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Published in:
Journal of Bacteriology

JOURNAL OF BACTERIOLOGY, Dec. 1989, p. 6791–6799
0021-9193/89/126791-09$02.00/0

Vol. 171, No. 12

Cloning of 1,2-Dichloroethane Degradation Genes of *Xanthobacter autotrophicus* GJ10 and Expression and Sequencing of the *dhlA* Gene

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Received 15 May 1989/Accepted 4 September 1989

A gene bank from the chlorinated hydrocarbon-degrading bacterium *Xanthobacter autotrophicus* GJ10 was prepared in the broad-host-range cosmid vector pLAFR1. By using mutants impaired in dichloroethane utilization and strains lacking dehalogenase activities, several genes involved in 1,2-dichloroethane metabolism were isolated. The haloalkane dehalogenase gene *dhlA* was subcloned, and it was efficiently expressed from its own constitutive promoter in strains of a *Pseudomonas* sp., *Escherichia coli*, and a *Xanthobacter* sp. at levels up to 30% of the total soluble cellular protein. A 3-kilobase-pair *Bam*HI DNA fragment on which the *dhlA* gene is localized was sequenced. The haloalkane dehalogenase gene was identified by the known N-terminal amino acid sequence of its product and found to encode a 310-amino-acid protein of molecular weight 35,143. Upstream of the dehalogenase gene, a good ribosome-binding site and two consensus *E. coli* promoter sequences were present.

Xanthobacter spp. are nitrogen-fixing bacteria that are able to grow autotrophically with a mixture of hydrogen and oxygen as an energy source (33). A member of this genus that is able to utilize several halogenated hydrocarbons as carbon sources has been isolated (15). The organism was obtained from an enrichment culture with 1,2-dichloroethane, which is an environmentally important compound with a production volume larger than that of any other industrial halogenated chemical. The 1,2-dichloroethane-degrading bacterium, designated strain GJ10, was found to degrade 1,2-dichloroethane via 2-chloroethanol, 2-chloroacetaldehyde, and chloroacetic acid to glycolate (Fig. 1) (13, 14). The dehalogenation steps in this sequence were found to be catalyzed by two different hydrolytic dehalogenases (14, 17).

Conversion of 1,2-dichloroethane was mediated by a haloalkane dehalogenase. This was the first enzyme found to catalyze hydrolytic dehalogenation of chlorinated hydrocarbons. The protein has been purified (17) and crystallized (26), and its three-dimensional structure is now under study. Chloroacetic acid hydrolysis was found to be mediated by a different enzyme. This haloacid dehalogenase has not been purified from strain GJ10, but much information is available about other dehalogenases of this class (22).

So far, haloalkane dehalogenases are the only enzymes known to be capable of direct hydrolytic dehalogenation of chlorinated and brominated hydrocarbons, without the requirement for coenzymes or oxygen. The enzyme of *X. autotrophicus* GJ10 is constitutively expressed to 2 to 3% of the soluble cellular protein (13, 17). It has a remarkably broad substrate range which includes terminally halogenated alkanes with chain lengths up to 4 carbons for chlorinated and up to at least 10 carbons for brominated alkanes. Other haloalkane dehalogenases of broad substrate range have been found in gram-positive haloalkane-utilizing bacteria (11, 28, 35).

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So far, no information is available about the genetics of haloalkane-utilizing organisms. Since the system is attractive both for studying the structure-activity relationship of dehalogenases and for opening up the possibility of using (modified) dehalogenases for the construction of organisms with new catabolic capabilities, an investigation of the genetics of 1,2-dichloroethane degradation in *X. autotrophicus* GJ10 was started.

Here, we report the isolation of several genes involved in 1,2-dichloroethane metabolism in this organism. The haloalkane dehalogenase gene, which we have designated *dhlA*, was sequenced, and its expression in other gram-negative bacteria was studied.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains of *X. autotrophicus* and other organisms used in this study are listed in Table 1. *Escherichia coli* HB101 (3) was used as the recipient in transduction and transformation experiments. Triparental matings were done with pRK2013 (5) as the helper plasmid delivering transfer functions. Plasmids pLAFR1 (6) and pLAFR3 (31) were used as cloning vectors.

Growth conditions. Strains containing plasmid pLAFR1 or its derivatives were cultivated on nutrient broth (NB) or LB plates containing 12 μg of tetracycline per ml as a selecting agent. The mineral medium (MMY) used for liquid cultures and citrate plates had the composition described elsewhere (13), but with 10 mg of yeast extract per liter replacing the vitamins. Carbon sources were added at 5 mM, unless stated otherwise. Liquid cultures with volatile substrates were carried out in closed flasks that were filled to one-fifth of their volume with medium. Cells were cultivated at 30°C with rotary shaking.

For enzyme assays, cultures were harvested at the end of the exponential growth phase and extracts were prepared as described previously (13).

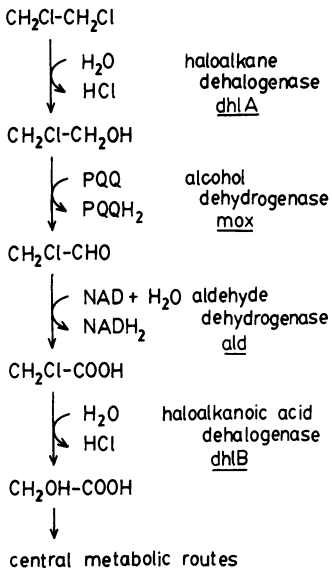

FIG. 1. Catabolic route for 1,2-dichloroethane of *Xanthobacter autotrophicus* GJ10 (13, 14).

Preparation of a gene bank. Total DNA was isolated from *X. autotrophicus* GJ10 grown on nutrient broth by using a described procedure (7). The DNA was partially digested with *Eco*RI and ligated in the cosmid cloning vector pLAFR1 (6). The vector was previously isolated from *E. coli* HB101 using the alkaline lysis method and cesium chloride gradient centrifugation and was digested with *Eco*RI (20). Ligated DNA was packaged into bacteriophage lambda core proteins according to protocol 2 as described by Maniatis et al. (20).

Restriction enzymes and T4 ligase were used according to the procedures supplied by the manufacturers. Preparation of protein extracts for in vitro packaging and transduction of phages to *E. coli* HB101 were performed as described by Maniatis et al. (20).

Triparental mating (5) was carried out by replica plating donor strains (pLAFR derivatives in HB101) on LB agar plates that were previously spread with 0.1 ml of an exponential culture (optical density at 450 nm of 0.6) of *E. coli* HB101(pRK2013). After overnight incubation, these plates were replica plated on NB agar that was previously spread with 0.1 ml of an overnight culture of the recipient strain in NB. The plates were incubated overnight at 30°C, and transconjugants were selected by replica plating on citrate plates containing 12 μg of tetracycline per ml. When a *Hyphomicrobium* sp. was the recipient strain, methanol instead of citrate was used as the carbon source in selective plates.

Enzyme activities and protein analysis. Colonies on agar plates were screened for dehalogenase activity by incubating a small amount of cells in a microdilution plate with 100 μl of a solution of 5 mM 1,2-dibromoethane in 50 mM Tris sulfate (pH 7.5). After incubation for 60 min at 30°C, 0.1 ml of 0.25 M $NH_4Fe(SO_4)_2$ in 6 M HNO_3 was added, followed by a drop of saturated $Hg(SCN)_2$ in ethanol. A red color indicated the presence of dehalogenase activity. For these tests, cells were grown on LB plates containing no NaCl.

Assays of enzyme activities were done immediately after preparation of the extracts. Haloalkane dehalogenase assays were carried out by incubating 0.1 ml of crude extract or an adequate dilution thereof at 30°C with 3 ml of 5 mM 1,2-dichloroethane in 50 mM Tris sulfate (pH 7.5). Halide liberation was followed colorimetrically (2). Chloroacetate dehalogenase activities were determined similarly but with 5 mM monochloroacetate in 50 mM glycine NaOH buffer (pH 9.0) as the substrate solution.

Assays for phenylmethane sulfonate (PMS)-dependent alcohol dehydrogenase and NAD-dependent aldehyde dehydrogenase have been described previously (13).

Protein was determined with the Folin phenol method, with bovine serum albumin as the standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on gels containing 10% polyacrylamide was performed as described previously (17). Marker proteins were ovotransferrin (molecular mass, 78 kilodaltons [kDa]) bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and myoglobin (17 kDa).

Nucleotide sequencing. The 3-kilobase (kb) *Bam*HI insert isolated from pPJ50 was digested with different restriction enzymes, and the resulting fragments were ligated into M13mp18 or M13mp19 vectors (34). All stretches of DNA were sequenced at least twice in both directions by using the dideoxyribonucleotide chain termination method (27). DNA sequences were analyzed with the PCGENE program (Genofit, Geneva, Switzerland) or the Staden package (30). Amino acid sequences were compared with the SWISS-PROT Protein Database (EMBL, Heidelberg, Federal Republic of Germany) release 9, with the program FASTP (18).

TABLE 1. Bacterial strains used

Organism	Relevant properties	Reference or source
E. coli HB101	$RecA^-$	3
X. autotrophicus		
GJ10	1,2-Dichloroethane-utilizing wild type	13, 14, 17
GJ10M41	Chloroacetaldehyde dehydrogenase-negative mutant of GJ10	12
GJ10M27	Chloroethanol dehydrogenase-negative mutant of GJ10	This paper
GZ29	Wild type, dehalogenase negative	33
7C	Wild type, dehalogenase negative	33
XD	Wild type, dehalogenase negative	13
Pseudomonas sp.		
GJ1	Utilizes 2-chloroethanol	15
GJ31	Utilizes chlorobenzene	25
Pseudomonas oleovorans TF41L	Utilizes *n*-alkanes	29
Hyphomicrobium sp. GJ21	Utilizes dichloromethane	D. B. Janssen

Analysis of chlorinated compounds. Culture fluids were analyzed for halogenated compounds by gas chromatography of diethyl ether extracts as described elsewhere (12).

Chemicals and enzymes. Restriction enzymes and T4 DNA ligase were obtained from Boehringer GmbH (Mannheim, Federal Republic of Germany) and Bethesda Research Laboratories (Neu Isenburg, Federal Republic of Germany). Chlorinated compounds were supplied by Janssen Chimica (Beerse, Belgium). Inorganic chemicals were of analytical grade.

RESULTS

Mutants affected in 1,2-dichloroethane utilization. In order to make it possible to screen for genes involved in 1,2-dichloroethane metabolism by complementation, it was desirable to isolate mutants that are defective in the catabolic route. Only a single mutant in 1,2-dichloroethane degradation has been described so far (13). This strain, designated GJ10M41, has lost chloroacetaldehyde dehydrogenase activity. Various attempts to obtain mutants that lack dehalogenase or chloroethanol dehydrogenase activity by penicillin or glycin contraselection were unsuccessful.

As an alternative approach, we attempted to isolate mutants by selecting for resistance toxic substrate analogs of 2-chloroethanol and methanol. 2-Bromoethanol was toxic at levels of 5 μM, presumably because of the formation of bromoacetaldehyde. Mutants resistant to this compound have been obtained in another organism and were found to lack alcohol dehydrogenase activity (12). Strain GJ10M27 was isolated by selecting for 2-bromoethanol resistance of strain GJ10 on citrate plates containing 20 μl of bromoethanol in the lid of the petri dish. The mutant did not utilize 1,2-dichloroethane, 2-chloroethanol, or methanol as a carbon source but was still able to grow with ethanol (slowly), 1-propanol, and 1-butanol. PMS-coupled methanol dehydrogenase activity could not be detected in extract of strain GJ10M27 grown in MMY medium supplemened with 5 mM citrate–5 mM methanol. Crude extract of strain GJ10 grown under the same conditions showed an activity of 153 mU/mg of protein. Thus, strain GJ10M27 is defective in methanol dehydrogenase activity.

Attempts to isolate dehalogenase negative mutants by selecting for 1,2-dibromoethane resistance (12) did not yield stable mutants that only lacked dehalogenase activities and could be used for screening of a gene bank by complementation.

Isolation of genes involved in 1,2-dichloroethane metabolism. A gene bank was constructed as described in Materials and Methods. Analysis of plasmids isolated from transduced HB101 clones showed that 3 out of 20 clones tested did not have inserts. The average total insert size was 12 ± 10 kb.

For screening of this gene bank, the general procedure was to individually transfer recombinant cosmids from transduced *E. coli* HB101 by triparental mating to appropriate recipient strains with selection on citrate plates containing tetracycline. The transconjugants were then tested for complementation or expression of the gene of interest.

Clones in *E. coli* HB101 containing the gene encoding haloalkane dehalogenase (*dhlA*) were identified by screening tetracycline-resistant transconjugants of *X. autotrophicus* GZ29 and XD. Conjugation of HB101 transformants with *X. autotrophicus* 7C, which produces much larger amounts of slime than the other organisms, was less efficient than that of strains GZ29 and XD. Screening of transconjugants for growth with 1-chlorobutane was not useful for identification of dehalogenase containing clones because of too much background growth of *Xanthobacter* strains on plain agar. However, by testing transconjugants of GZ29 and XD for dehalogenase activity by using the colony assay, clones expressing dehalogenase activity could easily be detected and the same donor strains gave positive results with either strain XD or GZ29 as the recipient. Out of 1,300 recombinants tested, three dehalogenase-positive clones were found (Table 2). Analysis of plasmids (pPJ10 to pPJ12) in these clones showed the presence of a common 8.3-kb *Eco*RI fragment.

TABLE 2. Plasmids containing *Xanthobacter* DNA in pLAFR

Plasmid[a]	Description	Screened in:
pPJ10, -11, -12	Haloalkane dehalogenase	GZ29, XD
pPJ13, -14, -15, -16	Halocarboxylic acid dehalogenase	GZ29
pPJ17, -25, -26	Methanol dehydrogenase	GJ10M27
pPJ19	Chloroacetaldehyde dehydrogenase	GJ10M41
pPJ20	*Eco*RI subclone of pPJ12	HB101
pPJ40	*Bam*HI deletion of pPJ20	HB101
pPJ50	*Bam*HI subclone of pPJ20 in pLAFR3	HB101
pPJ66	*Eco*RI subclone of pPJ14	GZ29

[a] All plasmids are pLAFR1 with different *Eco*RI inserts, unless stated otherwise.

A clone complementing the methanol dehydrogenase-negative mutant strain GJ10M27 was obtained by screening transconjugants of this organism for growth on methanol. Extract of cells of strain GJ10M27(pPJ17) grown on citrate plus methanol contained a methanol dehydrogenase activity of 502 mU/mg of protein, which was 3.5 times higher than the activity of the wild type on the same medium.

Screening for the NAD-dependent chloroacetaldehyde dehydrogenase gene was performed by using *X. autotrophicus* GJ10M41 (13) as a recipient in triparental matings and testing the resulting transconjugants for growth with chloroethanol. Out of 1,200 clones tested, 1 clone (pPJ19) complementing this mutant was obtained.

For selection of clones containing the chloroacetate dehalogenase gene (*dhlB*), we again used *X. autotrophicus* GZ29 and XD, the wild-type isolates lacking dehalogenase activities (13). Transconjugants were screened for dehalogenase by using a colony assay for chloroacetic acid, dichloroacetic acid, or bromoacetic acid as substrates, but without success. Screening transconjugants for chloroacetic acid utilization on plates, however, showed that four different HB101 transductants contained the chloroacetate dehalogenase gene (Table 2), and that it was expressed in transconjugants of both test strains.

Plasmids (pPJ13 to pPJ16) encoding halocarboxylic acid dehalogenase were isolated from the *E. coli* HB101 derivatives and digested with *Eco*RI. All four clones had a 7.0- and a 3.2-kb *Eco*RI fragment in common. Religation of digest from pPJ14, transformation to HB101, and screening for the presence of *dhlB* yielded four plasmids that all contained both *Eco*RI fragments as inserts (e.g., pPJ66).

None of the above clones were positive in more than one screening method. Furthermore, no common *Eco*RI fragments were found in plasmid preparations of clones from different classes. Thus, at least four unlinked different DNA segments were found to play a role in 1,2-dichloroethane metabolism (Fig. 1).

Physical mapping of the *dhlA* region. Subcloning of the

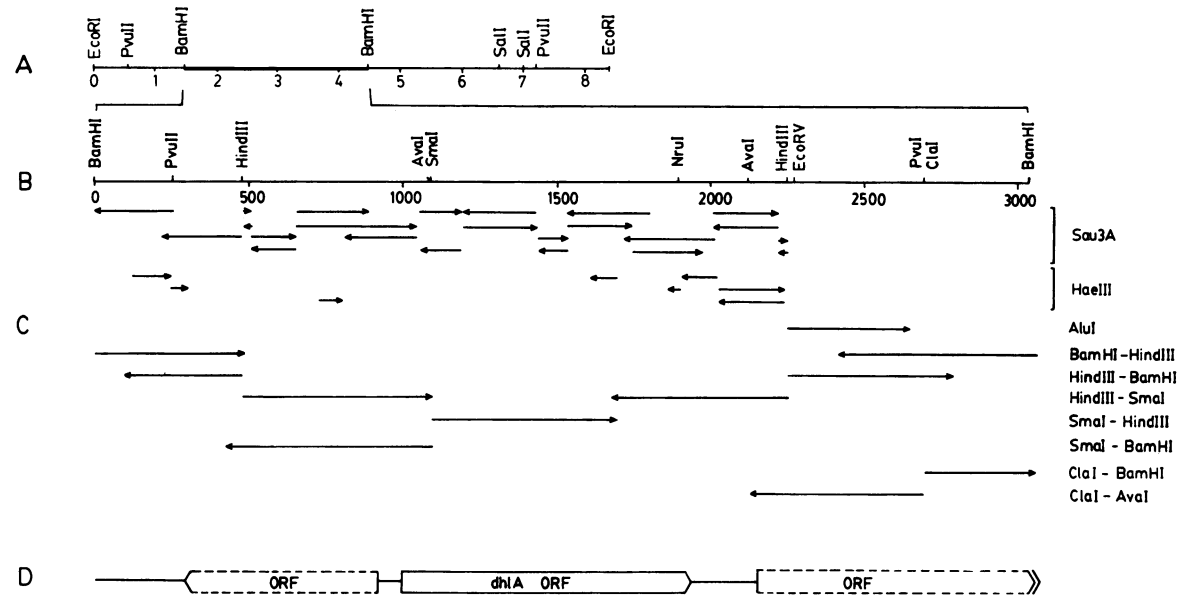

FIG. 2. Structure of the *Xanthobacter dhlA* region. (A) Restriction map of the 8.3-kb *Eco*RI insert of pPJ20 harboring the *dhlA* gene. (B) Restriction map of the 3-kb *Bam*HI fragment cloned in pPJ50, as deduced from the sequence. (C) Sequence strategy for the 3-kb *Bam*HI fragment harboring the *dhlA* region. Arrows indicate the DNA sequences determined with different clones in M13mp18 and M13mp19 vectors. (D) Location on the 3-kb *Bam*HI fragment of the three longest open reading frames (ORFs) found in the nucleotide sequence.

haloalkane dehalogenase region was facilitated by the fact that the dehalogenase was efficiently expressed in *E. coli* HB101. By using a colony assay, transformants could be tested quickly for the presence of the dehalogenase gene. From pPJ10 and pPJ12, subclones were prepared by using the restriction enzyme *Eco*RI. All four positive subclones contained a single 8.3-kb *Eco*RI insert, three of them oriented with the righthand *Eco*RI site (Fig. 2A) closest to the pLAFR1 *cos* site and one of them oriented in the opposite direction. A restriction map of this region was determined with pPJ20 (Fig. 2A).

Deletion plasmids, for example pPJ40, lacking the internal *Bam*HI fragment, no longer expressed dehalogenase activity. Several attempts to clone the 8.3-kb *Eco*RI or its internal *Bam*HI fragment in high-copy-number vectors such as pUC19 or pBR322 (20) were unsuccessful. Forced cloning of the flanking *Bam*HI-*Eco*RI fragments was possible. Cloning of *Eco*RI-*Sal*I fragments in pJRD158 (4) yielded only clones containing the smaller *Eco*RI-*Sal*I fragment, in some cases with an additional 0.4-kb *Sal*I insert, but none of these expressed dehalogenase activity. It was tentatively concluded that the dehalogenase gene is located at least partially on the internal 3-kb *Bam*HI region and that cloning of fragments containing this region in high-copy-number vectors was hindered, probably by the presence of a strong promoter causing overexpression of the dehalogenase gene.

The 3-kb *Bam*HI fragment of pPJ20 was isolated and ligated in the unique *Bam*HI restriction site of pLAFR3. Dehalogenase-positive, tetracycline-resistant transformants of *E. coli* HB101 were isolated and were indeed found to contain the *Bam*HI fragment. One such clone, pPJ50, contained the lefthand *Bam*HI site (Fig. 2B) closest to the *cos* site of the vector and was used for sequencing of *dhlA*.

Expression of the *dhl* genes in other bacteria. The expression of the dehalogenase genes in other gram-negative bacteria was examined by transferring plasmids to different gram-negative bacteria (Table 1) by triparental mating and testing the resulting transconjugants for dehalogenase activities.

TABLE 3. Expression of the *dhl* genes

Strain	Carbon source	Substrate for assay	Sp act (mU/mg)[a]
X. autotrophicus GJ10	Citrate	1,2-Dichloroethane	240
X. autotrophicus GJ10	Citrate	Chloroacetate	580
X. autotrophicus GJ10	Dichloroacetate	Chloroacetate	615
Pseudomonas GJ1(pPJ20)	Citrate	1,2-Dichloroethane	2,340
Pseudomonas GJ31(pPJ20)	Citrate	1,2-Dichloroethane	1,855
X. autotrophicus XD(pPJ20)	Citrate	1,2-Dichloroethane	659
X. autotrophicus GZ29(pPJ20)	Citrate	1,2-Dichloroethane	988
E. coli HB101(pPJ20)	LB	1,2-Dichloroethane	1,650
X. autotrophicus GJ10(pPJ20)	Citrate	1,2-Dichloroethane	788
X. autotrophicus XD(pPJ14)	Citrate	Chloroacetate	70
E. coli HB101(pPJ66)	LB	Chloroacetate	65
X. autotrophicus XD(pPJ14)	Dichloroacetate	Chloroacetate	3,178
X. autotrophicus XD(pPJ66)	Dichloroacetate	Chloroacetate	1,850

[a] Activities were measured in crude extracts from late-exponential-phase cultures grown at 30°C.

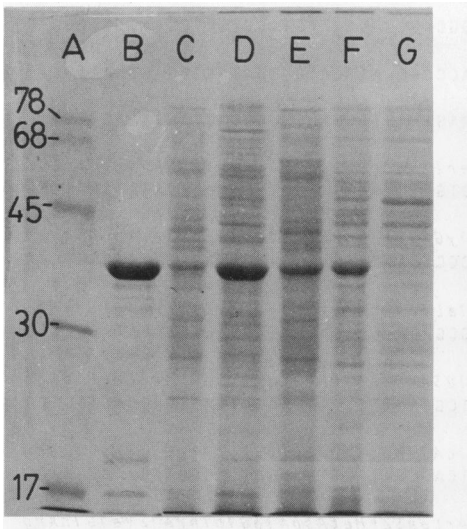

FIG. 3. Expression of the *dhlA* gene in different strains. Crude extracts prepared from different cultures were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes: A, molecular weight marker proteins; B, partially purified dehalogenase from *E. coli* HB101(pPJ20); C through G, crude extract from *X. autotrophicus* GJ10 grown on citrate (C), *Pseudomonas* sp. GJ1(pPJ20) grown on citrate (D), *X. autotrophicus* GZ29(pPJ20) grown on citrate (E), *E. coli* HB101(pPJ20) grown on LB (F), and *E. coli* HB101(pPJ66) grown on LB (G). Protein (20 to 30 μg) was applied to each slot.

When HB101(pPJ20) was used as the donor strain, transfer of tetracycline resistance appeared possible to *Hyphomicrobium* sp. strain GJ21, a dichloromethane-utilizing organism; *Pseudomonas* sp. strain GJ31, which is an organism utilizing chlorobenzene; *Pseudomonas* sp. strain TF41L, which utilizes n-alkanes; and two different strains of *X. autotrophicus*.

By using the colony test for dehalogenase activity, it was found that dehalogenase was expressed in *X. autotrophicus*, the three *Pseudomonas* strains, and *E. coli*, but it was not expressed or unstable in *Hyphomicrobium* sp. GJ21. Cells carrying dehalogenase genes were grown in medium containing no chlorinated substrates, and dehalogenase activities in crude extracts were determined. In *E. coli* and two *Pseudomonas* strains, haloalkane dehalogenase activities were much higher than in the wild-type strain *X. autotrophicus* GJ10, and levels up to 2.3 U/mg of protein were found. This implies that the dehalogenase amounted to up to 30% of the total cellular protein (Table 3). The dehalogenase protein was clearly visible as the most dominant protein band in crude extracts subjected to polyacrylamide gel electrophoresis (Fig. 3).

The introduction of the dehalogenase gene in the chloroethanol-utilizing bacterium *Pseudomonas* sp. GJ1 allowed this organism to degrade 1,2-dichloroethane, and all organic chlorine present was released as inorganic chloride (Table 4). Similarly, *X. autotrophicus* XD(pPJ20) and *Pseudomonas* sp. GJ31(pPJ20) utilized 1-chlorobutane for growth. Thus, the introduction of the haloalkane dehalogenase gene is sufficient for growth of these bacteria on 1-chlorinated alkanes that can be hydrolyzed by the dehalogenase.

The halocarboxylic acid dehalogenase gene was not constitutively expressed in *E. coli* or *X. autotrophicus* GZ29 after introduction of plasmid pPJ14 or its derivative pPJ66. Growth of strain XD containing either of these plasmids with dichloroacetic acid as a carbon source was possible, however, and led to induction of the synthesis of the enzyme to levels that were much higher than observed in *X. autotrophicus* GJ10 (Table 3). The haloacid dehalogenase was previously not found to be induced by dichloroacetate in *X. autotrophicus* GJ10, although increased levels have been found in mutant GJ10M41 (13).

Sequence of the haloalkane dehalogenase region. The 3.0-kb *Bam*HI fragment containing the complete *dhlA* gene was isolated from pPJ50, digested with different restriction enzymes, and ligated in the M13 sequencing vectors. The nucleotide sequence was determined from the series of fragments shown in Fig. 2C. The whole DNA sequence was determined independently in two directions, and the entire nucleotide sequence of the 3.0-kb *Bam*HI fragment (Fig. 4) and some restriction sites deduced from it are shown (Fig. 2B). The GC content of this 3,041-base-pair (bp) fragment was 56.9%.

Three open reading frames larger than 450 bp were found in this region (Fig. 4). The first part of the sequence of the 930-bp open reading frame located between the two *Hin*dIII sites was in agreement with the known N-terminal sequence of the dehalogenase protein (17). The molecular weight of the deduced protein was 35,143, and the number of amino acid residues was 310. The amino acid composition of the *dhlA* gene product was also in close agreement with the

TABLE 4. Degradation of chloroalkanes in *dhlA* containing bacteria[a]

Strain	Carbon source[b]		Product and substrate remaining[c]			
	Substrate 1	Substrate 2	Substrate 1 (mM)	Substrate 2 (mM)	$[Cl^-]$ (mM)	OD_{450}[d]
Pseudomonas sp.						
GJ1	1 mM DCE	1 mM CEO	0.81	<0.01	1.0	0.10
GJ1(pPJ20)	1 mM CEO		<0.001		1.0	0.106
GJ1(pPJ20)	1 mM DCE	1 mM CEO	<0.01	<0.001	3.0	0.206
GJ1(pPJ20)	1 mM DCE		<0.001		2.1	0.113
GJ31	2 mM ClBu	1 mM ClBz	0.40	<0.01	1.0	0.213
GJ31(pPJ20)	2 mM ClBu	1 mM ClBz	<0.01	<0.01	2.1	0.393
X. autotrophicus XD(pPJ20)	5 mM ClBu		<0.01		4.5	0.621

[a] Cultures were analyzed after 5 days of incubation at 30°C.
[b] Abbreviations: DCE, 1,2-dichloroethane; CEO, 2-chloroethanol; ClBu, 1-chlorobutane; ClBz, chlorobenzene. Concentrations added are given as if the compounds completely dissolved in the water phase.
[c] Concentrations of organic compounds represent actual values in the liquid phase, as measured by gas chromatography.
[d] OD_{450}, Optical density at 450 nm.

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GGATCCGATGAAGCCCGTTTTGCCATCACTCACCAGTATTGCGAAGCCTATGTCCGGCAGCAGGAAAACTACCTGCTGGAACTGTCGAAG     90

GACACCGTGCGAAGGTTATCGCCAGAACCGCAGGCCCTCGTCGGCTACTCTATTTACCCGCCATTCATGGGGATGGTTGAGGGAATGCAT    180

CCCCTTCGTACTTTGGATGCAGGCTGACCAACCTGGTCTTCCGCTGACTGTCTCCGAGTAAATTGGAGGCCGCCAGCTGCAAAGGTGTTA    270

                                                ***SerAsnSerIleArgLeuGlnGluLeuValValAsnThrLeu
AAACCAGGTTCCCGAAGACCATTTTTGCCAGAGGCCCATGCGATTTCATGAATTGCTGATGCGAAGCTGCTCCAACACCACATTGGTCAG    360

ThrGluSerIleThrAspIleSerGluProMetAlaIleAlaThrValSerTyrGlyGluValTyrProIleValLeuSerValAlaVal
CGTCTCGGAAATCGTATCAATGGATTCGGGCATTGCAATGGCCGTTACCGAATACCCCTCAACATAAGGAATAACCAAAGATACGGCCAC    450

AlaLeuAlaLysGluSerSerAlaLeuProAlaLeuLysGluAlaMetValGluAlaLeuAspArgTyrTyrSerLysLeuTyrGlyHis
GGCCAGCGCCTTTTCGCTGCTGGCCAGTGGCGCAAGCTTCTCAGCCATCACTTCGGCGAGATCCCGATAGTACGACTTGAGATAGCCGTG    540

ValThrGluAsnArgThrAlaIleAlaTrpTyrGluArgPheIleArgCysMetGluSerIleGluLeuGlyHisGlyLeuLeuGluArg
AACAGTTTCATTACGGGTGGCGATTGCCCAGTACTCCCTGAATATTCGACACATCTCGGAAATCTCAAGACCATGACCGAGCAGTTCTCG    630

LeuLeuAlaArgLeuGlnAlaHisGlnAspArgGlyAlaSerLeuProProHisGluAlaMetThrThrLeuCysArgGlnPheTyrArg
TAACAACGCTCTTAACTGGGCGTGTTGATCACGCCCTGCCGATAAGGGCGGATGCTCAGCCATGGTTGTCAGGCACCGTTGAAAGTAACG    720

AspAlaMetAlaValLeuLeuLeuAspGluSerLysPheTyrTyrGlnValAsnSerLeuSerMetAspAlaGlnThrAlaValGlnArg
GTCTGCCATGGCCACGAGGAGCAGGTCCTCGGACTTGAAATAGTACTGAACGTTGCTCAGGGACATGTCCGCTTGCGTTGCCACCTGCCT    810

MetThrLeuGlyPheGluGlyGluThrGluLeuLeuGluLeuAlaValAspLeuIleArgGluThrLysAlaAsnGlnLysMetGlyAsn
CATCGTCAAACCAAACTCACCCTCTGTCTCAAGCAATTCGAGCGCGACATCCAGGATTCGTTCGGTTTTGGCGTTTTGTTTCATTCCGTT    900

GluProGluPhePheThrSerMet                        -------                 ------          •• •
CTCCGGTTCAAAAAACGTTGACATTGTAGGTCAGGCGACCTACTTTGTCATTGCTAGGTCACCCGACCTAACTTTTGACAGACGCTTACG    990

••••   •MetIleAsnAlaIleArgThrProAspGlnArgPheSerAsnLeuAspGlnTyrProPheSerProAsnTyrLeuAspAsp
GAGGCTCTATGATAAATGCAATTCGCACCCCGGACCAACGCTTCAGCAATCTCGATCAGTATCCGTTCAGCCCCAACTACCTGGACGACC   1080

LeuProGlyTyrProGlyLeuArgAlaHisTyrLeuAspGluGlyAsnSerAspAlaGluAspValPheLeuCysLeuHisGlyGluPro
TCCCCGGCTACCCGGGATTGCGGGCACACTACCTCGACGAGGGCAATTCTGACGCTGAAGACGTTTTTCTCTGCCTTCATGGCGAGCCCA   1170

ThrTrpSerTyrLeuTyrArgLysMetIleProValPheAlaGluSerGlyAlaArgValIleAlaProAspPhePheGlyPheGlyLys
CCTGGAGTTACCTGTATCGCAAGATGATCCCGGTATTTGCTGAATCAGGCGCACGAGTTATTGCGCCAGACTTTTTTGGATTCGGAAAAT   1260

SerAspLysProValAspGluGluAspTyrThrPheGluPheHisArgAsnPheLeuLeuAlaLeuIleGluArgLeuAspLeuArgAsn
CCGACAAGCCAGTAGACGAAGAAGACTACACCTTCGAATTTCACCGCAACTTCCTGCTTGCACTAATCGAACGGCTTGACTTGCGCAACA   1350

IleThrLeuValValGlnAspTrpGlyGlyPheLeuGlyLeuThrLeuProMetAlaAspProSerArgPheLysArgLeuIleIleMet
TTACGCTGGTCGTTCAGGACTGGGGCGGATTTTTGGGGCTGACCTTACCGATGGCCGACCCTTCCCGCTTCAAGCGCCTGATCATCATGA   1440

AsnAlaCysLeuMetThrAspProValThrGlnProAlaPheSerAlaPheValThrGlnProAlaAspGlyPheThrAlaTrpLysTyr
ACGCCTGCTTGATGACCGACCCGGTCACCCAGCCTGCGTTTAGCGCCTTTGTCACCCAGCCTGCGGATGGCTTTACCGCCTGGAAATACG   1530

AspLeuValThrProSerAspLeuArgLeuAspGlnPheMetLysArgTrpAlaProThrLeuThrGluAlaGluAlaSerAlaTyrAla
ATCTGGTTACGCCATCAGACCTGCGCCTTGACCAGTTCATGAAGCGTTGGGCGCCCACACTGACCGAAGCTGAGGCCTCCGCGTATGCTG   1620

AlaProPheProAspThrSerTyrGlnAlaGlyValArgLysPheProLysMetValAlaGlnArgAspGlnAlaCysIleAspIleSer
CGCCTTTCCCTGACACTTCCTATCAGGCTGGTGTACGCAAGTTTCCCAAGATGGTCGCGCAACGCGACCAGGCCTGCATCGACATTTCAA   1710

ThrGluAlaIleSerPheTrpGlnAsnAspTrpAsnGlyGlnThrPheMetAlaIleGlyMetLysAspLysLeuLeuGlyProAspVal
CCGAAGCGATTTCGTTCTGGCAGAACGACTGGAATGGCCAGACCTTCATGGCCATTGGCATGAAAGACAAATTGCTGGGACCGGACGTCA   1800

MetTyrProMetLysAlaLeuIleAsnGlyCysProGluProLeuGluIleAlaAspAlaGlyHisPheValGlnGluPheGlyGluGln
TGTATCCTATGAAGGCGCTCATTAATGGCTGCCCGGAACCCCTCGAAATAGCGGACGCTGGCCATTTCGTACAGGAGTTTGGCGAGCAAG   1890

ValAlaArgGluAlaLeuLysHisPheAlaGluThrGlu***
TGGCTCGCGAGGCCCTGAAACACTTTGCCGAGACAGAATAGAGAATAGAGCGATGAACATTATGAACCTGGTATTCCACCCCAACCTCAG   1980

GGCAATCGCACTATCCCCTGACAATGATGGGATCGTCGGGCCGATGTGGTCGGGCCTGTCTGACACGCCGTGAACTCGTCCCTGAGGGCT   2070

                                                                                          Met
CGCTGCCGCCGTCCTGGCGGCAGACGGTCAGCCAAACCCGACCACATCACCCCTCGGGAAGCCCGGTGCCGGTACGAAAACTGCTTGATG   2160

SerValPheAlaGlyAlaAlaAlaMetLeuProAsnProLysProPhePheGluLeuIleProAspProArgArgAlaThrProAsnLys
TCGGTATTTGCCGGAGCCGCCGCCATGCTGCCGAACCCCAAGCCGTTCTTCGAATTGATCCCTGATCCGCGCCGGGCCACGCCCAATAAG   2250

LeuHisSerLeuSerAspIleLeuSerIleAlaLeuCysAlaValLeuSerGlyMetAspAspTrpGluAlaValAlaGluPheGlyArg
CTTCACTCGCTATCGGATATCCTCAGCATCGCCCTCTGTGCGGTACTCTCCGGCATGGATGACTGGGAAGCGGTGGCAGAGTTTGGCCGC   2340
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ThrLysGluGlyTrpLeuArgGlnPheLeuProLeuAlaAsnGlyIleProSerHisAspThrPheGlyArgValPheSerLeuIleAsp
ACCAAGGAGGGTTGGCTGCGACAATTCCTGCCGCTGGCCAACGGTATTCCCTCCCACGATACCTTTGGCCGCGTATTTTCCCTGATCGAC  2430

ProGluAlaPheGluAlaAlaPhePheAspTrpAlaAlaHisAlaArgIleGlyGlyAspValLeuAspGlnLeuAlaLeuAspGlyLys
CCGGAGGCGTTCGAGGCGGCGTTTTTCGACTGGGCCGCCCATGCCCGCATCGGCGGCGACGTTCTGGATCAACTGGCACTGGACGGCAAG  2520

ThrValArgArgSerHisArgGlySerAlaGlyArgAlaLeuHisLeuLeuHisAlaTrpSerCysGluThrArgLeuLeuValAlaGln
ACCGTCCGCCGCTCCCATCGCGGTTCGGCAGGTCGTGCGCTGCATCTGCTCCATGCCTGGTCCTGCGAGACGCGCTTGCTGGTGGCTCAA  2610

ArgArgValAspThrLysSerAsnGluIleThrAlaIleProAspIleLeuSerLeuPheAspLeuArgGlyValThrIleSerIleAsp
CGCCGGGTGGACACCAAATCGAACGAGATAACGGCGATTCCGGACATTCTGTCACTGTTCGATCTGCGAGGCGTCACCATCTCGATCGAT  2700

AlaIleGlyCysGlnLysAlaValAlaArgGlnIleThrGluAlaGlyGlyAspTyrValLeuAlaLeuLysGlyAsnGlnSerAlaLeu
GCCATCGGCTGCCAGAAAGCCGTAGCCCGGCAGATCACCGAGGCCGGGGGCGACTATGTGTTGGCCCTCAAGGGGAACCAGAGCGCGCTG  2790

HisAspAspValArgLeuPheMetGluThrGlnAlaAspArgHisProGlnGlyGlnAlaGluAlaValGluLysAspHisGlyArgIle
CATGATGACGTTCGCCTGTTCATGGAGACCCAGGCGGATCGGCATCCCCAAGGCCAGGCCGAGGCCGTGGAGAAAGACCACGGCCGGATC  2880

GluThrArgArgIleTrpValAsnAspGluIleAspTrpLeuThrGlnLysProAspTrpProGlyLeuLysThrLeuValMetValGlu
GAAACCCGTCGTATCTGGGTCAACGACGAGATCGACTGGTTGACACAGAAGCCGGACTGGCCGGGACTGAAGACGCTGGTGATGGTGGAG  2970

SerArgArgGluLeuAsnGlyGlnValSerCysGluArgArgCysPheIleThrSerHisThrAlaAspPro
AGCCGGCGCGAGCTCAACGGCCAGGTCTCGTGTGAGCGGCGCTGCTTTATCACTTCTCATACCGCGGATCC      3041
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FIG. 4. Complete nucleotide sequence of the haloalkane dehalogenase gene (*dhlA*) and its flanking regions. The deduced amino acid sequences of the three longest open reading frames found are indicated. The sequence of the first open reading frame (italics) is encoded in the reverse complement of the DNA sequence shown and is upstream of the *dhlA* gene. Its putative promoter is indicated (.....). The two possible promoter regions of the second open reading frame (*dhlA*) are also indicated: ——, first promoter; -----, second promoter. ●, Nucleotides that match the *E. coli* 16S rRNA 3′ terminal sequence; *, stop codons. (DNA and protein sequences reported in this paper have been deposited at GenBank under accession number M26950.)

composition of purified dehalogenase, apart from the number of cysteines, which appeared to be 4 rather than 1 (17).

A strong ribosome-binding site was found close to the start codon of the *dhlA* gene. Two consensus *E. coli* promoter sequences composed of a −10 and a −35 region were present (Fig. 4). According to the scoring method of Mulligan et al. (23), the first promoter site with a −35 sequence starting at 918 was a strong one, comparable to the *E. coli trp* promoter. The second, at position 945, was somewhat weaker but still contained the strongly conserved nucleotides of the *E. coli* consensus promoter sequences (TTGaca and TAtaaT at −35 and −10, respectively, with strongly conserved residues in capitals) (21).

Downstream of the dehalogenase region, an open reading frame of at least 294 amino acids was found. This fragment continued over the *Bam*HI site of pPJ50. Upstream of *dhlA*, a shorter hypothetical protein of 202 amino acids was found encoded on the complementary strand.

A 12-bp inverted repeat was detected between the dehalogenase gene and the open reading frame downstream of *dhlA*. No *rho*-independent termination site could be found. No clear *E. coli* promoter region or ribosome-binding site preceding the downstream open reading frame was found. A possible promoter sequence for the hypothetical upstream protein, encoded on the reverse complement, was present in the *dhlA* gene (Fig. 4).

DISCUSSION

The degradation of 1,2-dichloroethane by *X. autotrophicus* GJ10 proceeds through the concerted action of enzymes that are specific for halogenated xenobiotic compounds and enzymes that are involved in the metabolism of natural compounds, such as alcohols. The former are the dehalogenases that catalyze hydrolysis of 1,2-dichloroethane and chloroacetate. Both enzymes show a broad substrate range and are only produced by isolates that utilize halogenated substrates and, thus, can be considered enzymes that are acquired by this specific strain of *X. autotrophicus* during genetic adaptation to degradation of chlorinated hydrocarbons. In this paper, we describe the cloning of genes involved in 1,2-dichloroethane metabolism and present a further analysis of the haloalkane dehalogenase encoding gene *dhlA*.

The absence of dehalogenase activities in natural isolates of *X. autotrophicus* allowed the identification of clones containing the dehalogenase gene. Isolation of genes involved in methanol dehydrogenase and chloroacetaldehyde dehydrogenase activity was possible by screening for complementation of mutants lacking the dehydrogenase activities. In this way, four clones harboring genes involved in 1,2-dichloroethane metabolism were identified and the genes were localized on different DNA segments. No identical *Eco*RI fragments were present in the clones complementing haloalkane dehalogenase and halocarboxylic acid dehalogenase, indicating that these genes are not closely linked.

The biochemical defect causing loss of chloroethanol and methanol dehydrogenase activity (GJ10M27) has not yet been determined. The absence of methanol dehydrogenase activity in the chloroethanol utilization-negative mutant GJ10M27 confirms the involvement of the methanol dehydrogenase in chloroethanol and 1,2-dichloroethane metabolism (13). Methanol dehydrogenases of methylotrophic bacteria contain pyrolloquinoline quinone as a covalently bound cofactor. Specific other proteins are needed for assemblage of the active enzyme and electron transport. Up to 11 different genes distributed over 7 complementation groups were involved in methanol oxidation in *Xanthobacter* and *Methylobacterium* strains (1, 19, 24, 32). It may well be that the mutation in GJ10M27, complemented by pPJ17, is located in one of these groups.

The DNA segment harboring the dehalogenase gene was further characterized by DNA sequencing. The dehalogenase gene appeared to encode a 310-amino-acid polypeptide, with a composition and N-terminal sequence that are in

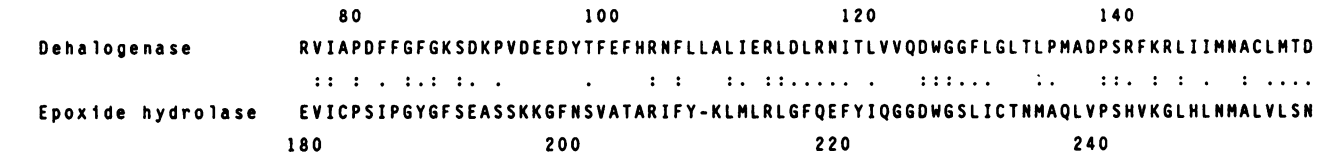

FIG. 5. Partial sequence homology of the dehalogenase protein and human epoxide hydrolase. Symbols: :, identical residues; ·, similar amino acids.

agreement with data that were obtained previously with the purified enzyme (17). Two other long open reading frames were found on the 3,041-bp *Bam*HI segment, but there is not yet any indication of their expression. All three open reading frames were compared to the SWISS-PROT protein data bank. No significant homologies were found for the upstream and downstream hypothetical proteins. The dehalogenase sequence also showed no overall similarity to other proteins, but a short stretch is significantly similar to human and rabbit epoxide hydrolases (9, 10) (Fig. 5). The calculation method of Goad and Kanehisa (8) showed a similarity of 46% with a statistical significance score of 8.9 for the dehalogenase segment 76 to 154 and the human epoxide hydrolase segment 179 to 256.

The efficient expression of the haloalkane dehalogenase gene in other gram-negative bacteria is not surprising in view of the fact that two regions with the consensus *E. coli* promoter sequence were present. Copy number probably also plays a role, since expression levels were higher in *X. autotrophicus* GJ10(pPJ20) than in the wild-type isolate GJ10. The *E. coli* consensus promoter sequence is known to stimulate transcription in *Pseudomonas* spp. (16), and our data suggest that it might also do so in *Xanthobacter* spp. In order to determine which of these sequences is the actual cause of the high expression and whether the promoter can be used for expression of other genes in *Xanthobacter* spp., it will be necessary to identify the transcription start site of the gene and to study expression of different genes linked to the promoter regions.

The haloalkane dehalogenase is an enzyme with broad substrate range, capable of hydrolyzing several chlorinated and brominated aliphatic hydrocarbons. Among the substrates are various environmentally important compounds that do not support growth. Introduction of dehalogenases in host strains that can utilize the products of dehalogenation could yield organisms with useful new degradative abilities. On the other hand, the substrate specificity of dehalogenases may limit the degradation of other chlorinated hydrocarbons. Using in vitro site-directed mutagenesis and in vivo selection of mutants, we are now attempting to obtain mutants with altered substrate range. The dehalogenase has been crystallized (26), and the future availability of the structure will give more insight into the structure-activity relationship of this protein. An intriguing questions is, for example, why the enzyme does not hydrolyze the environmentally important compound 1,2-dichloropropane although the structurally similar compounds 1,2-dibromopropane, 1,2-dichloroethane, and 1,3-dichloropropane are substrates.

ACKNOWLEDGMENTS

We thank G. Eggink and M. Kok for helpful suggestions and gifts of bacterial strains.

The work of D.B.J. has been made possible by a fellowship from the Royal Netherlands Academy of Sciences. Part of this work was financed by the Programma Commissie Industriële Biotechnologie.

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