Molecular biology of the 2-haloacid halidohydrolase IVa from *Pseudomonas cepacia* MBA4

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The structural gene (*hdl* IVa) for the *Pseudomonas cepacia* MBA4 2-haloacid halidohydrolase IVa (Hdl IVa) was isolated on a 1.6 kb fragment of *Ps. cepacia* MBA4 chromosomal DNA. The recombinant halidohydrolase was expressed in *Escherichia coli* and *Pseudomonas putida* and the structural gene was subcloned on to the *tac* expression vector pBTac1. High-level expression from the *tac* promoter was seen to be temperature-dependent, a consequence of the nucleotide sequence adjacent to the fragment encoding the halidohydrolase. The nucleotide sequence of the fragment encoding the Hdl IVa was determined and analysed. Three ATG codons were identified in one of the open reading frames and the one corresponding to the start of the *hdl* IVa structural gene was determined by comparison of the predicted amino acid sequences with the experimentally determined *N*-terminal sequences of halidohydrolase IVa. The *hdl* IVa gene encoded a 231-amino acid-residue protein of *M_r* 25 900. The sequence and predicted structural data are discussed and comparison is made with sequence data for other halidohydrolases.

INTRODUCTION

The dehalogenases are a family of enzymes that catalyse the cleavage of carbon–halogen bonds (Hardman, 1991). Three decades of work have resulted in the identification of microbial dehalogenases that catalyse the hydrolytic, oxidative and reductive dehalogenation of haloaliphatic and haloaromatic compounds. The most extensively studied dehalogenases have been those that catalyse hydrolytic dehalogenation, collectively termed the halidohydrolases (Goldman *et al.*, 1968), and of these the 2-haloacid halidohydrolases have received the most attention. The halidohydrolases that are active towards 2-haloalkanoic acids are divided into those active upon haloacetates (EC 3.8.1.3; Goldman, 1965) and those active upon haloacetates and 2-halopropionates (EC 3.8.1.2; Goldman *et al.*, 1968). The latter, the 2-haloacid halidohydrolases, can be placed into four groups on the basis of their reaction kinetics, sensitivity towards thiol-blocking reagents and chiral specificities (Weightman *et al.*, 1982; Hardman, 1991).

Pseudomonas cepacia MBA4 was isolated from soil by batch-enrichment culture and shown to possess two 2-haloacid halidohydrolases (Tsang *et al.*, 1988). The organism expressed only one enzyme in batch culture and, by using activity-stain PAGE, it was designated halidohydrolase IVa. Consideration of the insensitivity of this enzyme to thiol-blocking reagents and its chiral specificity towards L-2-monochloropropionic acid led us to place it in 2-haloacid halidohydrolase Group 1.

Physicochemical and biochemical studies of the 2-haloacid halidohydrolases have identified a large number of isoenzymic forms (up to ten), with many bacterial isolates possessing multiple forms (Hardman & Slater, 1981; Tsang *et al.*, 1988). All of these enzymes are active only towards α -substituted alkanolic acids and show no activity towards β -substituted acids, halo amides, halo aldehydes or haloalkanes. Where data are available these enzymes have been demonstrated to be dimeric proteins consisting of

identical subunits (*M_r* 23 000), with optimum catalytic activity in the pH range 9.0–10.

Isolation of so many isoenzymic forms from a wide variety of bacterial genera (Little & Williams, 1972; Slater *et al.*, 1979; Hardman & Slater 1981; Kawasaki *et al.*, 1981a; Leigh *et al.*, 1988) raises the question of their importance in the natural environment and the evolutionary relatedness of the different forms. The ubiquity of the haloacid halidohydrolases in natural bacterial isolates has led to the suggestion that they represent catabolic enzymes cleaving the halo substituents of halo metabolites as part of degradative pathways for the degradation of more complex halo-organic compounds.

A limited number of genetic studies, which consider the evolutionary mechanisms of halidohydrolases, have been reported in the literature. These reports have either been concerned with plasmid-encoded phenotypes (Kawasaki *et al.*, 1981b, 1982; Hardman *et al.*, 1986) or with the role of transposons in the expression of activities through decryptification (Slater *et al.*, 1985) or acquisitive evolution (Kawasaki *et al.*, 1985). To date, however, evolutionary relatedness of the different isoenzymes at the peptide or nucleotide sequence level in different bacterial isolates has not been reported. In order to enable such consideration we have cloned and sequenced the structural gene of the 2-halo acid halidohydrolase IVa from *Ps. cepacia* MBA4 (Tsang *et al.*, 1988). In the present paper we discuss the implications of the findings for studies on the evolution of the halidohydrolases and compare our findings with those reported for the halidohydrolases of *Pseudomonas* sp. CBS3 (Schneider *et al.*, 1991).

MATERIALS AND METHODS

Culture and maintenance conditions

The defined minimal salts medium and cultivation conditions were those previously described (Slater *et al.*, 1979). The halo-

Abbreviations used: *hdl* IVa, structural gene of halidohydrolase IVa; Hdl IVa, halidohydrolase IVa protein.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases.

generated carbon sources (monobromoacetic acid, monochloroacetic acid and 2-monochloropropionic acid) were added, where appropriate, to final concentrations of 15 mM. *Escherichia coli* strains were cultured in NaCl-free L broth (1% tryptone and 0.5% yeast extract). One-fifth-strength L broth supplemented with 2-monochloropropionic acid (15 mM) was used for the screening for halidohydrolase-producing (Hdl⁺) recombinant strains. Antibiotics were added where appropriate at 50 µg/ml except for rifampicin, which was used at 200 µg/ml.

Measurement of halidohydrolase activity in cell-free extracts

The preparation of cell-free extracts and the measurement of halidohydrolase activities were made as previously described (Hardman & Slater, 1981).

PAGE

Activity-stain PAGE was used to identify the recombinant halidohydrolase activity as that of the wild-type strain. The procedure was as described by Tsang *et al.* (1988).

Purification of halidohydrolase IVa

The purification of halidohydrolase IVa protein from cell-free extracts of *Ps. cepacia* MBA4 was achieved by using the method described by Tsang *et al.* (1988).

Immunoblot analysis

Immunoblot analysis of proteins on SDS/PAGE (gradient 7–18%) was also used to confirm the identity of the recombinant halidohydrolase. The proteins were transferred from the gel to a nitrocellulose sheet by electrophoresis (Towbin *et al.*, 1979). The proteins that were immunologically cross-reactive with Hdl IVa were detected by the method of Leary *et al.* (1983), with an antiserum raised against Hdl IVa purified from *Ps. cepacia* MBA4 and an anti-(rabbit IgG) antibody-alkaline phosphate conjugate.

Preparation of chromosomal and plasmid DNA

Chromosomal DNA was prepared from *Ps. cepacia* MBA4 using the method of Marmur (1961). The method of Holmes & Quigley (1981) was used for rapid small-scale plasmid preparations for screening transformants, and large-scale preparation was achieved by using the Brij lysis method (Clewell & Helinski, 1969) followed by CsCl/ethidium bromide buoyant-density centrifugation.

Construction of a *Ps. cepacia* MBA4 chromosomal DNA library and screening for Hdl⁺ phenotype

All DNA-modification enzymes were purchased from Boehringer Biochemicals (Mannheim, Germany) and used in accordance with the manufacturer's recommendations. A genomic library of *Ps. cepacia* MBA4 was constructed in the cosmid vector pMMB33 (Frey *et al.*, 1983) in accordance with the procedures of Franklin (1985). The library was constructed by using *Ps. cepacia* MBA4 DNA partially restricted with *Eco*RI and transfected into *E. coli* HB101 (Sm^r, *hsdR*, *hsdM*; RP4-5) (Burkardt *et al.*, 1979). Putative Hdl⁺ transfectants were screened by inoculating individual transfectants into 96-well micro-titre plates containing one-fifth-strength L broth supplemented with monochloroacetic acid and kanamycin (monochloroacetic acid was used because monobromoacetic acid was toxic to the *E. coli* host). The micro-titre plates were incubated for 2 days at 37 °C then AgNO₃ (10 µl of a 0.1 M solution) was added to each well. Putative Hdl⁺ transformants were identified by a white precipitate (AgCl) that formed from the free Cl⁻ ions released from the monochloroacetate by halidohydrolase activity.

Conjugal transfer of halidohydrolase activity from recombinant strains to *Pseudomonas putida* KT2441

Conjugation of recombinant Hdl⁺ *E. coli* with *Ps. putida* KT2441 (*hsdR*, Rif^r) was performed on L-broth/agar plates. Both donor and recipient bacteria were streaked separately on to the same plate and incubated at 37 °C for 3–4 h. Cells from each parental strain were then picked and mixed on a separate part of the plate and incubated overnight at 37 °C. Cells from the mixed culture were streaked on to antibiotic medium selective against both parental strains but not the desired exconjugants.

Nucleotide sequencing

The 1.6 kb *Eco*RI insert isolated from pUKC504 was digested with *Pvu*II and the resulting fragments were ligated into pGEM-3Z vector (Promega, Madison, WI, U.S.A.). In addition to the SP6 and T7 primers, several oligonucleotides were synthesized (in a Biosystems model 3810 apparatus) and also used as primers. Sequencing reactions were performed by using the dideoxy-ribonucleotide chain-termination method of Sanger *et al.* (1977) with Sequenase (Cambridge BioSciences, Cambridge, U.K.). Gels were electrophoresed at 55 °C so as to enhance separation in G + C-rich regions of the fragment. Nucleotide sequences were analysed with the UWGCG package (University of Wisconsin, Madison, WI, U.S.A.), the open reading frame analysis was achieved by using the FRAMES program and predicted amino acid sequences were compared with protein sequence database NBRF by using the FASTA program. Dot-matrix comparisons of the predicted peptide sequence of halidohydrolase IVa with those of other dehalogenases used the COMPARE and DOT-plot programs. Secondary-structure predictions were obtained by using the algorithms of Garnier *et al.* (1978), Chou & Fasman (1978) and Kyte & Doolittle (1982) in the PEPTIDE STRUCTURE and PLOTSTRUCTURE programs. Computer analyses were done through the SERC SEQNET facility.

N-Terminal amino acid sequence determination

The N-terminal amino acid sequence of purified recombinant Hdl IVa protein from *E. coli* (pUKC510) was determined by using automated Edman degradation and h.p.l.c. analysis with an automated protein sequencer (model 8710; Jaytee Biosciences, Herne Bay, Kent, U.K.).

RESULTS

Defining the 2-haloacid halidohydrolase IVa structural gene (*hdl IVa*)

A genomic library of *Ps. cepacia* MBA4 was constructed as described in the Materials and methods section. Screening of the *E. coli* HB101 (RP4-5) transfectants for the Hdl⁺ phenotype identified only one of 480 recombinant transfectants that gave a positive result. The Hdl⁺ clone was designated UKC500, and cell extracts prepared from cultures of this strain were shown to contain a halidohydrolase that was electrophoretically indistinguishable from that of the *Ps. cepacia* MBA4 enzyme (Fig. 1a); this identity was confirmed by immunoblot analysis (Fig. 1b). The purified recombinant protein also demonstrated substrate-activity profiles identical with that of pure Hdl IVa produced from *Ps. cepacia* MBA4, and the N-terminal sequences of the two proteins were identical over all of the first 20 amino acid residues sequenced.

The recombinant plasmid (pUKC500) contained a 35 kb fragment of *Ps. cepacia* MBA4 chromosomal DNA, confirmed by DNA–DNA hybridization between it and *Ps. cepacia* MBA4 chromosomal DNA after electrophoresis on agarose gel (results not shown). Restriction fragments generated by cleavage of

Fig. 1. Comparison of wild-type and recombinant proteins

(a) Comparison of the halidohydrolase profiles of cell-free extracts prepared from batch cultures of *Ps. cepacia* MBA4 and *E. coli* DH5 α (pUKC500). The halidohydrolase profile of a separate bacterial isolate (*Microbacterium* sp. G14; J. S. H. Tsang, A. T. Bull & D. J. Hardman, unpublished work) is included as a guide to similarity. (b) Immunoblot analysis of cell-free extract of *Ps. cepacia* MBA4 and of purified recombinant halidohydrolase extracted from *E. coli* DH5 α (pUKC510).

Fig. 2. Structure of the *Ps. cepacia* MBA4 *hdl* IVa region

Vector systems are given in parentheses. (a) Restriction map of the 9.0 kb *Pst*I insert of pUKC502 (pBR322); (b) restriction map of the 1.6 kb *Eco*RI insert of pUKC504 (pBR322); (c) restriction map of the 1.6 kb *Eco*RI insert of pUKC511 (pBTac1); (d) restriction map of the 1.6 kb *Eco*RI insert of pUKC512 (pBTac1); (e) restriction map of the 0.9 kb *Pvu*II-*Eco*RI insert of pUKC510 (Δ pBTac1); (f) sequencing strategy for the 1.6 kb *Eco*RI insert encoding the *hdl* IVa region.

pUKC500 with *Pst*I and *Eco*RI were subcloned into pBR322 and the recombinant plasmids were transformed into *E. coli* HB101. A *Pst*I digest resulted in a 9.0 kb insert from which the *Hdl*⁺ phenotype was expressed (pUKC502; Fig. 2a) and *Eco*RI digests demonstrated that a 1.6 kb *Eco*RI fragment (pUKC504) was sufficient for expression of the *Hdl*⁺ phenotype (Fig. 2b). Further restriction-endonuclease analysis identified three *Pvu*II and two *Xho*I sites within the 1.6 kb fragment (Fig. 2b).

Table 1. Halidohydrolase specific activities in cell-free extracts of *Ps. cepacia* MBA4 and recombinant strains cultured in the presence or in the absence of isopropyl β -D-thiogalactoside or monochloroacetic acid as an inducer

Strain	Specific activity (μ mol of monochloroacetate converted/min per mg of protein)	
	Uninduced	Induced
<i>Ps. cepacia</i> MBA4	0.005	1.00*
<i>E. coli</i> DH5 α (pUKC504)	0.04	0.04*
<i>Ps. putida</i> KT2441 (pUKC505)	0.06	0.10*
<i>E. coli</i> DH5 α (pUKC511)	0.20	0.40†
<i>E. coli</i> DH5 α (pUKC512)	0.04	0.04†

* Inducer: monochloroacetic acid.

† Inducer: isopropyl β -D-thiogalactoside.

Table 2. Effect of temperature on expression of recombinant halidohydrolase activity in *E. coli* DH5 α

Activity from pUKC510 was expressed constitutively.

Strain	Growth temperature (°C)	Halidohydrolase specific activity (μ mol of monochloroacetate converted/min per mg of protein)
<i>E. coli</i> DH5 α (pUKC511)*	37	0.40
<i>E. coli</i> DH5 α (pUKC511)*	30	0.36
<i>E. coli</i> DH5 α (pUKC510)	37	20.67
<i>E. coli</i> DH5 α (pUKC510)	30	2.26
<i>E. coli</i> DH5 α (pUKC513)	37	17.43
<i>E. coli</i> DH5 α (pUKC513)	30	0.51

* Inducer: isopropyl β -D-thiogalactoside.

Expression of the recombinant *hdl* IVa gene in *E. coli* and *Ps. putida* hosts

Regulation of expression of the isolated *hdl* IVa gene was investigated by preparing cell-free extracts of *E. coli* UKC504 from cultures grown in the presence or in the absence of monochloroacetate and assaying for halidohydrolase activity. The results (Table 1) show that low-level constitutive halidohydrolase activity was expressed from an internal promoter in pUKC504 and that the level of activity was 25-fold lower than that of the induced levels of activity in the parental strain *Ps. cepacia* MBA4.

As the expression of *Pseudomonas* genes in *E. coli* has previously been seen to be relatively inefficient (Jeenes *et al.*, 1986), the *hdl* IVa coding sequence was transferred into *Ps. putida* KT2441 by subcloning the 16 kb *Eco*RI fragment from pUKC504 into the *Eco*RI site of pMMB33 to form a plasmid (pUKC505) that was then transformed into *E. coli* HB101(RP4-5). The recombinant plasmid was then mobilized into *Ps. putida* KT2441 and exconjugants were selected. Cell extracts prepared from one of the exconjugants grown in the presence and in the absence of monochloroacetate as an inducer of halidohydrolase activity showed that the 'induced' level of activity was 1.7-fold greater than the activity in 'uninduced' cells, but only represented 10% of the induced parental activity (Table 1).

In order to enhance expression of the *hdl* IVa gene in the *E. coli* host the 1.6 kb fragment was subcloned into the *tac* expression vector pBTac1 (Boehringer). The 1.6 kb fragment was inserted in

Fig. 3. Nucleotide sequence of the 1.6 kb *EcoRI* fragment containing the halidohydrolase structural gene *hdi* IVa, together with the upstream region with the deduced amino acid sequence of the halidohydrolase and a second open reading frame

The three initiation codons within the *hdi* IVa coding sequence are marked (see the text for details). The underlined portion of the deduced protein sequence was confirmed by direct *N*-terminal sequencing. * represents base number 729, the end point of the deletion in pUKC510.

both orientations with respect to the *tac* promoter. When in the correct orientation (pUKC511; Fig. 2c) the induced level of expression was 10-fold greater than from pUKC504. However, in the opposite orientation (pUKC512; Fig. 2d), only low-level constitutive expression, equivalent to that for pUKC504, was observed (Table 2). During this work the recombinant plasmids were placed in a number of *E. coli* host strains (C600, HB101 and DH5 α) and the best expression of halidohydrolase activity was obtained in strain DH5 α . The data presented in Table 1 were obtained with strain DH5 α as the host, and the cultures were incubated at 37 °C.

During construction of pUKC511, a deletion derivative (desig-

nated pUKC510) was isolated which demonstrated a temperature-sensitive enhanced level of expression of halidohydrolase activity. The growth temperature had little effect on the halidohydrolase specific activity of UKC511 but, when *E. coli* DH5 α (pUKC510) was cultured at 37 °C, the level of expression was 10-fold greater than that expressed in cells grown at 30 °C (Table 2) the halidohydrolase being equivalent to 20% of the total cellular protein of the recombinant strain. Even at 30 °C the expression was greater than 2-fold of the wild-type strain. Sequence analysis (results not shown) of pUKC510 (Fig. 2e) indicated that a deletion event had occurred at the *PvuII* site situated towards the middle of the fragment resulting in the loss of the first 729 bp of

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      .       .       .       .       .       .
MVDSLRACVFDAYGTLTLDVHSAVMRNAMEVGASAEALSMLWRQRQLEYSWTRTLMHQYAD  Hdl IVa
M-DPIRACVFDAYGTLTLDVNTAVMKHAHDIGGCAEELSSLWRQRQLEYSWTRTLMGRYAD  DehC I
M-QEIRGVVFDLYGTLCDVHSAQLCGQYFPERGTEISLMWRQRQLEYSWRLSTMGQYVS  DehC II
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

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Fig. 4. Comparison of the deduced amino acid sequences of Hdl IVa and DehC I and DehC II from *Pseudomonas* sp. CBS3 (Schneider *et al.*, 1991) over the 60 N-terminal amino acid residues

Amino acids identical in Hdl IVa and one of DehC I or DehC II are indicated by ●, and residues identical in all three halidohydrolases are indicated by *.

the original fragment. This left a 0.9 kb fragment, which was sufficient to encode the halidohydrolase gene, removed the *PvuII* site and created a new *HindIII* restriction site (Fig. 2e). The deletion event also resulted in the loss of a 2.0 kb sequence of the vector (pBTac1). In order to confirm that the temperature-sensitive expression was a consequence of an intrinsic property of the 0.9 kb fragment, the insert was subcloned into the *lac* expression plasmid pUC9, and this recombinant plasmid was designated pUKC513. When expression levels were compared at the two temperatures activity was again elevated at the higher temperature (Table 2).

Nucleotide sequence of *hdl* IVa gene and flanking regions

The 1.6 kb *EcoRI* fragment containing the 0.9 kb fragment known to encode the Hdl IVa protein was sequenced according to the strategy in Fig. 2(f). The entire sequence of the 0.9 kb fragment was determined for both strands. The nucleotide sequence of the 1.6 kb fragment is given in Fig. 3. Open reading frame analysis using the FRAMES program identified an open reading frame extending from a position close to the *PvuII* site in the middle of the fragment to the *XhoI* site close to the *EcoRI* site sufficient to encode the Hdl IVa protein. The predicted amino acid sequence for this region is shown in Fig. 3. The open reading frame started from ATG at position 833–835 and ended with the TGA codon at 1526–1528. This latter sequence would encode a polypeptide of 231 amino acid residues with a predicted M_r of 25900. Within the same reading frame there were two other ATG codons, at positions 901–903 and 946–948, that could give rise to polypeptides with predicted M_r values of 23500 and 22000 respectively (Fig. 3).

In order to define which was the initiation codon for Hdl IVa translation the N-terminal amino acid sequence of the recombinant enzyme was determined. The results of this analysis showed that the N-terminal sequence was identical with that predicted for the translation of the full 696 bp open reading frame (underlined portion of the protein sequence in Fig. 3). The open reading frame encoding the halidohydrolase was preceded (–3 bp) by a sequence, AGGAG, that compares well with known strong ribosome-binding sites, and an inverted repeat sequence was observed in the –30 bp region. Since the predicted M_r of the protein is 29000 greater than that observed by SDS/PAGE (Tsang *et al.*, 1988), it is possible that the protein shows anomalous migration in the gels. The G + C content of the structural gene and upstream sequences of the 16 kb fragment was 55%, and in the third position of the triplet the value was 63%.

Secondary-structure analysis of the predicted protein sequence gave an indication of the presence of two structural domains in the protein. Residues 1–130 were predicted to be α -helical over about two-thirds of the sequence, in stretches of 7–40 residues, broken by three or four turns. Very little β -sheet structure was predicted. These residues were probably tightly folded since the Kyte–Doolittle hydrophobicity analysis suggests a mixture of short regions (20–40 residues) of alternating hydrophobicity and hydrophilicity. The sequence represented by residues 130–231,

by contrast, was predicted to be structured such that less than one-third was α -helical in nature and indeed would appear to have a significantly less ordered structure.

Schneider *et al.* (1991) reported the complete nucleotide sequences of structural genes of two haloacid halidohydrolases from *Pseudomonas* sp. CBS3, designated *dehC* I and *dehC* II. The nucleotide sequences of the two halidohydrolases indicated coding capacities of 227 and 229 amino acid residues, which are very similar to the 231 residues predicted for the *Ps. cepacia* MBA4 enzyme. Comparison of the amino acid sequences predicted for the two halidohydrolases with that predicted for Hdl IVa (Fig. 4) revealed an 81% similarity between Hdl IVa and DehC I with a 67% exact identity value. When compared with DehC II the respective values were 56% and 37%. Multiple alignment analysis of the primary sequences of the three proteins indicated a conserved region of ten amino acid residues at positions 41–50 (Fig. 4).

Comparison by dot-matrix analysis of the predicted peptide sequence of Hdl IVa with the haloalkane halidohydrolase of *Xanthobacter autotrophicus* (Janssen *et al.*, 1989) and the GSH-dependent dichloromethane dehalogenase of *Methylobacterium* sp. DM4 (La Roche & Leisinger, 1990) indicated that no statistically significant similarity existed between the 2-haloacid halidohydrolase and the other enzymes.

DISCUSSION

In this paper we have reported the cloning and subsequent genetic and biochemical analysis of the halidohydrolase IVa structural gene (*hdl* IVa) from *Ps. cepacia* MBA4. The halidohydrolase gene was isolated on a 1.6 kb *EcoRI* fragment and the nucleotide sequence of the whole fragment was determined, and the *hdl* IVa coding region was identified. The polypeptide predicted as being derived from the whole open reading frame was 231 amino acid residues in length (M_r 25900).

However, two other initiation codons present in the same reading frame could also yield proteins of comparable size if initiation started from these sites under certain circumstances. N-Terminal amino acid sequencing of the recombinant protein confirmed that halidohydrolase IVa was derived from the first initiation codon at the start of the open reading frame. Nevertheless, the presence of the other two initiation codons raises the possibility that initiation events from one or other of them could result in the production of a second protein that could be identified as a different halidohydrolase. *Ps. cepacia* MBA4 possesses a second halidohydrolase (Hdl III; Tsang *et al.*, 1988).

Analysis of the nucleotide sequence upstream of the coding region revealed the presence of a ribosome-binding site at –3 bp from the initiation codon, and there was a 7 bp inverted sequence situated in the –30 bp region. It is plausible that this latter sequence represents a binding site for a regulatory protein, which, in turn, would suggest that the *hdl* IVa gene was under positive regulatory control. Upstream of the initiation codon there are two putative –10 regions (at positions 770 and 793) that show a close match with the consensus sequence (TATAAT)

of the -10 region of *E. coli* promoters, and sequences with 50% and 67% identity with the -35 consensus sequence (TTGACA) are also to be found a further 15 and 18 bases upstream of the putative -10 regions. These putative promoter regions appear to be sufficiently similar to the *E. coli* sequences to enable them to act as promoters in the *E. coli* host cells and may result in the constitutive low-level expression of the halidohydrolase in *E. coli* (pUKC504). A recent compilation of the data on pseudomonad promoters (Deretic *et al.*, 1989) indicated that they could be placed into distinct groups. Under this scheme the *hdl* IVa promoter regions in *Ps. cepacia* MBA4 belong to the group A70, which is positively regulated and most probably utilizes a pseudomonad (σ^{70} (*rpo D*)) RNA polymerase.

Placing the 1.6 kb *Eco*RI fragment adjacent to the *tac* promoter (pUKC511) increased recombinant halidohydrolase IVa expression 10-fold, when induced with isopropyl β -D-thiogalactoside, over the original clone (pUKC504), induced by 2-monochloropropionic acid. The induced activity from pUKC504 was in turn equivalent to 40% of the induced level in the wild-type strain. The halidohydrolase expression from pUKC510 was shown to be constitutive. Although the reason for this could not be determined by reference to sequence analysis of the deletion plasmid, it is possible that the internal promoter and operator sequences located downstream of the *tac* and *lacUV5* on pUKC510 and pUKC513 respectively, as part of the cloned and deleted fragment, disrupt or even abolish the repressibility of the expression-vector promoters. Comparison of the specific activities of the *E. coli* strains containing pUKC510 with that containing pUKC504 (Tables 1 and 2) indicates that the expression of the halidohydrolase activity in UKC510 was driven by the expression-vector promoter. Halidohydrolase IVa expression from this plasmid was also found to be temperature-dependent, showing a 10-fold higher specific activity at 37 °C than at 30 °C in *E. coli* DH5 α (pUKC510). In different host strains a similar temperature-dependency was observed [*E. coli* C600 (pUKC510) 3-fold and *E. coli* HB101 (pUKC510) 4-fold at 37 °C], indicating that the effect was not host-strain-dependent. The fact that the deleted insert (0.9 kb) was responsible for the temperature-dependency in strain DH5 α was confirmed by subcloning the insert into the *lac* expression system (pUKC513). Shinedling *et al.* (1987) reported mutations that affected the translation of the bacteriophage T4 *rII*B gene in *E. coli* and found that the initiation-codon pair AUGAUA, when the AUG was only two nucleotide residues from the Shine-Dalgarno sequence, resulted in a cold-sensitive phenotype. Consideration of the sequence reported in this paper shows that the Shine-Dalgarno sequence is situated -3 bp upstream of the *hdl* IVa initiation codon. Our results support the hypothesis of temperature-sensitive expression put forward by Shinedling *et al.* (1987), that is at the elevated temperature (37 °C) ribosomal elements are able to interact with the Shine-Dalgarno sequence and the closely associated initiation codon, but at the lower temperature the AUG cannot interact with the initiator tRNA. In this latter situation initiation may occur from the weaker Shine-Dalgarno sequence (GGAG) situated -14 bp from the AUG, so resulting in the decreased expression of recombinant halidohydrolase activity.

The G+C content of the coding region and the upstream sequences was considerably lower than that previously reported for other genes isolated from *Ps. cepacia*, *Pseudomonas multivorans* and *Pseudomonas aeruginosa* (Ballard *et al.*, 1970). Although one of the lowest G+C contents reported in a pseudomonad (50%) was that in the pilin genes of *Ps. aeruginosa* strains PAK and PAO (Sastri *et al.*, 1985), the low G+C content of the halidohydrolase gene may be an indication that it originated in a different organism and that its presence in this isolate was the result of acquisitive evolution. Such an explanation has been

offered for the *Ps. cepacia* AC1100 2,4,5-trichlorophenoxyacetic acid-degradative genes (Tomasek *et al.*, 1989).

Comparison of the amino acid sequences predicted for the two halidohydrolases from *Pseudomonas* sp. CBS3, designated DehC I and DehC II, with that predicted for Hdl IVa (Fig. 4) revealed a high degree of similarity between Hdl IVa and DehC I, whereas there was significantly less similarity to DehC II. It is noteworthy that the DehC I protein demonstrated a higher degree of sequence similarity to the Hdl IVa protein than to the second halidohydrolase possessed by the CBS3 strain. Indeed the amino acid sequence similarity of the two CBS3 proteins was no greater than that demonstrated between DehC II and Hdl IVa. The high level of similarity of Hdl IVa and DehC I may reflect a common ancestry of the two enzymes, an explanation that would be enhanced if *Pseudomonas* sp. CBS3 was shown to be a strain of *Ps. cepacia*.

Janssen *et al.* (1989) reported the expression and sequencing of a halidohydrolase-type haloalkane dehalogenase from *X. autotrophicus* GJ10, and La Roche & Leisinger (1990) published the sequence of the GSH-dependent dichloromethane dehalogenase. Comparison of both of these sequences with that of *hdl* IVa failed to reveal significant similarity between the different dehalogenases (results not shown). The lack of similarity to the *X. autotrophicus* enzyme is consistent with the observed lack of similarity between the haloalkane halidohydrolases and the haloacetate halidohydrolases present in *X. autotrophicus* (Janssen *et al.*, 1985, 1989; Keuning *et al.*, 1985).

We have previously suggested that the diversity of 2-haloacid halidohydrolase forms might have arisen as a result of laboratory-induced environmental selection, which resulted in the specialization of existing hydrolases (Tsang *et al.*, 1988). As such enzymic forms could have evolved from different ancestral proteins, so the plethora of halidohydrolase forms could be explained. The observed lack of sequence similarity between the two enzymes in *Pseudomonas* sp. CBS3 and the observed complete lack of similarity of N-terminal amino acid sequences between Hdl IVa and a second enzyme, Hdl V, previously studied in our laboratory (results not shown), is consistent with this hypothesis. However, with this hypothesis in mind we compared the *hdl* IVa nucleotide and the predicted peptide sequences with the relevant databases and searched for sequence similarities, especially with other hydrolases. No significant similarities were identified, confirming the results reported by Schneider *et al.* (1991). These results contrast with the observation made by Janssen *et al.* (1989), who found that the *Xanthobacter* haloalkane halidohydrolase gene contained a nucleotide sequence of approx. 80 bp that showed significant sequence similarity to human and rabbit epoxide hydrolases.

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