

Cloning and Characterization of a Cryptic Haloacid Dehalogenase from *Burkholderia cepacia* MBA4

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Burkholderia cepacia MBA4 has been shown to produce a single dehalogenase batch culture. Moreover, other cryptic dehalogenases were also detected when the cells were grown in continuous culture. In this paper, we report the cloning and characterization of one of the cryptic dehalogenases in MBA4. This cryptic haloacid dehalogenase, designated Chd1, was expressed constitutively in *Escherichia coli*. This recombinant Chd1 had a relative molecular weight of 58,000 and existed predominantly as a dimer. The subunits had a relative molecular weight of 27,000. Chd1 exhibited isomer specificity, being active towards the L-isomer of 2-monochloropropionic acid only. The structural gene, *chd1*, was isolated on a 1.7-kb *Pst*I fragment. This fragment contains a functional promoter, because expression of *chd1* in *E. coli* is orientation independent. The nucleotide sequence of this fragment was determined and characterized. An open reading frame of 840 bp encoding a putative peptide of 280 amino acids was identified. This corresponds closely with the size of the subunit. The nucleotide sequence of *chd1* did not show any homology with those of other dehalogenase genes. Comparison of the predicted amino acid sequence, however, shows significant homology, ranging from 42 to 50%, with the amino acid sequences of many other dehalogenases. Chd1 is unusual in having a long leader sequence, a property of periplasmic enzymes.

2-Haloacid dehalogenases, or halohydrolyses, are hydrolytic enzymes that cleave the halogen-carbon bond(s) in halogenated aliphatic acids, yielding hydroxy- or oxoalkanoic acids from a substrate with a mono- or disubstitution, respectively (14, 39). *Burkholderia cepacia* MBA4 (formerly considered a *Pseudomonas* species) was isolated from soil by batch enrichment culture with monobromoacetic acid (MBA) as the sole carbon and energy source. MBA4 is able to grow on MBA, monochloroacetate (MCA), 2-mono-chloropropionate (2MCPA), and 2-monobromopropionate (2MBPA) (43). This bacterium produces a single dehalogenase (DehIVa) in batch culture, and this enzyme has been purified and characterized (43). The active enzyme is a dimeric protein of 45 kDa. The structural gene for DehIVa, *hdlIVa*, was isolated from a genomic library (42), and analysis of the DNA sequence revealed an open reading frame (ORF) for 231 amino acids and a putative protein of 25.9 kDa (32).

Cryptic genes are silent DNA sequences; i.e., they are not normally expressed in an individual (13). Previous studies suggest the presence of cryptic dehalogenases in the gene pools of some dehalogenase-producing bacteria (15, 19, 38, 40, 45). During the course of studying the physiology of MBA4 in continuous culture, other dehalogenases were also detected by activity-stained gel electrophoresis and by a change in specific dehalogenase activity. These cells grew in a fraction of the maximum specific growth rate (35a, 41). However, because of their lack of expression in normal batch culture, the availability of these cryptic dehalogenases is scarce. It is therefore difficult to characterize these cryptic dehalogenases unless sufficient amounts of the enzymes can be obtained, for example, by means of heterologous expression.

Producing an enzyme in a heterologous host requires the

isolation of the corresponding structural gene. Cloning and expression of the MBA4 *hdlIVa* gene in *Pseudomonas putida* and in *Escherichia coli* suggested that the promoter of the *Burkholderia* gene is not regulated in these foreign hosts (42). These results provide the basis for the hypothesis that genes normally silent in *Burkholderia* could be expressed constitutively, although at a basal level, in *E. coli*. In this study, we report the cloning and characterization of one of the cryptic dehalogenases found in *B. cepacia* MBA4.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *B. cepacia* MBA4 (43) was used as the source of chromosomal DNA. *E. coli* XL1-Blue MR (Stratagene) was used for library construction, and strain TOP10F' (Invitrogen) was used as a host strain for gene cloning and plasmid construction. Plasmid pSP73 (Promega) was used as a cloning vector. *B. cepacia* was grown in an MBA medium as described previously (43). Recombinant *E. coli* cells were grown at 37°C in Luria broth (1% tryptone, 0.5% yeast extract) with or without 0.5% NaCl and supplemented with 50 µg of ampicillin per ml. *E. coli* IT41 (20) is temperature sensitive and was grown at 30°C unless otherwise specified.

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, and sequencing oligonucleotide primers were obtained from Gibco-BRL and Advanced Biotechnologies. Calf intestine alkaline phosphatase was purchased from Boehringer Mannheim. Deoxynucleotides and a sequencing kit including Cy5-labelled fluorescent DNA were obtained from Pharmacia. MCA was obtained from Sigma. All other chemicals were of analytical grade.

DNA manipulation and transformation. Restriction endonuclease treatments, alkaline phosphatase treatments, and ligations were performed according to the suppliers' recommended protocols. Plasmid DNA was prepared with a Qiagen plasmid kit. For analytical purposes, the boiling method of Holmes and Quigley (17) was used. DNA fragments were purified with a GeneClean kit (Bio 101) after agarose gel electrophoresis. Transformation of *E. coli* TOP10F' with plasmid DNA was performed by the CaCl₂ method (8).

Construction of a genomic DNA library and screening of dehalogenase-producing clones. A genomic DNA library of *B. cepacia* MBA4 was constructed in the cosmid vector SuperCos1 according to the manufacturer's protocol (Stratagene). The library DNA was packaged in vitro with Gigapack II XL packaging extract and transfected into *E. coli* XL1-Blue MR. Putative dehalogenase-producing transfectants were screened with a 96-well microtiter plate with MCA as the reaction substrate as described previously (32, 42). MCA was used because MBA is toxic to *E. coli* cells and medium containing MBA inhibited the growth of the bacterium (32, 42).

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Preparation of total protein extract and activity-stained PAGE. Total protein extracts of the cells were prepared by sonication (MSE Soniprep 150) or by two passages through a French press (SLM Aminco) at 40,000 lb/in². The remaining whole cells and cell debris were removed by centrifugation at 48,400 × *g* for 45 min. The relative mobilities of the dehalogenases were analyzed by nondenaturing activity-stained polyacrylamide gel electrophoresis (PAGE) (43) with MCA as the reaction substrate. Crude protein extracts prepared from *B. cepacia* MBA4 were used as positive controls to determine the relative mobilities of the tested enzymes.

Dehalogenase and protein assays. The dehalogenase assay was routinely carried out with MCA as the substrate, owing to its better stability than that of MBA. A standard assay mixture (1 ml) consisted of 50 mM MCA, 20 mM Tris-sulfate buffer (pH 7.9), and enzyme and was incubated at 30°C. The halide ions released were measured with a Chloride Analyzer 925 (Corning). When the substrate concentration was below 1 mM, chloride ions were determined spectrophotometrically (3). Protein concentrations were determined with Bio-Rad protein assay reagent.

Enzyme purification. All steps were carried out at 0 to 5°C unless otherwise specified. All buffers contained 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA to prevent inactivation of the enzyme.

Step 1. Preparation of crude cell extracts. Total protein extracts were prepared as described above.

Step 2. Protamine sulfate precipitation. Nucleic acids were removed from the crude extract by protamine sulfate precipitation. Protamine sulfate (0.4% [wt/vol] final concentration) was added to the cell extracts, with stirring, for 20 min, and the precipitated nucleic acids were removed by centrifugation at 48,400 × *g* for 45 min. The supernatant was dialyzed overnight against 20 mM Tris-sulfate (pH 7.5).

Step 3. Ammonium sulfate precipitation. The dialyzed enzyme preparation was fractionated by stepwise addition of solid ammonium sulfate to 10 to 60% saturation. At each step, the precipitate was collected by centrifugation at 9,000 × *g* and dissolved in 20 mM Tris-sulfate (pH 7.5). The active fractions at 10 to 30% were dialyzed overnight against Tris-sulfate.

Step 4. Anion-exchange chromatography. The dialyzed enzyme was applied to a column (2.6 by 10 cm) of Sepharose Q Fast Flow (Pharmacia), equilibrated with 20 mM Tris (pH 8.2). The column was eluted at a flow rate of 7 ml/min with 1 liter of a linear gradient of 0 to 1 M NaCl in the same Tris buffer. Dehalogenase was eluted at 0.33 to 0.41 M NaCl. The active fractions were combined and dialyzed against 20 mM Tris-sulfate (pH 7.5).

Step 5. Hydroxylapatite chromatography. The enzyme was applied to a column (10 ml) of Hydroxylapatite Fast Flow (Calbiochem) equilibrated with 20 mM Tris-sulfate (pH 7.5). The column was washed with 100 ml of the same buffer and stepwise elution was carried out with a linear gradient of 5 to 90 mM trisodium phosphate in 20 mM Tris-sulfate (pH 7.5). The active fractions, which were eluted at 10 to 15 mM buffer concentrations, were pooled, dialyzed, and concentrated with Centricon 30 (Amicon). The purified enzyme was stored at -20°C, with a negligible loss of activity, for a period of over 1 month.

Molecular weight determination. The native molecular weight of the protein was determined by gel filtration on a High Prep 26/60 Sephacryl S-200 high-resolution column (Pharmacia). The medium was equilibrated with 20 mM Tris-sulfate, pH 7.9. Cell extracts were applied to the column and eluted with the same buffer at a flow rate of 1 ml/min. The column was calibrated with blue dextran (2,000 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). The molecular weight of the partially purified denatured dehalogenase was determined by sodium dodecyl sulfate (SDS)-PAGE on a 12% gel (26). Rainbow molecular weight markers (Amersham) were used as standards, and the gels were visualized with Coomassie blue R-250.

N-terminal amino acid sequence determination. Protein samples were transferred onto a Hybond-P polyvinylidene difluoride membrane (Amersham) from a denaturing gel by using a Bio-Rad electroblotting system. Electroblotting was carried out in 10 mM (cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 11, containing 10% (vol/vol) methanol for 1 h at 75 V. The amino acid sequence was determined with a G1000 protein sequencer (Hewlett-Packard).

Nucleotide sequence determination and alignment. An automated DNA sequencer (ALF-express; Pharmacia) was used to determine the DNA sequences of the inserts, using Cy5-labelled nucleotides according to the protocol provided by the supplier (Pharmacia). The 1.7-kb *Pst*I fragment in pHKU104 was initially sequenced with primers SP6 and T7. New oligonucleotide primers were then designed according to the obtained sequencing data. The designed primers used for sequencing in this study were as follows: S1, GGACA GCAGG GAAGC CGTCA C; S2, CCATC TATCT GCGTG TTTGA; S3, TATGG GACAC AATTG GTGCG; S4, TTTGC CAGGA GTCGC TTCCC; T1, GACTT TT GCG CCTGT CAGCA; T2, GAGTA CAAGA TAAGC TGATT; T3, ATATC CATAT CATTG CCGTC; and T4, TTTTT GTTAG TTAGA TATCC. The fragment was sequenced in both directions. The nucleotide sequences and the predicted amino acid sequences were analyzed by University of Wisconsin Genetics Computer Group (GCG) programs and by National Center for Biotechnology Information programs. Nucleotide sequences were compared with those in the EMBL nucleotide sequence data library. Amino acid sequences were compared with those in the SWISS-PROT protein database. Alignment of the

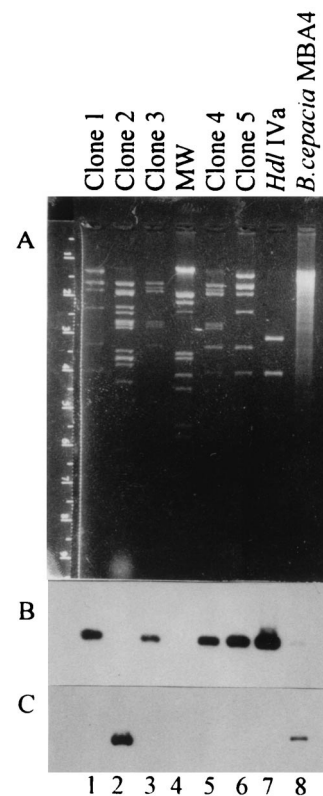


FIG. 1. Agarose gel electrophoresis of plasmids isolated from various clones. (A) Ethidium bromide-stained gel. (B) Southern blot hybridized with probes containing the *hdlIVa* gene. (C) Southern blot hybridized with probes containing the *chdI* gene. Lanes 1 to 3, 5, and 6 contain plasmid DNAs isolated from various clones and digested with *Eco*RI. Lane 7 contains a plasmid bearing the *hdlIVa* gene in a 1.6-kb *Eco*RI fragment. Lane 4 contains a molecular weight (MW) ladder (lambda DNA cut with *Hind*III and *Eco*RI). Lane 8 contains an MBA4 total DNA digested with *Eco*RI.

sequence of *Chd1* with those of other dehalogenases was performed with the GCG Pileup program.

Primer extension. The transcriptional start site was mapped by primer extension analysis (4). Total RNAs were isolated from cultures of HKU103 with the High Pure RNA isolation kit (Boehringer Mannheim). The Cy5-labelled primer (5'-GTCTCCGAACACACAGCTGA-3') was complementary to the sequence at positions 822 to 804. The RNA-primer mixture, containing 5 µg of total RNA and a 2 µM concentration of the primer, was incubated at 70°C for 10 min and quenched on ice for 1 min. The reverse transcription reaction was performed by first-strand cDNA synthesis with the SuperScript preamplification system (Gibco-BRL) according to the manufacturer's protocol. The size of the primer extension product was estimated by parallel sequencing reactions on pHKU103 by using the same primer. The transcriptional start site was determined with the ALF-express DNA sequencer.

Southern blot analysis. The enhanced chemiluminescence method (Amersham) was used for Southern hybridization. DNA fragments were labelled according to the manufacturer's protocol and hybridized to DNA-containing Hybond-N membranes.

Nucleotide sequence accession number. The nucleotide sequences presented here have been submitted to the EMBL database under accession no. AJ005843.

RESULTS

Cloning of the structural gene (*chdI*) for the cryptic haloacid dehalogenase from MBA4. Among 1,200 ampicillin-resistant transfectants, five putative dehalogenase-positive clones, which produced white precipitates upon addition of silver nitrate, were obtained. Total protein extracts were prepared from these putative dehalogenase-producing clones and analyzed by activity-stained PAGE. Clones 1, 3, 4, and 5 produced a dehalogenase migrating similarly to DehIVa. On the other hand, clone 2 produced a novel dehalogenase migrating

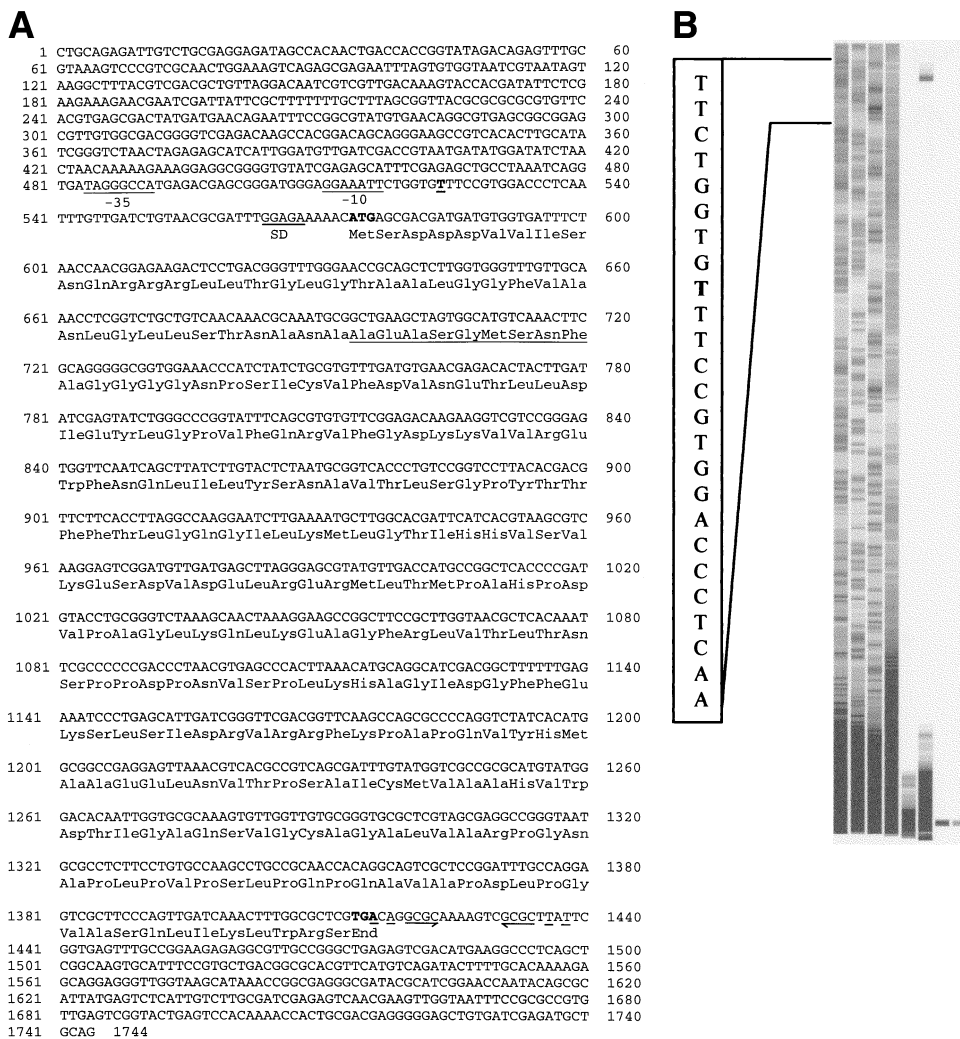


FIG. 2. Structure of the *chd1* gene. (A) Nucleotide sequence. The 1.7-kb *Pst*I fragment contains an ORF of 840 nucleotides putatively encoding a protein of 280 amino acids. The deduced amino acid sequence is shown below the nucleotide sequence. Possible -10 and -35 consensus sequences of *E. coli* and a possible Shine-Dalgarno (SD) sequence are indicated. The transcriptional start site is in boldface and underlined. The underlined portion of the deduced protein sequence was confirmed by direct N-terminal sequencing. (B) Mapping of the transcriptional start site of *chd1*. The products of primer extension and a parallel sequencing reaction were analyzed by the ALF-express DNA sequencer. The gel image was generated by the sequencer. The sequence illustrated represents positions 516 to 540. The end of the extension product is determined to be the T (in boldface) at position 524.

slower than DehIVa (data not shown). This dehalogenase was tentatively named Chd1 to indicate its cryptic nature.

The corresponding plasmids of the clones were isolated and cut with *Eco*RI restriction endonuclease. Figure 1A shows that they give different restriction patterns, indicating their independence in origin (lanes 1 to 3, 5, and 6). In order to eliminate DehIVa-producing clones, Southern blot analysis was carried out. Figure 1B shows that clones 1, 3, 4, and 5 (lanes 1, 3, 5, and 6, respectively) exhibited positive signals when hybridized with a probe containing the *hdIIVa* gene. This result suggested that four out of the five dehalogenase-producing clones contained DehIVa. Only clone 2 (lane 2) did not exhibit any hybridization signal. The structural gene for Chd1 was cloned (see below) and used as a probe. Figure 1C shows the specific hybridization of this *chd1*-containing probe with DNA of the various clones and with the total DNA of MBA4.

Subcloning of the DNA fragment for sequence analysis. The plasmid pHKU100, isolated from clone 2, was ca. 50 kb in size. In order to identify the location for the structural gene, *chd1*, of the cryptic dehalogenase, the plasmid was subjected to fur-

ther subcloning. Plasmid DNA was partially digested with *Sau*3AI and ligated to the *Bam*HI site of pSP73 (Promega). The ligation products were transformed into *E. coli* TOP10F', and transformants were screened for dehalogenase production. This produced plasmid pHKU101, which contains *chd1* in a 10-kb fragment. Plasmid pHKU101 was then subjected to *Eco*RI digestion, and a 3-kb fragment conferring dehalogenase activity was cloned into pSP73 to form pHKU102. This plasmid was then cleaved with *Pst*I, and a 1.7-kb fragment was cloned into pSP73 in both orientations to form pHKU103 and pHKU104. Subcloning of *chd1* was confirmed by the hybridization of the 1.7-kb *Pst*I fragment to DNA of all the sub-clones (data not shown). Cells harboring either pHKU103 or pHKU104 were dehalogenase positive (data not shown). This suggested that the 1.7-kb *Pst*I fragment contains a promoter that is functional in *E. coli*.

Nucleotide sequence of the *chd1* gene and flanking regions. The nucleotide sequence of the 1,744-bp insert in pHKU103 was determined on plasmid DNA templates by an automated DNA sequencer (Pharmacia). Primers SP6 and T7, annealing

TABLE 1. Purification of Chd1

Purification step	Total protein (mg)	Total activity (U)	Sp act (μmol of $\text{Cl}^-/\text{min}/\text{mg}$ of protein)	Yield (%)	Purification (fold)
Crude extract	2,880	63,360	22	100	
Protamine sulfate	2,323	58,075	25	92	1.1
$(\text{NH}_4)_2\text{SO}_4$	1,211	44,807	37	71	1.7
Sepharose Q	96	19,968	208	32	9.5
Hydroxylapatite	24	9,840	410	16	18.5

to the nucleotide sequences flanking the insert, were used. Other primers corresponding to sequences found within the inserts were also used. The nucleotide sequence is given in Fig. 2A. An ORF consisting of 840 nucleotides started from the ATG beginning at position 574 and ended with codon TGA beginning at position 1414. This sequence would encode a protein of 280 amino acid residues with a predicted molecular weight of 29,939.

Within the same reading frame another ATG codon starting at position 709 could give rise to a protein with a predicted molecular weight of 25,439. In order to define the initiation codon for Chd1, the N-terminal amino acid sequence of the recombinant protein was determined. The results showed that the nucleotide sequence corresponding to the N terminus started between the first and the second ATG codons (Fig. 2A).

A postulated Shine-Dalgarno (GGAGA) region was found close to the first ATG of the gene, beginning at position 564. Figure 2B is an image of the primer extension result showing the start of the transcript at the T at position 524. Possible -35 and -10 consensus sequences beginning at positions 484 (TA GGGCCA) and 512 (GGAAATT) were detected. Downstream of the structural gene, a putative stem-loop structure was detected between nucleotides 1416 and 1438, followed by a stretch of T residues.

Enzyme purification and biochemical characteristics. Total protein extracts were prepared from cells harboring pHKU103. The enzyme was purified 18.5-fold, with an overall yield of 15%, and the dehalogenase accounted for 5.5% of the total soluble cellular protein (Table 1). The purified enzyme preparation was considered to be 90% homogeneous when electrophoresed by denaturing PAGE (Fig. 3). The molecular mass of the dehalogenase was estimated to be 58 kDa by gel filtration chromatography on Sephacryl S-200, while in SDS-12% PAGE the apparent molecular mass was 27 kDa.

The purified enzyme was most active towards MBA, with specific activities of 5.44, 1.5, 4.56, and 1.95 μmol of halide released/min/mg of protein for MBA, MCA, 2MBPA, and 2MCPA, respectively. When activities towards D- and L-2MCPA were considered individually, the enzyme was found to be active towards the L-isomer only. The enzyme was not active towards dichloroacetate or chlorobutane. The Michaelis constant (K_m) for MBA at 25°C over the substrate range of 0.1 to 3.0 mM was 1.13 mM, and a V_{max} of 30 $\mu\text{mol}/\text{min}/\text{mg}$ was calculated.

The pH curve was bell shaped, with optimum activity at pH 6.5. The enzyme was also sensitive to heat treatment. A preincubation at 35°C for 15 min abolished 20 to 25% of the enzyme activity. The enzyme was not inactivated by incubation with 1 mM EDTA, CaCl_2 , MnCl_2 , NiCl_2 , ZnCl_2 , hydroxylamine, iodoacetate, N-ethylmaleimide, or FeSO_4 but was effectively inhibited by HgCl_2 . The enzyme was partially inhibited by CuSO_4 (41%), RbCl_2 (70%), and ρ -mercuric chlorobenzoate (87%).

DISCUSSION

In this paper, we have reported the cloning and characterization of a cryptic dehalogenase, Chd1, from *B. cepacia* MBA4. This dehalogenase was not detected previously in MBA4 as visualized by activity-stained PAGE, which can differentiate dehalogenases according to their electrophoretic mobilities. In previous experiments using continuous-culture techniques, at least two other dehalogenases were detected (41). The genes for these previously identified dehalogenases were not isolated in the present study. It is likely that many of the bacterial promoters did not function properly in *E. coli*, leading to a lack of expression. It is common for dehalogenases expressed in soil isolates to not be expressed in *E. coli* (16, 25). Moreover, many natural isolates do contain unexpressed cryptic dehalogenases (16, 19, 25).

We have purified and characterized Chd1. It is a 2-haloacid dehalogenase with substrate specificity towards the L-isomer only. This protein, however, has some unique properties. Unlike previously isolated dehalogenases for which the optimal pH is alkaline (9, 11, 12, 24, 27, 30, 31, 46), the present enzyme is most active at pH 6.5. Comparison of the ORF for Chd1 with those for other dehalogenases (Fig. 4) shows that Chd1 possesses a long N-terminal peptide not found in other dehalogenases. Primer extension results confirmed that the transcript is sufficient to provide a polypeptide to start at the first AUG of the ORF. N-terminal amino acid sequence determination also confirmed that the protein starts upstream of the second in-frame methionine. The putative N-terminal sequence also exhibited the signal peptide property of periplasmic enzymes at Ser-Thr-Asn-Ala-Asn-Ala-↓-Ala-Glu-Ala, where the arrow indicates the postulated cleavage site (34). This fits well with the N-terminal amino acid sequence of the purified protein, which starts at Ala-Glu-Ala. Periplasmic proteins were isolated from cells harboring pHKU103 by a quantitative method (1). This protein fraction was shown to contain dehalogenase activity (data not shown). Plasmid pHKU103 was also transformed into *E. coli* strain IT41 (20), which contains a temperature-sensitive leader peptidase gene. Figure 5 shows that precursor molecules of Chd1 were detected, with a concomitant decrease in the cleaved protein, in cells grown at a non-

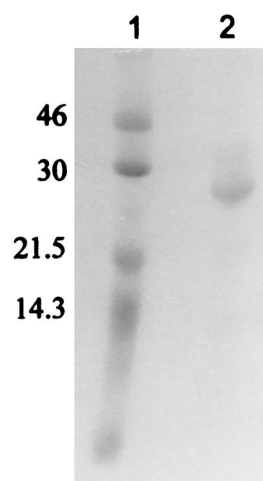


FIG. 3. SDS-PAGE of the purified dehalogenase on a 12% gel. Lane 1, molecular mass standards. Lane 2, 15 μg of the purified enzyme preparation. The gel was stained with Coomassie blue R-250. Numbers on the left are molecular masses, in kilodaltons.

HdlIva	1MVDLSLRACV
DehCI	1YDPIIRACV
HadL	1MKNIQGIV
DhlVII	1MKNIQGAV
DehH109	1MQPIEGIV
LdexYL	1MDYIKGIA
DehH2	1MKKIEAIA
DehCII	1MQEIRGVV
DhlB	1MLKAVV
DehCrp1	1	MSDDDVVISNQRRRLLTGLGTAALGGFVANLGLLSTNANAAEASGMSNFAGGGGNPISICV
HdlIva	10	FDAYGTL L DVHSA V M R NA EV GASAE ALS ML WR Q R Q LE YSW TR TL M HQ Y AD FW Q L T DE AL
DehCI	9	FDAYGTL L DVNTA V M K HA HD IGC AE EL SSL WR Q R Q LE YS W TR TL M G RY AD FW Q L T DE AL
HadL	9	FDLYGTL Y DVHSV V Q ACE EV Y PG Q G DA IS RL W R Q Q LE Y T W L RS L M G RY V N F E K ATE D AL
DhlVII	9	FDLYGTL Y DVNSV A Q V C EE V Y SG H G DS IS RL W . Q K Q LE Y T W L RS L M G RY V N F E K ATE D AL
DehH109	9	FDLYGTL Y DVHSV V Q ACE S A Y PG Q GE AI S RL W R Q Q LE Y T W L S S L M G RY AS F E Q R T E AL
LdexYL	9	FDLYGTL F DVHSV V G R C DE A F P G R GRE IS AL W R Q Q LE Y T W L RS L M N RY V N F Q Q ATE D AL
DehH2	9	FDLYGTL Y DVHSV V D ACE K Q Y PG K G K D IS V L W R Q Q LE Y A W L R C L M G Q Y I K F E E AT A N A L
DehCII	9	FDLYGTL C DVHSV A Q L C G Q Y F PER G TE IS L M W R Q Q LE Y S W L RS L M G Q Y V S F P Q A T E D A L
DhlB	7	FDAYGTL F DVQ S V AD A T E R A Y P G R GE Y I T Q V W R Q Q LE Y S W L R A L M G RY AD FW G V T R E A L
DehCrp1	61	FDVNET LL DIE Y L GP V F Q R V FG D K.. K V V RE W F N Q L L D Y S NA V T L S G P Y T T F T L G O G I L
HdlIva	70	T F A L R T Y H L E D R K G L K D R L M S A Y K E L S A Y P D A A E T L E K L R S A G Y I V A I L S N G N D E M L Q A A
DehCI	69	D F A L E S F G L L E R T D L K N R L L D A Y H E L S A Y P D A V G T L G A L K A A G F T T A I L S N G N N E M L R G A
HadL	69	R F T C T H L G L S L D D E T H Q R L S D A Y L H L T P Y A D T A D A V R R L K A A G L P L G I S N G S H C S I E Q V
DhlVII	68	R F T C T H L G L S L D D E T H Q R L S D A Y L H L T P Y A D T A D A L R R L K A A G L P V G I S N G S H C S I E Q V
DehH109	69	R Y T C K H L G L A T D E T L R Q L G O A Y L H L A P H P D T T A L R R L K A S G L P M A I S N G S H S I E Q V
LdexYL	69	R F T C R H L G L D D A R T R S T L C D A Y L R L A P F S E V P D S L E L R R R G L K L A I L S N G S P O S I D A V
DehH2	69	T Y T C N Q M K L D C D E G S A M R L T E Y L R L K P F E V R G A L R A L R Q R G M R L A I L S N G S T E T I H D V
DehCII	69	V E F C N A L N L K L R E D T R I A L C N E Y L N I K P Y R E V R S A L E S L R S G A V P L A I L S N G S A H S I Q S V
DhlB	67	A Y T L G T L G L E F D E S F L G M A Q A Y N R L T P Y P D A A Q C L A E L.. A P L K R A I L S N G A P D M L Q A L
DehCrp1	119	K M L G T I H H V S V K E S D V D E L R R E R M L T M P A H P D V P A G L K Q L K E A G F R L V T L T M S P D P N V S P
HdlIva	130	L K A S K L D R V L D S C L S A D L K I Y K P D R I Y Q F A C D R L G V N P N E V C F V S S N A W D L G A G K F G
DehCI	129	L R A G N L T E A L D Q C I S V D E I K I Y K P D R V Y Q F A C D R L D V R P S E V C F V S S N A W D I G A G A F G
HadL	129	V T N S E M N W A F D Q L I S V E D V Q V F K P D S R V S L A E K R M G F P K E N I L F V S S N A W D A S A A S N F G
DhlVII	128	V T N S E M N W A F D Q L I S V E D V Q V F K P D S R V S L A E K R M G F P K E N I L F V S S N A W D A S A A S N F G
DehH109	129	V S H S D M G W A F D H L I S V E T V K V F K P D N R V S L A E Q T M A T P R D R L F V S S N S W D A T G A R H F G
LdexYL	129	V S H A G L R D G E D H L S V D P V Q V Y K P D N R V Y E L A E Q A L G L D R S A I L F V S S N A W D A T G A R Y F G
DehH2	129	V H N S G V E G E F F E H L I S V D S A R A Y K P H P L A Y E L G E A F G I S R E S I L F V S S N F P D V S C A K A F G
DehCII	129	V G N A G I E H F F S H L I S A D E V S V S K P S P A A Y E L A E K R L K V R S K L L F V S S N A W D A S G A R H F G
DhlB	125	V A N A G L T D S F D A V I S V D A K R V F K P H P D S Y A L V E E V L G V T P A E V L F V S S N C F D V G A K N F G
DehCrp1	179	L K H A G I D G F F E K S L S I D R V R R F K P A P Q V H M A A E L N V T P S A I C M V A A H V W D T I G A Q S V G
HdlIva	190	F N T V R I N R Q G N P P E Y E.. F A P L K H Q V N S L S E L W E L L A K N V T K A A*.....
DehCI	189	F N T V R I N R I N K P Q E Y S.. F A P Q R H Q L S S L S E L F Q L L R L T Q*.....
HadL	189	F P V C W I N R.. Q N G A F D E L D A K P H V V R N L A E M S N W L V N S L D.....
DhlVII	188	F P V C W I N R.. Q N G A F D E L D A K P H V V R N L A E M S N W L V N S L D.....
DehH109	189	F P V C W I N R.. Q N G A F D E L G A T P T R E V R D L G E M S D W L D
LdexYL	189	F P T C W I N R.. T G N V F E M G Q T P D W E V T S L R A V V E L F E T A A G K A E G*.....
DehH2	189	Y Q V C W I N R.. Y G F A F D E L G Q T P D F T V P V M D A I V H L I A V.....
DehCII	189	F Q V C W I N R.. S R N T F E Q L G E R P D H V I S G L D E L P N L I N F A S A D R *.....
DhlB	185	F S V A R V A R L S Q E A L A R E L V S G T I A P L T M F K A L R M R E E T Y A E A P D F V V P A L G D L P R L V R G M
DehCrp1	239	C A G A L V A R F G N A P L P V P S L P Q E Q A V A P D L P G V A S Q L I K L W R S*.....
HdlIva	
DehCI	
HadL	
DhlVII	
DehH109	
LdexYL	
DehH2	
DehCII	
DhlB	245	AG A H L A P A V
DehCrp1	

FIG. 4. Comparison of haloacid dehalogenases. The amino acid sequences of haloacid dehalogenases from *B. cepacia* MBA4 (DehIVa and Chd1 [DehCrp1]) (accession no. Q51645 [29; also this study]), *Pseudomonas* sp. strains CBS3 (DehCI and DehCII) (accession no. P24069 and P24070 [33]) and YL (LdexYL) (accession no. Q53464 [30]), *P. putida* AJ1 (HadL) (accession no. A44830 [18]) and 109 (DehH109) (accession no. Q59728 [19]), *X. autotrophicus* GJ10 (DhlB) (accession no. Q60099 [41]), *P. fluorescens* (DhlVII) (accession no. Q59666 [16]), and *Moraxella* sp. strain B (DehH2) (accession no. Q01399 [20]) are aligned. The numbers on the left are the residue numbers of each amino acid sequence. The conserved residues are boxed and shaded.

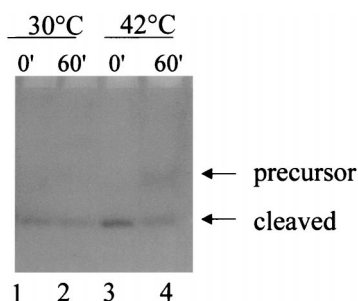


FIG. 5. Accumulation of precursor proteins in a temperature-sensitive leader peptidase mutant. IT41(pHKU103) cells were grown at 30°C until an optical density at 600 nm of 0.55 was reached and then were divided into two aliquots. The two cultures were then incubated at 30 or 42°C. Total protein extracts were prepared from samples taken at 0 and 60 min and analyzed on an activity-stained gel. The substrate used for visualization of the dehalogenase was MBA. "Cleaved" refers to cleaved dehalogenase.

permissive temperature (compare lanes 3 and 4). These data strongly suggested that Chd1 is a periplasmic protein.

Activity-stained PAGE analysis indicated that Chd1 has a smaller relative mobility value than does DehIVa of MBA4. DehIVa is a dimeric protein of 45 kDa with a subunit size of 23 kDa (43). SDS-PAGE analysis of purified Chd1 suggested that it has a subunit size of ca. 27 kDa. Its shorter migration distance in activity-stained PAGE and the gel filtration chromatography results indicate that Chd1 is most likely a dimer consisting of two identical subunits. Thus far, most of the dehalogenases isolated are dimers (21, 22, 29, 30, 43), although monomeric and tetrameric dehalogenases have also been detected in nature (21, 24, 28, 44).

The GCG program FASTA has been used to search for sequences similar to the *chd1* gene sequence. No significant homology was discovered among sequences in the EMBL database. However, when the National Center for Biotechnology Information program BLASTP was used to search for similarity with the predicted sequence of Chd1, a few dehalogenases showing significant amino acid sequence homology were obtained. The observed lack of DNA sequence homology among the dehalogenases is consistent with the hypothesis that the enzymes resulted from specialization of existing hydrolases (23, 25, 43, 46).

The protein sequences of these dehalogenases were then compared with the predicted Chd1 amino acid sequences by using the GCG program BESTFIT. The gap creation penalty and the gap extension penalty set for the BESTFIT program were 3 and 1, respectively. The similarities with the other dehalogenases are as follows: *B. cepacia* MBA4 (32), 49% (HdIVa); *Pseudomonas* sp. strain CBS3 (36), 50% (DehCI) and 42% (DehCII); *Pseudomonas* sp. strain YL (33), 48%; *P. putida* AJ1 (21), 47% (HadL); *P. putida* 109 (22), 45% (DehH109); *Xanthobacter autotrophicus* GJ10 (44), 49% (DhIB); *Pseudomonas fluorescens* (18), 47% (DhIVII); and *Moraxella* sp. strain B (23), 45% (DehH2). Phylogenetic analysis was performed by using the PHYLIP package (version 3.5). A phylogenetic tree of haloacid dehalogenases was constructed by the neighbor-joining method (35), with 100 bootstrapped replicates (10). All the dehalogenases analyzed were closely related to each other, with Chd1 most closely related to DehCI of *Pseudomonas* sp. strain CBS3 and to DehIVa (data not shown).

Haloacid dehalogenases have also been isolated in other types of bacteria, such as *P. putida* (2), *Rhizobium* (7), *Alcaligenes xylosoxidans* subsp. *denitrificans* (6), and *Agrobacterium tumefaciens* (37). However, no significant homologies were de-

tected by comparing the nucleotide or the deduced amino acid sequences of these enzymes with those in the EMBL database (5, 7). The molecular characterization of dehalogenases awaits future investigation.

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