-digestion of pFL45 and religation yielded pFL451 with a 4.2 kb fragment flanked by *Hin*dIII and *Eco*RI sites (position 2.28 to 6.5, Figure 1a). However, no expression of *dhlIV* could be achieved from this DNA fragment inserted in pUC18. Subcloning the same 1.9-kb*Hin*dII-fragment as found in pFL48 from pFL451, succeeded in restoring the ability of *E. coli* RRI to grow on haloalkanoic acids, probably due to the right orientation of P_{lac}. These findings are in good agreement with the results from shotgun cloning experiments, yielding no degradation activity from recombinant cells transformed with *Hin*dIII fragments from pFL40 into pME285, where no external promoter was present.

No dehalogenase activity was measured using pFL458 with a 3.9 kb *Xba*I fragment (position 1.31 to 5.18), although P_{lac} was located upstream of *dhlIV*, nor from pFL453 with a 5.7 kb *Hin*dIII-*Eco*RI fragment (position 0.81 to 6.5). These results indicate that an activating regulatory element ($dhlR_{IV}$) is located in a region ranging approximately 3 to 1.5 kb upstream of the *dhlIV* gene.

A region of the transposable element *DEH* (Topping et al. 1995), containing the D,L-haloalkanoic acid dehalogenase gene *dehI* and its regulatory gene $dehR_I$ from *P. putida* PP3 (Weightman et al. 1979) was compared with a region of pFL45. Significant homologous positions of some restriction enzyme sites of $dehR_I$ were found 3.5 to 1.5 kb upstream the *dhlIV* gene (Figure 3). Initial sequencing of this region (positions 0.6–2.5, data not shown) from subclones of pFL45 resulted in DNA sequence similarities of about 95% to the regulatory gene $dehR_I$, encoding a σ^{54}-dependent activator protein (Topping et al. 1995).

A further homologous region between *DEH* and pFL45 on basis of the positions of some restriction enzyme sites was also found within both dehalogenases *dehI* and *dhlIV*. Gaps between these dehalogenases and their associated regulatory genes were markely different, 0.28 kb between $dehR_I$ and *dehI* versus 1.5 kb between $dhlR_{IV}$ and *dhlIV* (Figure 3).

Further subcloning of the dehalogenase gene

To determine the complete nucleotide sequence of the 1.9 kb *Hin*dII fragment comprising *dhlIV*, subsequent shorter DNA fragments were obtained by digestion with various restriction enzymes, sometimes chosen from initial sequencing data. A complete set of overlapping deletion fragments were generated and their DNA sequence was analysed. Like the 1.9 kb *Hin*dII fragment three of these deletion fragments were able to express the D,L-dehalogenase in *E. coli*: a 1.6 kb *Sfu*I-*Hin*dII fragment in pFL481, a 1.3 kb *Sfu*I-*Ssp*I fragment in pFL489 and pFL 482 with a 1.1 kb *Dra*I/*Ssp*I insert, obviously all under the control of P_{lac} (Figure 1b).

The dehalogenases expressed by these clones were compared to that of the parent strain *A. xylosoxidans* ABIV and found to be identical. This was proved by patterns of substrate specificity in crude extracts, activity staining technique, estimation of molecular weight by SDS-PAGE of partially purified enzymes and in Southern blots, using a 0.55 kb *Bst*EII fragment from pFL483, which comprised an inner fragment of the open reading frame of *dhlIV* (Figure 1b).

Nucleotide sequence determination

By sequencing a set of overlapping subclones of pFL48, the complete nucleotide sequence of the 1.9 kb *Hin*dII fragment was obtained. Together with the deduced amino acid sequence of *dhlIV* it is presented in Figure 4. One open reading frame (ORF) with a predicted length of approximately 900 nucleotides was detected, possessing two possible starting points at nucleotides 639 and 660 with the initiation codon ATG and ending with a termination codon TAA at position 1548. Therefore this ORF encodes a protein with 303- respectively 296- amino acid residues with calculated molecular masses of 33.6 or 32.8 kD, comparable to the molecular mass of DhlIV (32 kD), estimated by SDS-PAGE. Because of two reasons the start of the biological active protein is thought to be located at position 660. Firstly the 14 residues from the N-terminal amino acid sequence from purified DhlIV halidohydrolase have been determined and found to match exactly those deduced amino acids residues starting at the second start position. Secondly only one putative ribosomal binding site, GGAGA, was found in this region at position 649, and therefore downstream the first putative starting codon.

A putative -35 and -10 consensus sequence of a σ^{70} *E. coli* promoter was detected at positions 519 (TTCATA) and 545 (TAAATT), spaced by 19 nucleotides. Both sequences comprised 4 out of 6 conserved nucleotides. The proposed function of these sequences was not found, since there was no expression of the dehalogenase, if the vector encoded promoter P_{lac} was located downstream *dhlIV* (Figure 1b).

In addition a putative σ^{54} (RpoN) promoter consensus sequence of the -12/-24 type was detected at position 283 and a palindromic nucleotide sequence with a proposed function as a binding site for regulatory proteins (Morett & Segovia 1993) was located at position 212 (Figure 4).

The G+C content of 54.3% of the *dhlIV* gene and 52.7% of the complete nucleotide sequence of the 1.9 kb *Hin*dII fragment appeared to be significantly lower than the G +C content found in *A. xylosoxidans ssp. denitrificans* genomic DNA (63.9-68.9%) (Kersters & De Ley 1984), but more similar to that of *E. coli* DNA.

Sequence comparison

With one exception we could not find significant homologies by comparison the DNA sequence or the deduced amino acid sequence of *dhlIV* with any sequence listed in the EMBL database respectively the SWISS-PROT protein data bank. Comparing the deduced amino acid sequences of the nine published L-specific halidohydrolases and the α/β hydrolase fold dehalogenases (Janssen et al. 1994) with *dhlIV*, no sequences with 5 or more continuous identical amino

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   1  GG TGG CCA ACA TTT GAA AGC TCA GGT CAT TGG CTT CGA GCA GGT GAC   47
  48 GAA ACC GAA GAA TGG TGC TCT CGT CGG GCA GGT GGT CTT CTC CCA CGT   95
  96 CCA GTC CAG CAA ACT CAC GGA ACA TCG GTG TGT CGT ACA GCG CCT CTT  143
 144 CCA TGG CTG GGT CAG ACA GGT TGA ACC AGT GTT GCA AGA AGT GGA TGC  191
 192 GAA GCA TCA TCT GCA CGG GAA ATG GCG GGC GCC CGC CCT TGG TGC CAC  239
                                                          -24
 240 GTG CCG GCG CGT GGG GTG CAA TCA GCG CCA CCA GTT CGG CCC ATG GCA  287
                     -12
 288 CCA CCA GGT TCA TCT CGT CAA GAA ATT CGG CTT GCG CGT TCT CTT GGT  335
 336 CTT GCG TTC GAA TCC GGT GTC CCC GAG GCT CAT CTG TTT CAT GTC TGA  383
 384 ACT GTC TCA CAT CCG GTG CGC CGC GCC GAG GTT TTG CAG ACT TTC CTT  431
 432 AAC CAT AGG CGC AAT TGA CAG TCA GGT CAA ATA TGG TTT GCG CGG ATC  479
                                                      -35
 480 AGA AAT GAA CCT AAC ACA GTT GCT GAA TGA CTG GCT GTC TTT CAT AAG  527
                  -10
 528 CAT TTG ATT TTA AAG AGT AAA TTT ATA TGG CAC GGG CCT TGC GAT ATA  575
 576 AGC TGG GCG TCC AAT ATG ACG CCC CTG ACT CGG CGA TTT TGC TGA CTT  623
                              S/D
 624 ACG GAC CAA GCA TTT ATG GAT AGG AGA GCC CAC ACA ATG ACC AAC CCT  671
   1                     Met Asp Arg Arg Ala His Thr Met Thr Asn Pro    4

 672 GCA TAC TTT CCG CAA CTT TCC CAG CTT GAT GTG TCT GGA GAA ATG GAA  719
   5 Ala Tyr Phe Pro Gln Leu Ser Gln Leu Asp Val Ser Gly Glu Met Glu   20

 720 TCG ACC TAT GAA GAT ATT CGC CTA ACG TTA CGC GTG CCT TGG GTC GCC  767
  21 Ser Thr Tyr Glu Asp Ile Arg Leu Thr Leu Arg Val Pro Trp Val Ala   36

 768 TTC GGT TGC CGA GTG CTT GCT ACA TTT CCA GGT TAC CTG CCA CTT GCA  815
  37 Phe Gly Cys Arg Val Leu Ala Thr Phe Pro Gly Tyr Leu Pro Leu Ala   52

 816 TGG CGC CGC AGC GCA GAA GCA CTC ATT ACC CGC TAC GCT GAG CAA GCC  863
  53 Trp Arg Arg Ser Ala Glu Ala Leu Ile Thr Arg Tyr Ala Glu Gln Ala   68

 864 GCT GAC GAG CTG CGC GAG CGC TCC CTA CTC AAC ATC GGT CCA TTG CCG  911
  69 Ala Asp Glu Leu Arg Glu Arg Ser Leu Leu Asn Ile Gly Pro Leu Pro   84

 912 AAC TTA AAA GAA CGG TTG TAC GCT GCA GGA TTC GAT GAC GGA GAA ATT  959
  85 Asn Leu Lys Glu Arg Leu Tyr Ala Ala Gly Phe Asp Asp Gly Glu Ile  100

 960 GAG AAG GTT AGA CGC GTG CTT TAT GCG TTT AAC TAT GGT AAT CCA AAA 1007
 101 Glu Lys Val Arg Arg Val Leu Tyr Ala Phe Asn Tyr Gly Asn Pro Lys  116

1008 TAT CTG TTG CTC ATT ACC GCG TTG AGT GAA AGC ATG CAG ATG CGG CCG 1055
 117 Tyr Leu Leu Leu Ile Thr Ala Leu Ser Glu Ser Met Gln Met Arg Pro  132

1056 GTG GGA GGA GCT GAG GTT TCG TCC GAG CTT CGA GCA TCC ATC CCG AAG 1103
 133 Val Gly Gly Ala Glu Val Ser Ser Glu Leu Arg Ala Ser Ile Pro Lys  148

1104 GGG CAT CCA AAA GGT ATG GAT CCG CTT TTG CCG CTT GTC GAT GCC ACC 1151
 149 Gly His Pro Lys Gly Met Asp Pro Leu Leu Pro Leu Val Asp Ala Thr  164

1152 AAG GCA TCC ACC GAG GTT CAA GGG CTC CTT AAG CGG GTG GCT GAC CTT 1199
 165 Lys Ala Ser Thr Glu Val Gln Gly Leu Leu Lys Arg Val Ala Asp Leu  180

1200 CAC TAT CAT CAC GGT CCG GCA AGT GAT TTC CAA GCG CTG GCC AAT TGG 1247
 181 His Tyr His His Gly Pro Ala Ser Asp Phe Gln Ala Leu Ala Asn Trp  196

1248 CCG AAG GTA CTG CAG ATT GTT ACA GAT GAA GTG CTC GCA CCG GTT GCC 1295
 197 Pro Lys Val Leu Gln Ile Val Thr Asp Glu Val Leu Ala Pro Val Ala  212

1296 CGC ACC GAG CAG TAT GAT GCC AAG TCA CGG GAG CTG GTA ACC CGG GCG 1343
 213 Arg Thr Glu Gln Tyr Asp Ala Lys Ser Arg Glu Leu Val Thr Arg Ala  228

1344 CGG GAA CTG GTG CGT GGA CTG CCC GGC TCT GCT GGT GTT CAG CGG TCG 1391
 229 Arg Glu Leu Val Arg Gly Leu Pro Gly Ser Ala Gly Val Gln Arg Ser  244

1392 GAG CTA ATG TCC ATG CTG ACA CCG AAC GAG CTT GCC GGT CTG ACT GGT 1439
 245 Glu Leu Met Ser Met Leu Thr Pro Asn Glu Leu Ala Gly Leu Thr Gly  260

1440 GTC CTG TTC ATG TAT CAG CGC TTC ATC GCT GAC ATC ACA ATT AGC ATC 1487
 261 Val Leu Phe Met Tyr Gln Arg Phe Ile Ala Asp Ile Thr Ile Ser Ile  276

1488 ATT CAT ATA ACA GAG TGT TTG GAC GGC GCG GAA GCA GCG TCT AAG TCG 1535
 277 Ile His Ile Thr Glu Cys Leu Asp Gly Ala Glu Ala Ala Ser Lys Ser  292

1536 CCT TTT CCT ATC TAA CTT ATT GAG AAT CAC CTA TGA ATG GCA TGT AGA 1583
 293 Pro Phe Pro Ile ***                                            297

1584 GCC CGA GAA ACC AAG AGC CCG CAT GCC ATG TAT TGT CTA AAT GTT TAT 1631

1632 CAA ATA TTG CGT TTC GCA CTA ATC GGA GAT TTT ATT ATG GCC TTA GAC 1679

1680 AAG TTC TCG TTA ATA AAT TAT AGC GGC GGC AAT AGC GCT TAT TGT TTC 1727

1728 ATC TGT TGT TGT TCT AAT CGT TAG CGC TTG CAC GGT CGG CGC ACT GGT 1775

1776 CCG GGG GGC TTT GCT TAA GCC AAA CAC GCA ACG CCT CGC GGA TGG GAA 1823

1824 ACC CGG CGA TAA CGG TGC AGG CGA CGA AAC ACG GCT GCT GTG AAG GGG 1871

1872 CAT AGA AGT AGT AAC CAG AGT TAA TTG GCT AGC ATT GGT TTT CTA GAG 1919

1920 ACT CGT C                                                      1926
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Figure 4. Nucleotide and deduced amino acid sequence of *dhlIV*: The putative σ^{54} (-24/-12) and σ^{70} (-35/-10) promoter consensus sequences and Shine- Dalgarno-box (S/D) are indicated. A palindromic nucleotide sequence upstream of the putative σ^{54} promoter sequence is indicated by arrows. The amino acid residues from the first start codon of the ORF are typed in Italic (not found in the biological active protein). The determined N-terminal amino acid residues are underlined.

acid residues were found. The only exception is a motif of 7 amino acids, at position 129 to 135 in the sequence of the D-specific halidohydrolase *hadD* (Barth et al. 1992), identical to the amino acids in position 112 to 118 from *dhlIV*. A further sequence of 11 amino acid residues (position 80 - 90) of *hadD* shows similarity to those of *dhlIV* (position 66 - 76) (Figure 5). The total maximum homology between both amino acid sequences was estimated to be approximately 15%.

Comparing the DNA sequences in the most homologous domain and its flanking region, it is obvious that guanine and cytosine of *hadD* are often substituted by adenine and thymine in *dhlIV*, which is in accordance to the lower G + C content of this gene.

Discussion

We have cloned and sequenced a D,L-halidohydrolase gene from *A. xylosoxidans* ssp. *denitrificans* ABIV located on pFL40. The sequence of the open reading frame that has been determined shows no significant homologies to known L-specific halidohydrolases, compared by Nardi-Dei et al. (1994) nor to the conserved regions of the α/β-hydrolase fold group (Janssen et al. 1994). This is in concordance with the classification of halidohydrolases, where D,L-halidohydrolases are separated from L-specific dehalogenases and the α/β- hydrolase fold group, until now based upon biochemical characteristics (Janssen et al. 1994; Slater et al. 1995).

Only a short continuous sequence of 7 amino acids of *dhlIV* shows homology to the *hadD* dehalogenase. This sequence contains no Arg, Asp, Cys or His, amino acid residues which are probably involved in the reaction of hydrolytic dehalogenases (Little & Williams 1971, Weightman et al. 1982). The second short sequence of 11 amino acid residues from *dhlIV* with similarity to *hadD* does contain Arg and Asp. Whether these motifs are preserved regions as a result of involvement in the dehalogenation reaction or it is a fortuitous homology can not be decided by the data available up to now, since mechanistic information is lacking for both enzymes.

Nardi-Dei et al. (1994) found some similarities between the L-DexYL dehalogenase from *P. putida* YL and two hypothetical *E. coli* proteins and Kawasaki et al. (1992) also described amino acid sequences of *dehHI* homologous to *Pseudomonas* sp. hydrolases. In contrast to these observations, but in agreement with Barth et al. (1992), we failed in detecting any proteins related to DhlIV.

As there are significant differences in the G + C content of *dhlIV* from pFL40 and the chromosome of its host *A. xylosoxidans* ABIV, we suggest that this strain is not the original host of the self transmissible plasmid pFL40. This argument is underlined by the isolation of three different bacterial strains from DCPA contaminated soils, expressing halidohydrolases with the same biochemical characteristics as determined for DhlIV (Schwarze et al. 1996). Each of these strains harboured a plasmid nearly identical to pFL40 in restriction enzyme patterns and provided significant homology in DNA hybridization experiments with the *dhlIV* specific gene probe (Brokamp et al. 1996). However the origin and evolution of the *dhlIV* halidohydrolase gene still remains unclear.

DNA sequences of further D,L-halidohydrolases are still unknown, however something is known about the genetic organisation of the class 2R-halidohydrolase DehI (Slater, 1994). Two initiation codons and a strong Shine-Dalgarno sequence 8 bases upstream the second start codon have been identified. The translation is starting at this second methionine, analogous to the start of translation described for *dhlIV* (Figure 4). A σ^{54}- dependent activator protein *dehRI* is located upstream of the *dehI* gene (Topping et al. 1995). There are strong indications that also upstream the *dhlIV* gene a highly homologous activating regulatory gene is located in the same orientation as found on the transposable element *DEH*, but the gap between the regulatory gene and the corresponding dehalogenase is different.

There are also remarkable similarities in the positions of some restriction enzymes within the *dehI* and *dhlIV* genes. Although there are further considerable similarities between DhlIV and DehI, even in substrate specifity and sensitivity to sulfhydryl reagents (Table 2) there are some differences between these enzymes as well as in the organisation of their genes. *dehI* and $dehR_I$ are encoded by a chromosomal mobile element, leading to movements of the *DEH* element to different plasmids and back to the chromosome (Thomas et al. 1992). *dhlIV* and its associated regulatory gene are plasmid-borne, transposition events or high frequencies of recombination has never been found. Comparing both dehalogenases, the estimated molecular sizes are significantly different, 32 kD for DhlIV and 46 kD for DehI (Slater 1994).

According to our results DhlIV acts on haloalkanoic acids by inversion of the optical configuration

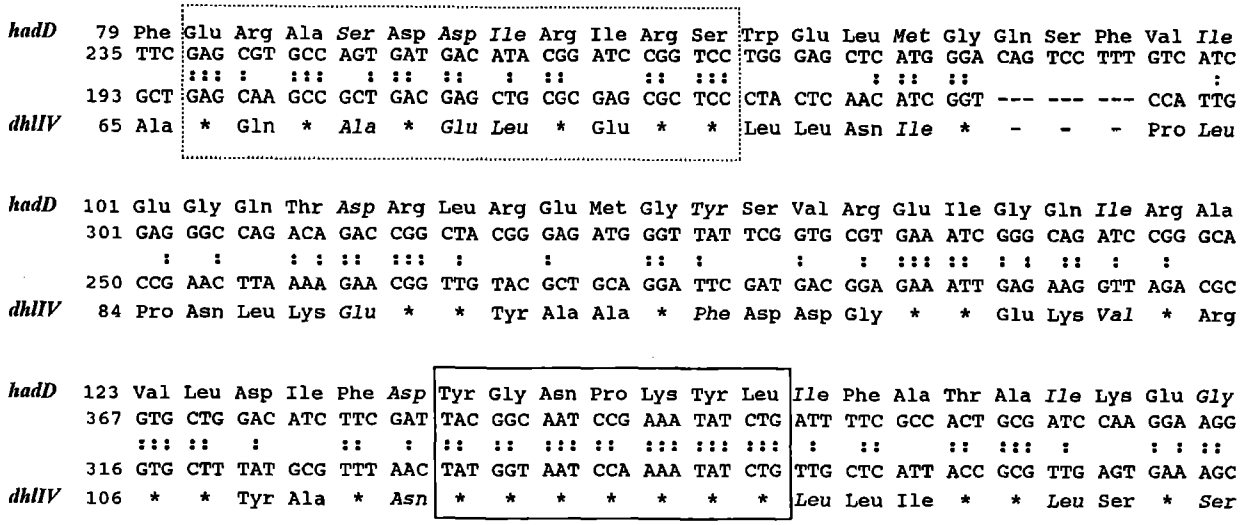

Figure 5. Homologous regions between the halidohydrolases *dhlIV* and *hadD*. The nucleotide- and amino acid residue sequences of homologous regions of *hadD* and *dhlIV* are shown. The 7 and the 11 amino acids homologous domains are boxed. Matching nucleotides are indicated by colons, matching amino acid residues are characterized by an asterisk, amino acid residues of the same homologous group are typed in Italic.

(class 2I), whereas DehI maintains the optical configuration of substrate-product (class 2R). As outlined by Weightman et al. (1982) there are two different catalytic mechanisms involved in these dehalogenation reactions, one is a generalized base catalysis reaction which inverts substrate product configuration, the other mechanism comprises an active -SH group from a cysteine residue leading to retention of configuration. Whereas the first mechanism is thought not to be affected by thiol reagents, the second is vigorously inhibited. However there are also enzymes among the class 2I dehalogenases that are more or less affected by thiol reagents (Table2). Differences in inhibition by thiol reagents were also found among the L-isomer specific halidohydrolases (Nardi-Dei et al. 1994). Janssen et al. (1994) pointed out, that this effect should be handled with care in proposing a specific reaction mechanism, since there are some dehalogenases inhibited, although no cysteine is involved in the dehalogenating reaction. The amino acid sequence of *dhlIV* contains two cysteine residues at positions 39 and 282, but it is still unclear if they are involved in the mechanism of catalysis.

Evaluation of these data led us prefer to place DhlIV among the class 2I-dehalogenases preliminarily according to the classification established by Slater et al. (1995). Considering the differences in the proposed reaction mechanisms between DhlIV and DehI it is questionable, whether these two D,L-halidohydrolases are homologous.

There are no genetical data of further dehalogenases inverting substrate-product configuration available (Table 2). We also failed up to now to clone and express such enzymes from own isolates (Brokamp et al. 1996), differing in biochemical characteristics to DhlIV. From hybridization experiments with a inner fragment of *dhlIV* no positive signals were obtained from these strains. Nevertheless a possible homology can not be excluded from these results if DNA sequence similarities are in a range below 70%, as found between different L-isomer specific dehalogenase genes (Kawasaki et al. 1994), resulting in no positive hybridization signals among each other. So accurate classification of D,L-halidohydrolases and identification of their evolutionary relationship needs additional sequence data of other D,L-halidohydrolases.

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