First publ. in: Journal of Bacteriology 170 (1988), 12, pp. 5698-5704

Dichloromethane Dehalogenase with Improved Catalytic Activity Isolated from a Fast-Growing Dichloromethane-Utilizing Bacterium

RUDOLF SCHOLTZ,[1] LAWRENCE P. WACKETT,[2] CHRISTINE EGLI,[1] ALASDAIR M. COOK,[1] AND THOMAS LEISINGER[1]*

Department of Microbiology, Eidgenössische Technische Hochschule-Zentrum, CH-8092 Zurich, Switzerland,[1] and Gray Freshwater Biological Institute and Department of Biochemistry, University of Minnesota, Navarre, Minnesota 55392[2]

A methylotrophic bacterium, denoted strain DM11, was isolated from groundwater and shown to utilize dichloromethane or dibromomethane as the sole carbon and energy source. The new isolate grew at the high rate of 0.22 h^{-1} compared with 11 previously characterized dichloromethane-utilizing bacteria (μ_{max}, 0.08 h^{-1}). The dichloromethane dehalogenase from strain DM11 (group B enzyme) was purified by anion-exchange chromatography. It was shown to be substantially different from the set of dichloromethane dehalogenases from the 11 slow-growing strains (group A enzymes) that had previously been demonstrated to be identical. The V_{max} for the group B enzyme was 97 mkat/kg of protein, some 5.6-fold higher than that of the group A enzymes. The group A dehalogenases showed hyperbolic saturation with the cosubstrate glutathione, whereas the group B enzyme showed positive cooperativity in glutathione binding. Only 1 of 15 amino acids occupied common positions at the N termini, and amino acid contents were substantially different in group A and group B dehalogenases. Immunological assays demonstrated weak cross-reactivity between the two enzymes. Despite the observed structural and kinetic differences, there is potentially evolutionary relatedness between group A and group B enzymes, as indicated by (i) hybridization of DM11 DNA with a gene probe of the group A enzyme, (ii) a common requirement for glutathione in catalysis, and (iii) similar subunit molecular weights of about 34,000.

Dichloromethane (DCM) is a major haloalkane pollutant that serves as the sole carbon and energy source for a number of facultative methylotrophic bacteria (10), and the compound has been shown to be amenable to biotreatment (7). The ability to grow on DCM requires the biosynthesis of a DCM dehalogenase (19), which was first purified and characterized from a *Hyphomicrobium* sp. by Kohler-Staub and Leisinger (11). In all of the aerobic DCM-utilizing bacteria examined, the reaction is catalyzed by an inducible, glutathione (GSH)-dependent enzyme with a subunit molecular weight of 33,000 (10). Eleven DCM-degrading isolates from The Netherlands, Germany, and Switzerland were shown to contain a DCM dehalogenase. In the four cases examined in detail, the dehalogenases were identical with respect to their N-terminal amino acid sequences and very similar with respect to specific activity, inducibility, subunit molecular weight, and immunological cross-reactivity (10). The relatively low catalytic activity of the enzyme was compensated for in all of the strains by high levels of DCM dehalogenase (15 to 20% of total soluble protein). These observations led us to speculate that DCM dehalogenase is a recently evolved enzyme which was distributed by horizontal gene transfer (8) and whose catalytic activity is still in the process of being improved.

We have isolated a DCM-degrading methylotrophic bacterium that grows more rapidly on DCM than do the previous isolates, and we can explore the evolutionary improvement of DCM dehalogenase in nature. This organism contained a DCM dehalogenase with increased specific activity. In the study reported here, we compared the molecular and catalytic properties of two previously described low-specific-activity DCM dehalogenases (group A) with those of the highly active group B dehalogenase. This comparison revealed structural and functional differences between the two DCM dehalogenase types.

* Corresponding author.

MATERIALS AND METHODS

Materials. The inoculum for enrichments was groundwater from five wells in the vicinity of an industrial plant from which DCM had leaked for several years. The sources of chemicals are given elsewhere (14, 15). Horseradish peroxidase-coupled goat antibody to rabbit immunoglobulin G was from Calbiochem-Behring, La Jolla, Calif. [α-^{32}P]dCTP and the nick translation kit were from Amersham, Buckinghamshire, England.

Mono Q HR16/10 (Pharmacia, Uppsala, Sweden), DEAE 3-SW (Toyo Soda, Tokyo, Japan), and TSK G-4000 SW (Toyo Soda) columns were used in fast-protein liquid chromatography procedures. RP300 high-performance liquid chromatography columns were from Brownlee Labs, Inc., Santa Clara, Calif. Hybond-N membrane was purchased from Amersham.

Unless otherwise stated, all experiments with volatile substrates were done in screw-cap vessels closed with Mininert valves (Precision Sampling, Baton Rouge, La.).

Apparatus and analytical methods. Gas chromatography and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were done as described elsewhere (14). Protein bands in SDS-PAGE gels were quantified by scanning (Shimadzu, Tokyo, Japan) at 550 nm with an area integration program. Fast-protein liquid chromatography procedures used either the LKB (Bromma, Sweden) system or the Pharmacia system. N-terminal amino acid sequences were analyzed by an automated Edman procedure with a 470A ABI Sequenator (Beckman Instruments, Inc., Palo Alto, Calif.) with on-line phenylhydantoin and 120A

phenylhydantoin amino acid analysis (Beckman). Portions (10 to 15 pmol) of enzyme purified on preparative DEAE 3-SW were used. Total amino acid composition was determined as described elsewhere (14).

Protein was assayed by the method of Bradford (2), with bovine serum albumin or carbonic anhydrase as the standard; similar results were obtained with both standards. Halide release in the growth medium and the Gram reaction were determined as previously described (15).

Enzyme assays and steady-state kinetic determinations. DCM dehalogenase activities were determined by assaying the rate of formaldehyde production as described by Kohler-Staub and Leisinger (11), with the following modifications. (i) The incubation buffer used was either 50 mM Tris sulfate (pH 8.2) or 100 mM potassium phosphate (pH 8.0). (ii) For the chemical determination of formaldehyde, the incubation mixture (1.2 ml) contained 40 μl of crude extract (10 to 15 mg of protein per ml) or 8.3 μg of the purified enzyme. (iii) In the coupled assay with formaldehyde dehydrogenase (total volume, 0.5 ml), 1.72 μg of the purified protein or an appropriate amount of crude extract was used. Kinetic determinations in the coupled assay were done with phosphate buffer only. Steady-state kinetic data were analyzed by double reciprocal plots for substrates showing hyperbolic saturation effects and by Hill plots for substrates that displayed a sigmoidal curve in a plot of velocity versus substrate concentration (16).

Organisms, enrichment cultures, growth conditions, and storage of cells. The sources and the growth conditions for the bacterial strains *Pseudomonas* sp. strain DM1, *Hyphomicrobium* sp. strain DM2, *Methylobacterium* sp. strain DM4 (formerly *Pseudomonas* sp. strain DM4), and *Hyphomicrobium* sp. strain GJ21 are described elsewhere (10). Enrichment and cultivation of strain DM11 were done in a mineral salts medium (KH_2PO_4, 6.8 g/liter; $(NH_4)_2SO_4$, 0.2 g/liter; $MgSO_4$, 0.1 g/liter) which was autoclaved and supplemented with a 0.25% $Ca(NO_3)_2$ solution (1 ml/liter) and trace elements (1 ml/liter) and brought to pH 7.15 with 1 M NaOH. The previously described trace element solution (19) was modified by addition of $NiSO_4$ (0.1 g/liter) and concentrated H_2SO_4 (5 ml/liter) and removal of $Ca(NO_3)_2$.

Enrichment cultures were inoculated by addition of 1 or 10 ml of untreated groundwater to sterile medium (50 ml in 300-ml flasks) containing 5 mM DCM as the sole carbon and energy source. Growth was determined by monitoring turbidity, DCM utilization, and chloride release. Positive cultures were subcultured twice and purified on DCM indicator agar (8). In addition, 0.1-ml samples of groundwater were plated directly on DCM indicator agar.

Organisms were grown routinely in 30-ml portions with 8 mM DCM, as described elsewhere (15). Large quantities of cells from 1-liter cultures were obtained by growing the bacteria with 24 mM DCM, which was added in three portions at 8 mM each after the pH had been adjusted with 1 M NaOH to 7.15. The incubation conditions were the same as those described for the 30-ml cultures. Cells were harvested as described elsewhere (14). Growth kinetics and yields were measured as previously described (15).

All strains could be maintained in mineral salts medium or on DCM agar plates at 4°C for at least 2 months. All strains except DM11 could be maintained in mineral salts medium containing 50% (vol/vol) glycerol at −80°C.

Purification of enzymes. DCM dehalogenase from *Methylobacterium* sp. strain DM4 was purified by liquid chromatography (11) from 5 g (wet weight) of cells suspended in 10 ml of buffer; glycerol was omitted from all buffers. The enzyme from strain DM11 was purified by two methods.

(i) Two-step procedure. Crude extract (10 to 20 mg of protein per ml) was prepared and treated with protamine sulfate at 4°C (14). Supernatant fluid was loaded in 1-ml portions on a preparative DEAE 3-SW fast-protein liquid chromatography colum (15 cm by 21.5 mm [inside diameter]) equilibrated with buffer A (50 mM potassium phosphate [pH 7.0] containing 0.5 mM disodium EDTA and 2 mM dithiothreitol) and chromatographed at 25°C with 4 ml of buffer A per min for 9 min. A linear gradient of KCl in buffer A from 0 to 0.7 M KCl was then applied over 30 min. Protein in active fractions was concentrated by ultrafiltration (PM10 membrane; Amicon Grace, Danvers, Mass.) and desalted by high-performance liquid chromatography (RP300 column; 30 by 2.1 mm [inside diameter]) at a flow rate of 0.5 ml/min and 25°C as follows: 0 to 5 min, eluent A (10 μl of 2-mercaptoethanol per liter, 0.1% [vol/vol] trifluoroacetate); 5.1 to 25 min, linear gradient from 100% eluent A to 100% eluent B (1-propanol, 10 μl of 2-mercaptoethanol per ml, 0.1% trifluoroacetate). DCM dehalogenase eluted between 30 and 32% 1-propanol as a narrow, symmetrical peak. This preparation was used for total amino acid composition and N-terminal sequence determinations.

For analytical comparisons, crude extracts were subjected to protamine sulfate treatment and then chromatographed on an analytical DEAE 3-SW fast-protein liquid chromatography column (15 cm by 7.5 mm [inside diameter]). The buffers and the KCl gradient were identical to those of the two-step procedure. The flow rate was 0.8 ml/min, and the temperature was 25°C.

(ii) One-step procedure. Crude extract (2.3 ml) prepared in buffer C (50 mM Tris sulfate [pH 7.0], 25% [vol/vol] glycerol) was loaded directly on a Mono Q HR16/10 column equilibrated with buffer C. The column was eluted at 6 ml/min with buffer C for 20 min and then with a linear gradient of 0 to 0.4 M KCl in buffer C for 67 min. The chromatography was performed at 25°C. This preparation was used for kinetic experiments.

Molecular weight determinations. Molecular weights of subunits of DCM dehalogenases were examined under denaturating conditions by SDS-PAGE with 12% acrylamide (14).

Preparation of antisera. Electrophoretically pure dehalogenase from strain DM4 was used to prepare rabbit antisera. In the first injection, 400 μg of enzyme was emulsified in Freund complete adjuvant and injected subcutaneously in several locations. Subsequently, four booster injections of 100 μg of dehalogenase each were administered at 1- to 2-week intervals. The antisera obtained 1 week following the last injection showed a titer above 2,048 by the ring precipitin test and a titer of >100,000 by enzyme-linked immunosorbent assay (ELISA) (6).

Immunodiffusion and ELISA. Ouchterlony immunodiffusion experiments were done (6) with 1.5% (vol/vol) agarose in gels of 15-mm diameter and wells of 2-mm diameter. Center wells contained final antisera as described above or preimmune serum as a control. Outer wells contained crude extract prepared from strains DM4 and DM11 at 18 and 21 mg of protein per ml, respectively, or at serial dilutions of extract solutions.

ELISA experiments (6) were done with horseradish peroxidase-coupled goat antibody to rabbit immunoglobulin G and *o*-dianisidine as the colorimetric reagent. Both direct-binding and competition assays were conducted. In the direct-binding assay, DM4 and DM11 dehalogenases, re-

TABLE 1. Growth and quantitative substrate utilization by strains DM11, DM4, and DM2

Substrate	Strain DM11			Strain DM4			Strain DM2[a]		
	Y_m (g of protein/mol of C)	μ (h^{-1})	Concn (mM)	Y_m (g of protein/mol of C)	μ (h^{-1})	Concn (mM)	Y_m (g of protein/mol of C)	μ (h^{-1})	Concn (mM)
Methanol	8.5	0.20	10	5.8	0.17	10	9.2	0.10	10
DCM	6.5	0.22	10	3.0[a]	0.09[a]	10	7.6	0.07	10
Dibromomethane	4.8	0.14	6	—[b]	—	6	—	—	10

[a] These are previously published data (7, 19; R. Gälli, Ph.D. thesis, Eidgenössische Technische Hochschule, Zurich, Switzerland, 1986).
[b] —, No quantitative substrate utilization and poor growth.

spectively, were added to wells at a concentration of 5×10^{-7} M. Calculations were based on subunit molecular weights of 33,000 and DCM dehalogenase concentrations in the crude extract of 16 and 8% for the DM4 and DM11 dehalogenases, respectively. A series of 1:2 dilutions was made from the concentrated antigen solutions. Wells containing buffer only were prepared for control incubations. The extent of binding of the antibody to the serially diluted antigens was evaluated by the procedure of Dunbar (6). In the competition assay, all wells were coated with a 1-mg/ml solution of DM4 extract and then treated with serially diluted antiserum in the presence of different concentrations of DM4 and DM11 extract to compete with antibody binding to the antigen coated on the plate. Further development of the plate was performed as for the direct-binding assay.

DNA hybridization experiments. Total cellular DNAs from strains DM1, DM2, GJ21, DM4, and DM11 were isolated as described by Hintermann et al. (9), digested with *Bam*HI, and after separation on a horizontal 0.6% agarose gel, blotted to Hybond-N (Amersham) by the method of Smith and Summers (17). Prehybridization, hybridization, and autoradiography were done as described by Maniatis et al. (12). Nick translation was done with [α-^{32}P]dCTP with the nick translation kit from Amersham. The hybridization probe was plasmid pME1528, which carries in pUC18 (13) a 2.2-kilobase (kb) fragment with the DM4 DCM dehalogenase structural gene (S. La Roche, unpublished data) subcloned from plasmid pME1510 (8).

RESULTS

Enrichment, isolation, and characterization of strain DM11. Groundwater exposed to DCM was examined for the presence of microorganisms able to degrade DCM by plating out on DCM indicator agar. Five different colony morphologies were observed on all plates within 7 days, and between 10 and 16 days acid release was indicated. The decrease in pH correlated with the presence of an organism that formed orange colonies. It was present at 90 to 990 CFU/ml of groundwater (DCM indicator agar). Colonies with different morphologies from the selective plates were transferred to DCM-salts liquid medium, but only the organism that gave rise to orange colonies grew reproducibly in the selective medium. Liquid cultures inoculated with groundwater showed turbidity and DCM disappearance after 7 to 14 days. From these enrichments, the same orange-pigmented organism was isolated after multiple transfers through liquid and solid DCM-salts media. It was named strain DM11. The purity of strain DM11 was confirmed by colony homogeneity on acetate-salts agar and succinate-salts agar.

Strain DM11 was a gram-negative, motile rod (1 by 2 to 3 μm) with a single polar flagellum. The organism utilized methanol, DCM, bromochloromethane, dibromomethane, acetate, and succinate but was unable to grow on formate, diiodomethane, chloroform, ethanol, and a number of C-2 chloroalkanes. It is thus a facultative methylotrophic organism (18). No further taxonomic studies were conducted.

Growth physiology. Strain DM11 grew much more rapidly in DCM-salts medium than did strains DM1 to DM10 (Table 1). Substrate utilization was quantitative, and recovery of the halogen substituents as halides was stoichiometric. Strain DM11 is the first organism which has been demonstrated to utilize dibromomethane quantitatively (Table 1). The growth yield of strain DM11 utilizing DCM (effectively formaldehyde) or methanol (Table 1) was typical of other methylotrophs grown on methanol (1). The lower growth yield with dibromomethane was attributed to the lower growth rate (20).

Purification of the DCM dehalogenase of strain DM11. The dehalogenase was purified in two steps by anion-exchange chromatography after protamine sulfate treatment of the crude extract (Table 2). The active enzyme eluted between 420 and 460 mM KCl with a single distinct peak. The enzyme yield could be increased by (i) reducing the ratio of protamine sulfate to bacterial protein at the cost of incomplete removal of nucleic acids and (ii) taking a larger portion of the

TABLE 2. Purification of DCM dehalogenase from strain DM11

Enzyme fraction	Vol (ml)	Total protein (mg)	Total activity (nkat)	Sp act[a] (mkat/kg of protein)	Yield (%)	Purification factor
Two-step procedure						
Crude extract	15.7	118	387	3.28	100	1
Protamine sulfate	18.0	50	245	4.9	63	1.5
DEAE 3-SW	27.4	2.3	97	42.2	25	12.9
One-step procedure						
Crude extract	2.3	46	160	3.48	100	1
Mono Q	5.0	2.2	93.3	43.4	58	12.5

[a] Assays were done in 50 mM Tris sulfate buffer.

TABLE 3. Properties of DCM dehalogenases from strain DM11, *Methylobacterium* sp. strain DM4, and *Hyphomicrobium* sp. strain DM2[f]

DCM dehalogenase source	Substrates	GSH dependence	Sp act (mkat/kg of protein) of:		k_{cat} (s^{-1})	Enzyme as % of soluble protein	M_r(s) Subunit	K_m (μM) of DCM[a]	K_m or $S_{0.5}$ of GSH (μM)[b]
			Crude extract	Pure enzyme					
Strain DM11	CH_2Cl_2, CH_2Br_2, CH_2I_2	Sigmoidal	8.1[c]	97.0[c]	3.28[c]	8	34,000[d]	51	83
Strain DM4	CH_2Cl_2, CH_2Br_2, CH_2I_2	Hyperbolic	4.3	25[e]	0.87[e]	16[f]	35,000[d]	20	62
Strain DM2	CH_2Cl_2, CH_2Br_2, CH_2I_2	Hyperbolic	3.5	17.3	0.55	16	33,000; 35,000[d]	30	320

[a] GSH concentration, 5 mM.
[b] DCM concentration, 1 mM.
[c] These enzyme activities were measured in 100 mM potassium phosphate (pH 8.0).
[d] These data were obtained from parallel determinations on the same SDS-PAGE gel.
[e] These values for the pure enzyme were estimated from the specific activity in crude extracts and the percentage of total soluble protein that was DCM dehalogenase.
[f] Previously published data (10, 11).

enzyme peak from the DEAE column at the cost of a further purification step.

The low yield of the two-step procedure led us to purify the DCM dehalogenase without protamine sulfate treatment. Crude extract at a high protein concentration (20 mg/ml) was loaded directly on a preparative Mono Q column. Most of the activity could be collected in a single fraction which eluted at 200 mM KCl. A purification factor of 12.5 to obtain homogeneity indicated that the dehalogenase represented about 8% of the total soluble protein, which is consistent with data obtained from the two-step purification method (7.7%) and from densitometer scanning of an SDS-PAGE gel loaded with crude extract (6.7%). This method afforded one-step, 1-h purification of DM11 dehalogenase, yielding a pure enzyme which could be used to study enzyme kinetics without an additional concentration step. The purity of the enzyme was confirmed by SDS-gel electrophoresis and by gel filtration chromatography, in which the enzyme eluted as a single symmetrical peak.

The enzyme was identified as a DCM dehalogenase by its catalysis of the strictly GSH-dependent dehalogenation of its halogenated substrates (Table 3) to 2 mol of a halide ion and 1 mol of formaldehyde. However, unlike the identical dehalogenases in strains DM2 and DM4, the DM11 enzyme had a significantly higher specific activity. The DM11 dehalogenase showed a 2.5-fold higher specific activity then did the DM2 or DM4 enzyme when both were assayed in Tris buffer. In phosphate buffer, the difference was more significant; the DM11 enzyme catalyzed dehalogenation at a 5.6-fold greater rate (Table 3). Furthermore, the enzyme made up a smaller portion of the total soluble protein in strain DM11 than did the comparable enzymes in strains DM2 and DM4 (Table 3).

Physical properties of the enzyme. The subunit molecular weight of the DM11 enzyme was very similar to that of the DM2 or DM4 enzyme, although it reproducibly migrated slightly faster in 12% SDS-PAGE. These determinations showed molecular weights of 34,000 for the DM11 dehalogenase and 35,000 for the DM2 or DM4 enzyme (Table 3).

The amino acid compositions of the DM11, DM2, and DM4 enzymes were examined (Table 4). In five cases, the DM11 enzyme differed by about 50% in the frequency of a constituent from the DM2 and DM4 enzymes: aspartate, threonine, serine, glutamate, and phenylalanine.

The relatively low level of basic amino acids found in the DM11 enzyme was consistent with the ready purification (Table 2) of this enzyme on an anion-exchange resin. The DM11 enzyme was eluted as a narrow peak at a high salt concentration and separated from other proteins (Fig. 1C), whereas the DM2 and DM4 enzymes were eluted as broad peaks with many other proteins at low salt concentrations (Fig. 1A and B). There was negligible interaction between the dehalogenases when, e.g., extracts from DM4 and DM11 were mixed (Fig. 1D).

The N-terminal amino acid sequence of DM11 dehalogenase was determined and compared with the sequence (10) of the DM2 and DM4 enzymes (Fig. 2). No direct homology was observed between the N termini, with the exception of the histidine residue in position 9; the amino acid pair L-R was observed in both sequences (positions 4 and 5 in DM11 and 14 and 15 in DM4). These data confirm the differences between the DM11 (group B) enzyme and the other type represented by DM2 and DM4 (group A). Both sequences were compared with proteins of the database in Wisconsin (sequence analysis software package, version 5.1, October 1987 [4]) and showed no homology with any other protein.

Immunological properties. Ouchterlony immunodiffusion analysis of the crude extract from strain DM4 and purified

TABLE 4. Amino acid compositions of DCM dehalogenases from strain DM11, *Methylobacterium* sp. strain DM4, and *Hyphomicrobium* sp. strain DM2[a]

Amino acid	No. of residues/mol		
	DM11	DM4	DM2
Aspartic acid	33	23	23
Threonine	22	16	16
Serine	19	12	11
Glutamic acid	28	39	39
Proline	12	14	14
Glycine	25	25	23
Alanine	22	23	22
Half-cystine	2	3	3
Valine	20	18	17
Methionine	5	3	3
Isoleucine	12	13	13
Leucine	21	17	17
Tyrosine	12	13	14
Phenylalanine	9	14	17
Histidine	7	8	8
Lysine	12	16	17
Arginine	12	16	15

[a] The values shown were measured at 24 h and corrected to zero time for hydrolysis. Cysteine was determined directly, presumably underestimating the cysteine levels. Methionine was determined as methionine sulfone. Tryptophan was not determined.

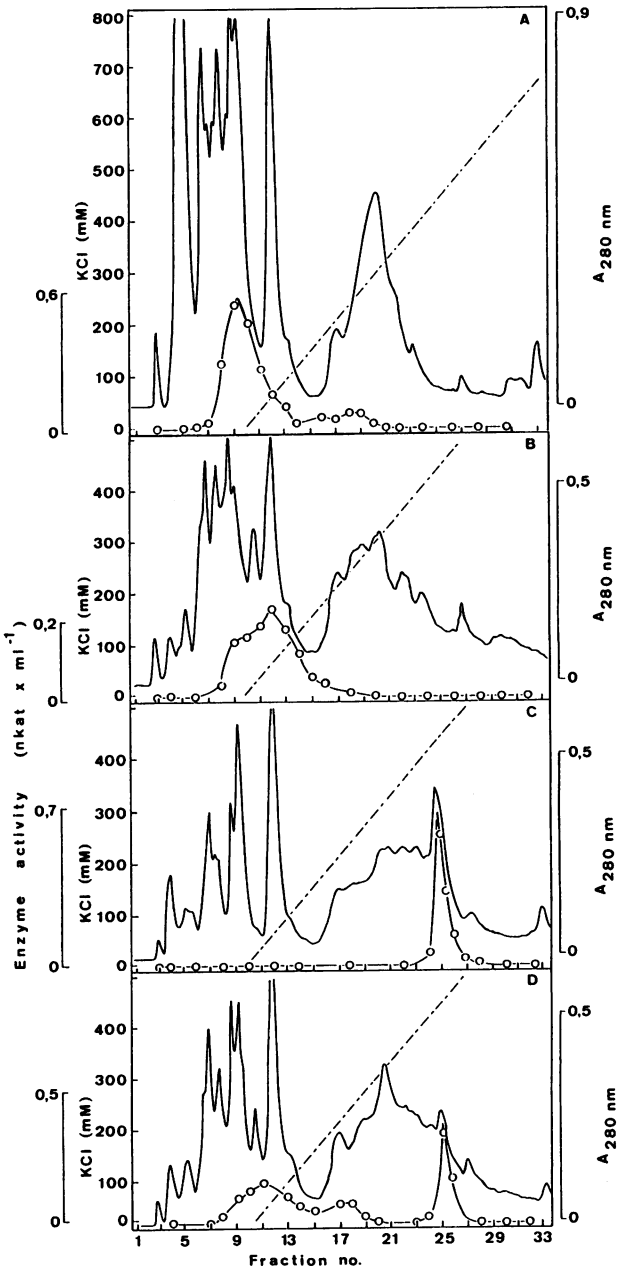

FIG. 1. Elution profiles from an analytical DEAE 3-SW column of DCM dehalogenases in protamine sulfate-treated crude extracts from strain DM11, *Methylobacterium* sp. strain DM4, and *Hyphomicrobium* sp. strain DM2. Panels: A, DM2; B, DM4; C, DM11; D, DM11 and DM4. —, Protein; ---, KCl gradient; O, enzyme activity.

dehalogenase from strain DM4 showed precipitin lines with rabbit antisera but not with serum obtained before injection of DM4 dehalogenase. When crude extract and purified dehalogenase from strain DM4 were in adjacent wells, immunological identity was indicated by fusion of the precipitin lines and the absence of a spur. In contrast, however, no reaction was observed between DM11 extract and antisera prepared against DCM dehalogenase from strain DM4.

To follow up these data, an ELISA was used as a more sensitive indicator of potential immunological cross-reaction of DM11 antigen with antisera prepared against DM4 dehalogenase. In the direct-binding assay we found a strong

Position:	1	5	10	15
DM11:	?-T-K-L-R-Y-L-H-H-P-A-S-Q-P-P			
DM2 and DM4:	M-S-P-N-P-T-N-I-H-T-G-K-T-L-R			

FIG. 2. N-terminal amino acid sequences of strain DM11 and *Hyphomicrobium* sp. strain DM2 and *Methylobacterium* sp. strain DM4.

reaction between DM4 antigen and its antisera, even at an antiserum dilution of 10^5. A similar reaction with the DM11 antigen was observed at an antiserum dilution of 4.5×10^2, but at dilutions greater than 10^3 the extent of reaction diminished. Thus, although the DM11 antigen did cross-react, it required a much greater antiserum concentration (3 orders of magnitude higher) to demonstrate a similar degree of reaction. To confirm this result, we conducted a competition assay in which DM11 antigen was used to block the DM4 antigen-antibody reaction. In this experiment, we could effectively block the reaction with soluble DM4 antigen in the reaction wells at 1:3, 1:9, and 1:27 dilutions, whereas these same concentrations of DM11 antigen did not block the DM4 antigen-antibody reaction. In conclusion, these immunological comparisons demonstrated only a very weak immunological relatedness between dehalogenases obtained from strains DM4 and DM11.

Kinetic properties of DCM dehalogenase from strain DM11. Preliminary kinetic experiments indicated that the enzyme displayed a 2.3-fold-greater specific activity when assayed in 100 mM potassium phosphate buffer (97 mkat/kg of protein; Table 3) at pH 8.2 than when assayed in Tris sulfate buffer at the same pH (42.2 to 43.4 mkat/kg of protein; Table 2). Thus, all initial-rate data were obtained with phosphate buffer in the assay mixture. By these data, the DM11 dehalogenase exhibited a 5.6-fold greater V_{max} than did the enzyme from DM2 or DM4 (Table 3). DCM dehalogenase prepared from strain DM4 did not show a marked difference in reaction velocity in assays with phosphate or Tris buffer.

To assess further differences and similarities between dehalogenases, the concentrations of GSH and DCM that sustain reaction rates of 0.5 V_{max} were determined for the DM11 dehalogenase. Initial-rate data that had been obtained by varying the concentration of DCM at a fixed GSH concentration (5 mM) showed hyperbolic saturation in a plot of velocity versus substrate concentration. An apparent K_m for DCM of 51 μM was obtained from a linear least-squares fit of rate data ($r = 0.997$) in a double-reciprocal plot (Table 3). The converse experiment was performed in which the GSH concentration was varied at a fixed concentration of DCM (1 mM). On the basis of these data, a plot of reaction velocity versus GSH concentration yielded a sigmoidal saturation curve. The initial rates determined at GSH concentrations of 40 to 134 μM were treated by the logarithmic form of the Hill equation to give a linear plot ($r = 0.997$), with $S_{0.5} = 83$ μM and $n_H = 1.93$. At a low GSH concentration (≤20 μM), the n_H was 0.99.

Hybridization experiments. Figure 3 shows hybridization of a ^{32}P-labeled gene probe of DCM dehalogenase from strain DM4 to *Bam*HI digests of total cellular DNAs from five different methylotrophic bacteria. Digests of strains DM1, GJ21, DM2, and DM4, representing the group A dehalogenases, showed a discrete band at 4.5 kb. This result is consistent with earlier experiments by Gälli and Leisinger (unpublished data), who found a single hybridization signal at 4.5 kb with DNA of 10 DCM-utilizing bacteria. Digests of DNA from strain DM11 also exhibited hybridization to the

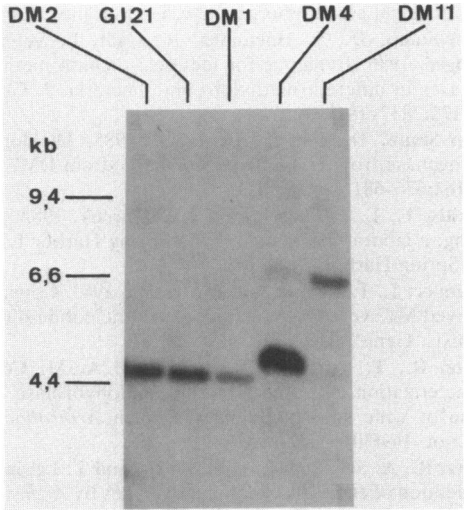

FIG. 3. Southern blot hybridization of ^{32}P-labeled pME1528 to *Bam*HI-digested total cellular DNAs from *Pseudomonas* sp. strain DM1, *Hyphomicrobium* sp. strain GJ21, *Hyphomicrobium* sp. strain DM2, *Methylobacterium* sp. strain DM4, and strain DM11.

DM4 gene probe. The hybridization band had a size of about 6.6 kb, indicating a different locus for the *Bam*HI restriction site(s) in the DNA of strain DM11. The intensity of the hybridization signal of the 6.6-kb DM11 fragment was somewhat lower than with the other digests, so in these experiments we used twice as much DNA from strain DM11 as from the other strains.

DISCUSSION

All previous studies on methylotrophic bacteria that utilize DCM as a sole carbon source indicate growth rates of 0.05 to 0.11 h^{-1} (Table 1). Further, methylotrophic strains DM1, DM2, DM4, and GJ21 contain identical DCM dehalogenases to convert DCM into formaldehyde (10). Our new isolate, strain DM11, grows significantly faster in DCM-salts medium than any previously described isolate (Table 1). Preliminary SDS-PAGE experiments with crude extract showed that a smaller percentage of total soluble protein migrated at the position previously demonstrated to represent the subunit of DCM dehalogenase. These observations suggested that strain DM11 contains a new DCM dehalogenase and that the turnover number for this enzyme is greater than that of the group studied previously. In this context, the dehalogenase from strain DM11 (group B DCM dehalogenase) was purified and its properties were compared with those of the group A enzyme obtained from strains DM2 and DM4.

In common with the group A dehalogenase, the group B enzyme has an obligate requirement for GSH, and the subunit molecular weight of 34,000 is similar to the molecular weight of 35,000 observed for the group A enzymes (Table 3). However, the possibility that the group B enzyme is a truncated form of the group A enzyme is ruled out by the following observations of structural differences between the two groups. (i) The 15 amino acids at the N termini were distinctly different, (ii) a summation of the variance between like components on the basis of the data in Table 4 gave a difference of 62 amino acids, and (iii) the elution volumes of the two proteins on an anion-exchange column were dramatically different (Fig. 1), which demonstrated a significant difference between the surface charges of the proteins. Further, the two enzyme groups are not closely related immunologically, as evidenced by the failure of antisera prepared against the group A DCM dehalogenase to cross-react with the group B enzyme by Ouchterlony analysis. Only the more sensitive ELISA methodologies (3) show the group A and B enzymes to possess weak immunological cross-reactivity.

The most significant distinction between these DCM dehalogenases, germane to the issue of enhanced DCM biodegradation rates, was the difference in kinetic properties. Under conditions of substrate saturation, the group B enzyme was significantly faster in dechlorination than the group A enzyme. Further study of the structural and catalytic properties underlying this difference in V_{max} should prove instructive in elucidating the mechanism of DCM dehalogenation. A second major kinetic difference between these two classes is the observed positive cooperativity in GSH binding by the DM11 DCM dehalogenase. The slope of the Hill plot derived from steady-state rate determinations yielded an n_H of $\approx$2. This result could be indicative of two equivalent substrate binding sites with strong cooperativity or a greater number of binding sites with less interaction between sites (16). Further kinetic studies and additional insights into the oligomeric structure of the DM11 enzyme are required to resolve this issue. An additional observation was made that the slope of the Hill plot approaches 1 (n_H = 0.99 obtained experimentally) at low concentrations. This indicates that only one GSH-binding site needs to be occupied before any reaction occurs.

Allosteric effects in enzymes offer greater metabolic control over the reaction rate (5), and this may constitute the evolutionary basis of the observed positive cooperativity in GSH binding. In the case of the group B DCM dehalogenase, a change in reaction rate of 0.1 to 0.9 V_{max} requires only a ninefold increase in the GSH concentration. In contrast, the same velocity change in enzyme reactions showing hyperbolic saturation typically requires substrate concentration changes on the order of 100-fold. In this context, it would be of interest to examine GSH levels in vivo and their effects on metabolic control of DCM dehalogenase during growth of strain DM11 in DCM medium.

Despite the clear structural and catalytic differences between the group A and group B enzymes, a gene probe prepared with cloned DNA from strain DM4 hybridized with DNA from strain DM11. There is precedence for using DNA probe methodologies that display greater sensitivity in detecting gene relatedness than immunodiffusion techniques that demonstrate antigenic relatedness beween the corresponding gene products. For example, antiserum prepared against methanol dehydrogenase from *Methylobacterium organophilum* does not show cross-reaction by Ouchterlony analysis with the enzyme from several other methylotrophic bacteria, although a DNA probe for methanol dehydrogenase from *M. organophilum* is capable of hybridizing with the respective genes from those methylotrophs (B. Bratina and R. S. Hanson, personal communication). The cloning of genes that encode the group A and group B DCM dehalogenases paves the way for DNA sequencing and, thus, a complete primary structure comparison between these two enzyme types. Further studies of this kind will allow an extension of the current investigation to lead into detailed mechanistic and evolutionary questions concerning bacterial DCM catabolism.

ACKNOWLEDGMENTS

We are grateful to Peter James for N-terminal sequencing and to Monica Wirth for determining the amino acid compositions. We acknowledge the expert technical assistance of Steve Householder in the growth of strain DM11 and purification of the enzyme. We thank Salome La Roche for providing the DM4 gene probe.

This work was supported by a grant from the Swiss Federal Institute of Technology, Zurich, Switzerland.

LITERATURE CITED

1. **Anthony, C.** 1982. The biochemistry of the methylotrophs, p. 245–268. Academic Press, Inc., London.
2. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Clausen, J.** 1981. Immunochemical techniques for the identification and estimation of macromolecules, p. 47–71. *In* T. S. Work and E. Work (ed.), Laboratory techniques in biochemistry and molecular biology. Elsevier Biochemical Press, Amsterdam.
4. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
5. **Dixon, M., and E. C. Webb.** 1979. Enzymes, p. 399–467. Academic Press, Inc., New York.
6. **Dunbar, B. S.** 1987. Two-dimensional electrophoresis and immunological techniques. Plenum Publishing Corp., New York.
7. **Gälli, R., and T. Leisinger.** 1985. Specialized bacterial strains for the removal of dichloromethane from industrial waste. Conserv. Recycling **8**:91–100.
8. **Gälli, R., and T. Leisinger.** 1988. Plasmid analysis and cloning of the dichloromethane utilization genes of *Methylobacterium* sp. DM4. J. Gen. Microbiol. **134**:943–952.
9. **Hintermann, G., R. Crameri, T. Kieser, and R. Hütter.** 1981. Restriction analysis of the *Streptomyces glaucescens* genome by agarose gel electrophoresis. Arch. Microbiol. **130**:218–222.
10. **Kohler-Staub, D., S. Hartmans, R. Gälli, F. Suter, and T. Leisinger.** 1986. Evidence for identical dichloromethane dehalogenases in different methylotrophic bacteria. J. Gen. Microbiol. **132**:2837–2843.
11. **Kohler-Staub, D., and T. Leisinger.** 1985. Dichloromethane dehalogenase from *Hyphomicrobium* sp. strain DM2. J. Bacteriol. **162**:676–681.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26**:101–106.
14. **Scholtz, R., T. Leisinger, F. Suter, and A. M. Cook.** 1987. Characterization of 1-chlorohexane halidohydrolase, a dehalogenase of wide substrate range from an *Arthrobacter* sp. J. Bacteriol. **169**:5016–5021.
15. **Scholtz, R., A. Schmuckle, A. M. Cook, and T. Leisinger.** 1987. Degradation of eighteen 1-monohaloalkanes by *Arthrobacter* sp. strain HA1. J. Gen. Microbiol. **133**:267–274.
16. **Segel, I. H.** 1976. Biochemical calculations. John Wiley & Sons, Inc., New York.
17. **Smith, G. E., and M. D. Summers.** 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. Anal. Biochem. **109**:123–129.
18. **Stanier, R. Y., J. L. Ingraham, M. L. Wheelis, and P. R. Painter.** 1986. The microbial world, p. 395–401. Prentice-Hall, Inc., Englewood Cliffs, N.J.
19. **Stucki, G., R. Gälli, H.-R. Ebersold, and T. Leisinger.** 1981. Dehalogenation of dichloromethane by cell extracts of *Hyphomicrobium* DM2. Arch. Microbiol. **130**:366–371.
20. **Tempest, D. W., and O. M. Neijssel.** 1984. The status of Y_{ATP} and maintenance energy as biologically interpretable phenomena. Annu. Rev. Microbiol. **38**:459–486.