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Complete Nucleotide Sequences and Comparison of the Structural Genes of Two 2-Haloalkanoic Acid Dehalogenases from *Pseudomonas* sp. Strain CBS3

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The nucleotide sequences of two DNA segments from *Pseudomonas* sp. strain CBS3 that code for two different haloalkanoic acid halidohydrolases were determined. Two open reading frames with coding capacities of 227 amino acids (corresponding to a molecular mass of 25,401 Da) and 229 amino acids (corresponding to a molecular mass of 25,683 Da) were identified as structural genes of 2-haloalkanoic acid dehalogenases I (*dehCI*) and II (*dehCII*) by comparison with the N-terminal amino acid sequences of these enzymes. Comparison of the two sequences revealed 45% homology on the DNA level and 37.5% homology on the amino acid level. No homology with other known protein or nucleotide sequences was found.

Chlorinated hydrocarbons are widely used as herbicides, fungicides, and insecticides (20). A variety of microorganisms that contain enzymes which remove the halogen substituent from various halogenated compounds has been isolated (4, 19–21, 28). In the case of chlorinated alkanoic acids, these bacteria very often contain several isoenzymes with the ability to dehalogenate these substrates. Generally, two (9, 30) or three (16) of these enzymes were detected in a single strain. Hardman (5a) has suggested that microorganisms that express two or more independently regulated dehalogenases have growth advantages under fluctuating environmental conditions.

One explanation for the evolution of such multiple enzymes suggests that gene duplications and subsequent mutations account for the differences in the enzymes (30).

We have isolated a bacterial strain that utilizes 4-chlorobenzoate as the sole carbon and energy source (11). In addition to the enzyme that dehalogenates 4-chlorobenzoate (27) to 4-hydroxybenzoate, this strain contained several other dehalogenating enzymes. A two-component enzyme system termed 4-chlorophenylacetic acid 3,4-dioxygenase dehalogenated 4-chlorophenylacetic acid (12) to 3,4-dihydroxyphenylacetate. Two 2-haloalkanoic acid dehalogenases which catalyze hydrolytic dehalogenation of monochloroacetate (MCA) and 2-monochloropropionate have been found in this strain. One of these enzymes has been purified to homogeneity (10). Both enzymes dehalogenate only L-2-monochloropropionate to D-lactate, but neither enzyme showed activity with D-2-monochloropropionate; however, the conversion rates for several substrates were different for the two enzymes. These enzymes seem to represent ideal models for derivation of further evidence concerning the origin and evolution of isofunctional dehalogenases.

In another report, we describe the cloning of the two 2-haloalkanoic acid dehalogenase genes from *Pseudomonas* sp. strain CBS3 and their expression in *Escherichia coli* (21a). Here we report the subcloning, complete nucleotide sequences, and comparison of the nucleotide and amino acid sequences of these enzymes.

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MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, and alkaline phosphatase were from Gibco-BRL (Eggenstein, Federal Republic of Germany [FRG]); Cyclone I Biosystem was from IBI (New Haven, Conn.); the T7 Sequencing kit and Deaza T7 Sequencing mixes were from Pharmacia LKB (Freiburg, FRG); [α-^{35}S]dATP was from Amersham (Braunschweig, FRG); MCA sodium salt was from Riedel-de Haën AG (Seelze, FRG).

Bacterial strains and plasmids. High-copy-number plasmid pUC18 was used as a cloning vector (31). *E. coli* TG1 (42) was the recipient strain in transduction and transformation experiments. M13 vectors mp18 and mp19 were used for DNA sequence analysis (31). The sources of DNA for subcloning and nucleotide sequencing were plasmids pUK1035 and pUK1164.

Growth conditions. *E. coli* clones were grown at 37°C in LB broth (18) or on LB agar plates supplemented with ampicillin (100 μg/ml). For protein expression, cells were grown in modified LB broth in which NaCl had been replaced by 0.3% MCA.

DNA manipulations and transformations. Preparative amounts of plasmid DNA were obtained by the method of Clewell and Helinski (1). For analytical purposes, plasmid DNA was isolated by the boiling method of Holmes and Quigley (6). Agarose gel electrophoresis, DNA digestions with restriction enzymes, treatments with alkaline phosphatase, and ligations were done by standard procedures (18). DNA fragments were isolated from agarose gels by the freeze-squeeze method (26). Transformation of *E. coli* with plasmid DNA was performed by the $CaCl_2$ procedure (17).

Recombinant plasmid construction. Restriction fragments of plasmids pUK1035 and pUK1164 were inserted separately into vector pUC18 (Fig. 1). After transformation of *E. coli* TG1, ampicillin-resistant white colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates were screened for the presence of dehalogenase I or II activity.

Identification of dehalogenase I- and II-containing clones. To check *E. coli* clones for the presence of dehalogenase activities, cells were incubated in liquid medium containing MCA. Positive clones were identified by an increase in chloride concentration during incubation. Free chloride ions were determined by a Marius Chlor-O-Counter (Labo International, Delft, The Netherlands) as described by Slater et al. (23).

Measurement of dehalogenase activity in crude extracts. Preparation of crude extracts and measurements of dehalogenase activities against MCA in crude extracts were carried out as described by Slater et al. (23). Positive clones were grown in liquid medium with or without MCA as an inducer. Cultures were harvested at the end of the exponential growth phase.

DNA sequence analysis. The plasmid DNAs of active dehalogenase I- or II-producing clones were isolated for subcloning into M13mp18 and M13mp19. The insert DNAs of these clones and fragments of these inserts generated by digestion with different restriction enzymes were introduced into vector M13. Additionally, two sets of deletion clones were constructed from the *Bam*HI fragment that codes for dehalogenase II with the Cyclone I Biosystem according to manufacturer instructions. Transfection of *E. coli* with M13 phage DNA, preparation of single-stranded DNA from selected phage plaques, and dideoxy sequencing reactions were performed according to the instruction manual provided by Pharmacia with the sequencing kit for the use of [α-^{35}S]dATP. All stretches of DNA were sequenced in both directions by the dideoxynucleotide sequencing method of Sanger et al. (22). Sequence ladders were resolved on gels containing 4 to 8% polyacrylamide.

The nucleotide sequences were analyzed with the GEN MON program (Gesellschaft für Biotechnologische Forschung, Braunschweig, FRG). Amino acid sequences were compared with the SWISS-PROT protein data base. Nucleotide sequences were compared with the European Molecular Biology Laboratory nucleotide sequence data library.

Purification, electroblotting, and N-terminal amino acid sequence determinations of both haloalkanoic acid dehalogenases. Dehalogenase I was isolated as previously described (10). Partially purified dehalogenase II was kindly provided by F. Mörsberger (Institut für Grenzflächen- und Bioverfahrenstechnik, Fraunhofer-Gesellschaft Stuttgart, Stuttgart, FRG). Dehalogenase II was localized after electrophoresis on 8% nondenaturing polyacrylamide gel as described by Weightman and Slater (29). Active protein was eluted from this gel by incubating the gel slices at 5°C in 50 mM potassium phosphate buffer (pH 7.5) for 15 h. Protein samples were then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli (14) with a 12.5% polyacrylamide gel.

After electrophoresis, proteins were transferred onto a polyvinylidine difluoride Immobilon membrane (Millipore, Eschborn, FRG) by electroblotting, as described by Kyhse-Anderson (13), in an LKB Multiphor Nova Blot apparatus. Electroblotting was done at 1 mA/cm^2 for 2 h.

Proteins were stained with 0.2% (wt/vol) Coomassie brilliant blue R250 (Serva, Heidelberg, FRG) in 45% (vol/vol) methanol and 10% (vol/vol) acetic acid for 5 min. Excess stain was removed in H_2O, followed by destaining in 45% (vol/vol) methanol containing 10% (vol/vol) acetic acid. The membranes were dried, and visible protein bands were subjected to N-terminal amino acid sequencing by the Edman method (2, 3) with an Applied Biosystems 471A gas phase protein sequencer with a 140A solvent delivery system (Applied Biosystems, Weiterstadt, FRG).

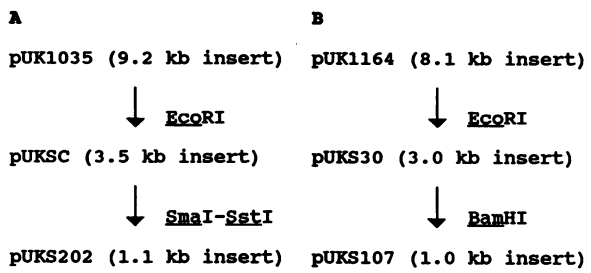

FIG. 1. Subcloning of dehalogenase I gene *dehC*I (A) and dehalogenase II gene *dehC*II (B) from *Pseudomonas* sp. strain CBS3.

Nucleotide sequence accession number. The nucleotide sequences presented here have been submitted to the GenBank data base under accession no. M37618 (*dehC*I) and M37619 (*dehC*II).

RESULTS

Subcloning of the structural genes of the two haloalkanoic acid dehalogenases from *Pseudomonas* sp. strain CBS3. The two 2-haloalkanoic acid dehalogenase genes from *Pseudomonas* sp. strain CBS3 were cloned by using broad-host-range cosmid pMMB33. The gene for dehalogenase I (*dehC*I) was obtained on plasmid pUK1035 in a 9.2-kb insert, whereas the insert on plasmid pUK1164 that codes for dehalogenase II (*dehC*II) was 8.1 kb long.

To subclone these two dehalogenase genes, we digested these inserts with various restriction enzymes and ligated the fragments obtained into plasmid vector pUC18. The subclones obtained were checked for dehalogenating activity. For *dehC*I, a 1.1-kb *Sma*I-*Sst*I fragment expressing dehalogenating activity was obtained, whereas the smallest insert containing intact *dehC*II was a 1-kb *Bam*HI fragment. Figure 1 shows the subcloning procedures by which these fragments were obtained.

In subclones containing these fragments, expression of the dehalogenases was constitutive and no longer induced by MCA. Significant dehalogenase activities were detected in crude extracts when these subclones were grown with or without MCA. This suggests that the regulatory sequences were removed during the subcloning procedures and that expression of these enzymes in the parent clones is under negative control.

Further subcloning for sequencing analysis. For determination of the complete nucleotide sequences, the two dehalogenase genes containing fragments had to be resolved into several smaller fragments. No active dehalogenase was expressed from any fragments obtained by further subcloning. Digestion of the *dehC*I-containing *Sma*I-*Sst*I fragment with *Sau*3A resulted in formation of four fragments of 400, 270, 200, and 170 bp. A second set of fragments of 370 and 720 bp was generated by digestion with *Hin*dIII-*Sma*I and *Hin*dIII-*Sst*I.

Treatment of the *dehC*II-containing 1-kb *Bam*HI fragment with *Sau*3A led to four fragments of 520, 340, 100, and 80 bp. To combine the sequence data obtained from these fragments, sequential series of overlapping clones were prepared by generating deletion subclones from M13 recombinants. The sequencing strategies for both dehalogenase genes are outlined in Fig. 2.

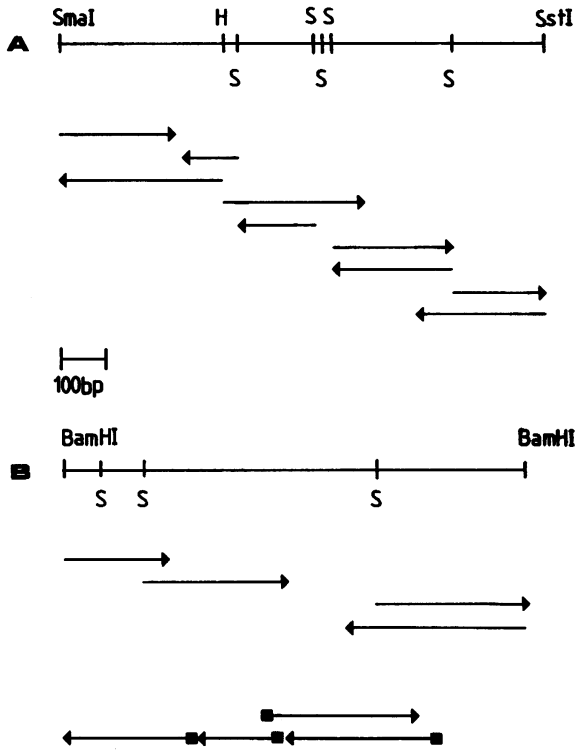

FIG. 2. Restriction map of the 1.1-kb *Sma*I-*Sst*I fragment that encodes the dehalogenase I structural gene (A), restriction map of the 1-kb *Bam*HI fragment that encodes the dehalogenase II structural gene (B), and summaries of the sequencing strategy. The arrows represent the direction and extent of sequencing from particular restriction sites. Arrows starting with squares indicate DNA fragments obtained from deletion subclones. S, *Sau*3A; H, *Hin*dIII; bp, base pairs.

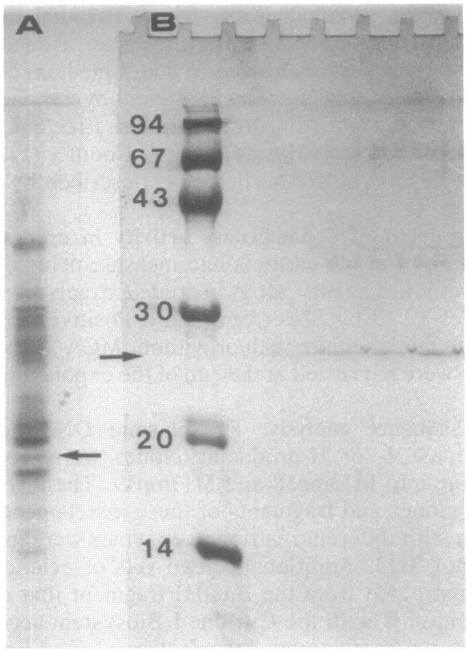

FIG. 3. Gel electrophoresis of dehalogenase II. (A) Nondenaturing polyacrylamide gel of the partially purified enzyme (20 μg). (B) SDS-polyacrylamide gel electrophoresis of eluted dehalogenase II from a nondenaturing gel. Proteins of the Pharmacia Low-Molecular-Weight Calibration Kit served as markers. Molecular weight standards are shown in thousands. Dehalogenase II is indicated by arrows.

Determination of the N-terminal amino acid sequence of dehalogenase I. The N-terminal amino acid sequence obtained from purified dehalogenase I was NH_2-Met-Asp-Pro-Ile-X-Ala-X-Val-Phe-Asp-Ala-Tyr-Gly-Thr-Leu-Leu-Asp-Val-Asn-Thr-Ala-Val-Met. Amino acids 5 and 7 were not identified.

Determination of the N-terminal amino acid sequence of dehalogenase II. After purification on a nondenaturing gel (Fig. 3A), dehalogenase II migrated on an SDS-polyacrylamide gel as a band with a molecular mass of about 28 kDa (Fig. 3B). The N-terminal amino acid sequence determined for dehalogenase II was NH_2-Met-Gln-Glu-Ile-X-Gly-Val-Val. Amino acid 5 was not identified.

Nucleotide sequence of *dehC*I. The nucleotide sequence of the *dehC*I-containing 1.1-kb *Sma*I-*Sst*I fragment and the deduced amino acid sequence of dehalogenase I are shown in Fig. 4A. Only one open reading frame of appropriate length was found in this fragment. The first 23 amino acids of the deduced protein sequence beginning at nucleotide 153 corresponded to the N-terminal amino acid sequence determined by amino acid sequence analysis of purified dehalogenase I. The number of amino acids in the deduced protein was 227, and the molecular mass was 25,401 Da, which corresponded well to the 28 kDa determined by SDS-polyacrylamide gel electrophoresis (10).

A postulated Shine-Dalgarno (GGAC) region was found close to the GTG start codon of the *dehC*I coding region. Possible −10 and −35 consensus sequences of *E. coli* at positions 125 (CAGATT) or 126 (AGATTT) and 104 (TTAACC) were detected. Expression of dehalogenase I in *E. coli* was due to its own promoter, since the dehalogenase was produced when the 1.1-kb fragment was inserted into pUC18 in the opposite orientation relative to the *lac* promoter from pUC18.

Downstream of the dehalogenase structural gene, beginning at position 869, a rho-independent G+C-rich terminator sequence of imperfect dyad symmetry, followed by a stretch of T residues, was found.

The dehalogenase I structural gene showed a G+C content of 56%, which is lower than the 63% G+C content of the total DNA of the parent strain. In the third position, the codons are dominated by G and C (64.5%).

Nucleotide sequence of *dehC*II. The nucleotide sequence of the dehalogenase II-encoding 1-kb *Bam*HI fragment and the predicted amino acid sequence of dehalogenase II are presented in Fig. 4B. Comparison of the only open reading frame of appropriate length with the first eight amino acids of the N-terminal amino acid sequence of dehalogenase II showed that the DNA segment starting at nucleotide 72 coded for the enzyme. The *dehC*II structural gene encoding 229 amino acids starts with an ATG initiation codon and is preceded by a postulated Shine-Dalgarno (GAGG) sequence at nucleotide 61. The molecular mass of the protein predicted by the nucleotide sequence was calculated to 25,683 Da and corresponded well to the molecular mass determined by SDS-polyacrylamide gel electrophoresis. Downstream of the stop codon, a putative stem-loop structure was detected between nucleotides 762 and 790, followed by a stretch of T residues. Upstream of the coding sequence, possible −10 and −35 consensus sequences of *E. coli* were identified at nucleotide positions 45 (TTGTTT) and 22 (TTGGCA). De-

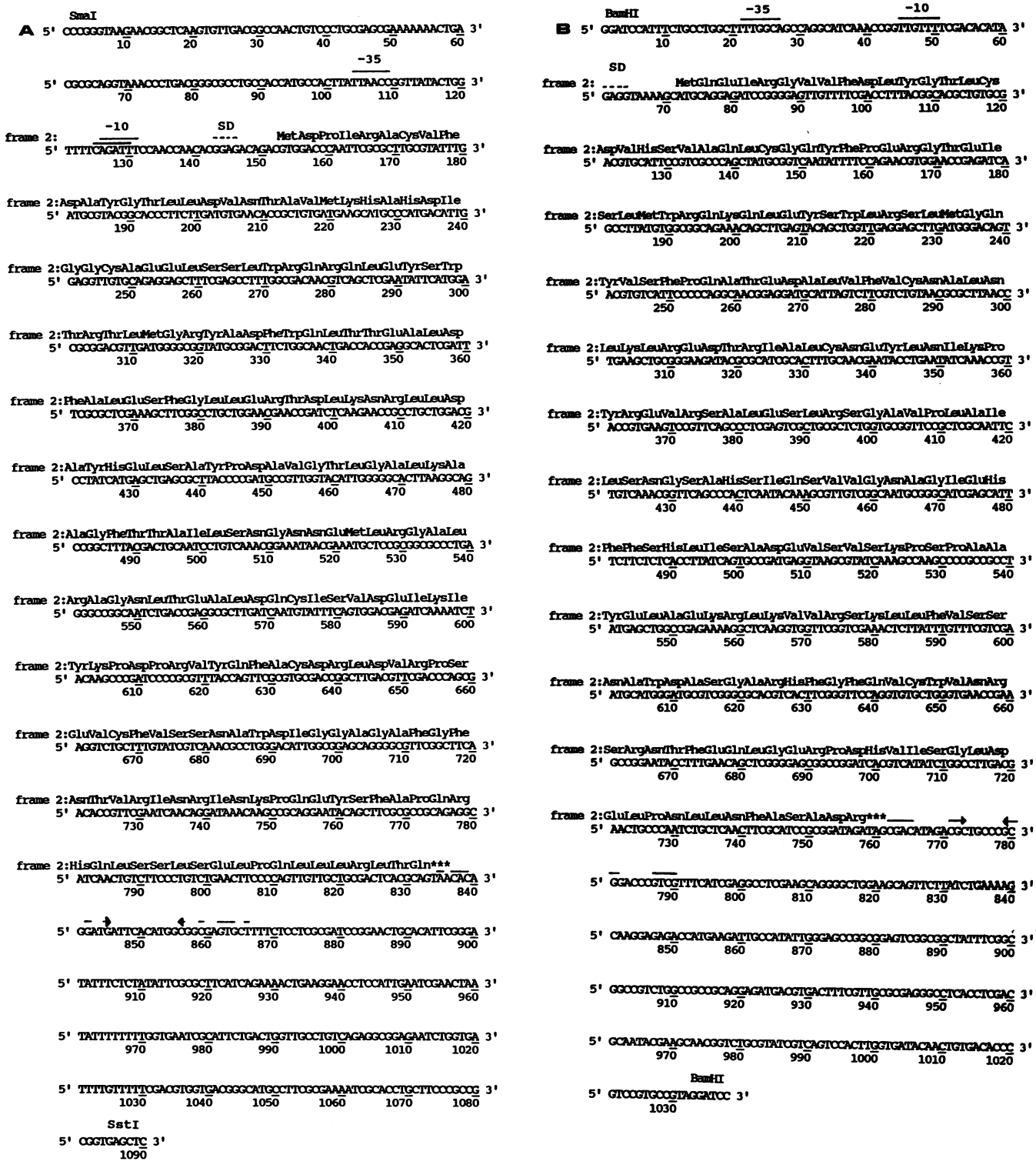

FIG. 4. Nucleotide sequences of the 1.1-kb *Sma*I-*Sst*I fragment containing the dehalogenase I structural gene (A) and the 1-kb *Bam*HI fragment containing the dehalogenase II structural gene (B). The deduced amino acid sequences are shown above the nucleotide sequences. Possible −10 and −35 consensus sequences of *E. coli* are marked by continuous lines, and possible Shine-Dalgarno sequences are indicated by dashed lines above the sequences. Putative stem-loop structures are shown by arrows. Asterisks indicate translation stop codons.

letion of the first 64 bp of the *Bam*HI fragment inserted in the opposite orientation relative to the *lac* promoter in M13 prevented expression of dehalogenase II. When the same insert was introduced into M13 in the correct orientation, dehalogenase II formation was inducible by addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside. This suggests that in the clone containing the 1-kb *Bam*HI fragment, dehalogenase II was expressed from its own promoter, which was

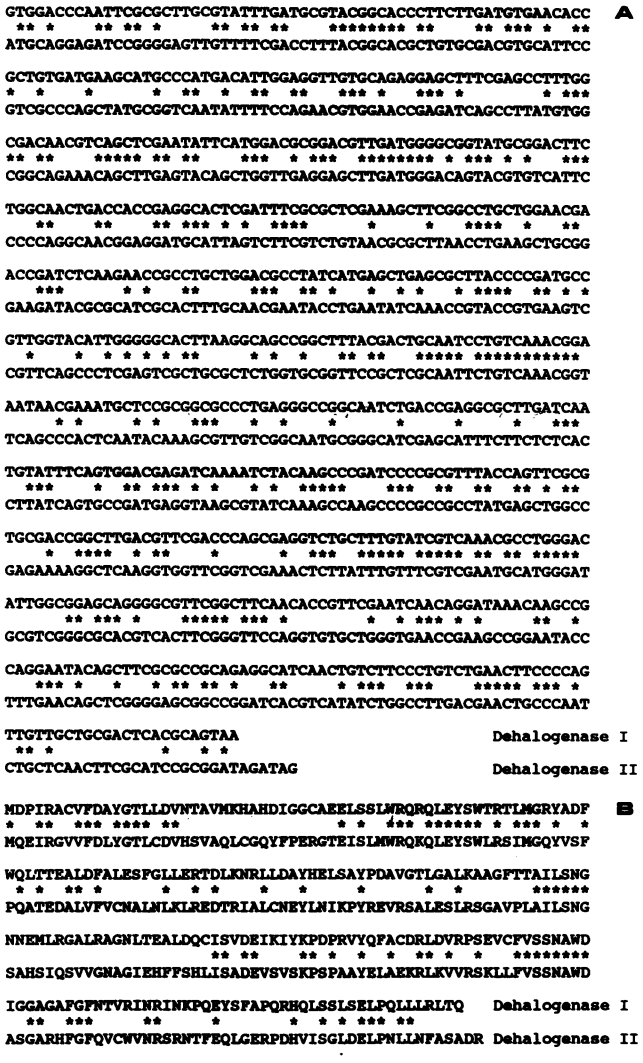

FIG. 5. Comparison of the nucleotide sequences (A) and deduced amino acid sequences (B) of dehalogenases I and II. Identical nucleotides and amino acids are indicated by asterisks.

located between nucleotides 1 and 64. *E. coli* clones in which nucleotides 740 to 1038 had been deleted showed no expression of dehalogenase II, whereas a deletion extending from nucleotides 870 to 1038 did not affect expression of the enzyme.

*dehC*II showed a G+C content of 54.6%, and in position 3 the codons were dominated by G and C (63.9%), as in *dehC*I.

Between nucleotides 30 and 106, a 31-bp inverted repeat was detected. Another 11-bp inverted repeat exists between nucleotides 373 and 440.

Sequence comparison of *dehC*I and *dehC*II. Nucleotide sequence comparison and comparison of the deduced amino acid sequences of *dehC*I and *dehC*II are shown in Fig. 5. The two genes showed homologies of 45% on the base sequence level, 37.5% on the identical amino acid level, and more than 70% on the similar amino acid level. Hydropathy plots (data not shown) indicated the overall hydrophilic character of both enzymes. Comparison of the deduced amino acids showed five regions containing highly conserved amino acid residues. These regions extended from amino acid 4 to 18, 40 to 55, 115 to 120, 173 to 189, and 210 to 222. The relative amounts of Phe, Leu, Trp, and Tyr were especially high in these conserved sequences. It is interesting that cysteine was not present in these conserved sequences.

Comparison of the DNA sequences with the European Molecular Biology Laboratory nucleotide sequence data base and of the amino acid sequences with the SWISS-PROT protein data bank revealed no significant homologies with other sequences.

DISCUSSION

In clones expressing the 2-haloalkanoic acid-dehalogenating enzymes from *Pseudomonas* sp. strain CBS3, the structural genes were identified unequivocally. Possible consensus *E. coli* promoter sequences were detected by similarity to known sequences. The data derived from the DNA sequences corresponded well to the protein data on the enzymes. This is the first time that the complete sequence of a 2-haloalkanoic acid dehalogenase has become available.

Similar enzymes have been cloned from other bacterial strains. A 2-haloalkanoic acid dehalogenase from *Pseudomonas putida* PP3, which was located on the chromosome and shown to be associated with mobile genetic elements, transposed onto plasmid RP4, where its gene was identified (24). The two plasmid-encoded 2-haloalkanoic acid dehalogenases (HI and HII) from a *Moraxella* species (8) have been cloned successfully on plasmid pBR322 in *E. coli*, but recombinants containing the gene for dehalogenase HII were unstable and could not be maintained. None of these dehalogenase genes had previously been sequenced.

So far, *Pseudomonas* sp. strain CBS3 is the only bacterial strain known whose two haloalkanoic acid dehalogenases have been cloned separately.

Both enzymes converted L-2-chloropropionate to D-lactate but did not convert D-2-chloropropionate. The sequence comparison of these two isoenzymes revealed interesting results. On the DNA level, a rather low degree of homology was observed. Even in stretches of complete amino acid homology, the nucleotide sequence homology was as low as 59%.

It is interesting that both of the enzymes described here contained no cysteines within the conserved regions. This amino acid is supposed to be essential in the active site of 2-haloalkanoic acid dehalogenases, converting the substrate under retention of the configuration (5, 30).

The similarities on the amino acid level were high. Some parts of the amino acid sequence were completely conserved, but no homology with dichloromethane dehalogenase from *Methylobacterium* sp. strain DM4 (15), haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (7), or other sequenced proteins was found.

The amino acid homology of two dehalogenases from *Pseudomonas* sp. strain CBS3 might be attributed to the functional requirements for stereospecific conversion of the L isomer of the substrate to the D isomer of the product. Comparison of the above-described sequences with those of enzymes of the same type from other organisms, which will become available soon, will show whether these enzymes form a separate class of dehalogenases and whether they are similar to enzymes that convert only the D form of the substrate or both (16, 25, 30) stereoisomers.

The regulatory sequences were removed from both of the dehalogenases described here during subcloning, whereas the promoters, which lead to expression of the enzymes in *E. coli*, were retained.

Still, we are left with the intriguing question of why

Pseudomonas sp. strain CBS3 contains, in addition to 4-chlorobenzoate dehalogenase and 4-chlorophenylacetic acid 3,4-dioxygenase, two aliphatic dehalogenases not necessary for growth of *Pseudomonas* sp. strain CBS3 on 4-chlorobenzoate, the substrate on which this bacterial strain was originally isolated. Cloning and sequencing of the other dehalogenases from *Pseudomonas* sp. strain CBS3 is in progress in our laboratory.

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