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Isolation and Characterization of the *Methylophilus* sp. Strain DM11 Gene Encoding Dichloromethane Dehalogenase/Glutathione *S*-Transferase

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The restricted facultative methylotroph *Methylophilus* sp. strain DM11 utilizes dichloromethane as the sole carbon and energy source. It differs from other dichloromethane-utilizing methylotrophs by faster growth on this substrate and by possession of a group B dichloromethane dehalogenase catalyzing dechlorination at a fivefold-higher rate than the group A enzymes of slow-growing strains. We isolated *dcmA*, the structural gene of the strain DM11 dichloromethane dehalogenase, to elucidate its relationship to the previously characterized *dcmA* gene of *Methylobacterium* sp. strain DM4, which encodes a group A enzyme. Nucleotide sequence determination of *dcmA* from strain DM11 predicts a protein of 267 amino acids, corresponding to a molecular mass of 31,197 Da. The 5′ terminus of in vivo *dcmA* transcripts was determined by primer extension to be 70 bp upstream of the translation initiation codon. It was preceded by a putative promoter sequence with high resemblance to the *Escherichia coli* σ^{70} consensus promoter sequence. *dcmA* and 130 bp of its upstream sequence were brought under control of the *tac* promoter and expressed in *E. coli* to approximately 20% of the total cellular protein by induction with isopropylthiogalactopyranoside (IPTG) and growth at 25°C. Expression at 37°C led to massive formation of inclusion bodies. Comparison of the strain DM11 and strain DM4 dichloromethane dehalogenase sequences revealed 59% identity at the DNA level and 56% identity at the protein level, thus indicating an ancient divergence of the two enzymes. Both dehalogenases are more closely related to eukaryotic class theta glutathione *S*-transferases than to a number of bacterial glutathione *S*-transferases.

The first step in the utilization of the xenobiotic dichloromethane (DCM) as a carbon and energy source by methylotrophic bacteria is catalyzed by DCM dehalogenase. This enzyme has a strict requirement for glutathione (GSH). It catalyzes the formation of an *S*-chloromethyl GSH conjugate which is assumed to undergo nonenzymatic hydrolysis to *S*-hydroxymethyl GSH. Decomposition of the latter then leads to formaldehyde, a central metabolite of methylotrophic metabolism, and to the regeneration of GSH (17, 24). The amino acid sequence deduced from *dcmA*, the structural gene of DCM dehalogenase, revealed that this enzyme is related to the GSH *S*-transferase enzyme family (21).

DCM dehalogenases have been purified and characterized from five facultative methylotrophs isolated from different environments contaminated with DCM (16, 17, 41). They were found to be very similar with respect to subunit molecular mass, obligate requirement for GSH in catalysis, and a substrate range restricted to dichloro-, dibromo-, and diiodomethane. However, with respect to other criteria such as N-terminal amino acid sequences, kinetic properties, and immunological relatedness, they clearly fall into two classes. One class is formed by the group A enzymes, the DCM dehalogenases of the unidentified methylotrophic bacterium DM1, of the *Hyphomicrobium* spp. strains DM2 and GJ21, and of *Methylobacterium* sp. strain DM4. The other class consists of the group B enzyme from the methylotrophic bacterium strain DM11, which has recently been identified as a *Methylophilus* sp. strain (7). The most significant difference between the two groups lies in their kinetic properties. Under conditions of substrate saturation, the group B enzyme is significantly faster in dechlorination than group A enzymes. This is reflected in a more than twofold-increased growth rate of *Methylophilus* sp. strain DM11 on DCM compared with other DCM-utilizing methylotrophs and in a smaller fraction (7% versus 15 to 20%) of the total soluble protein representing DCM dehalogenase (41). In the study reported here, we characterize the group B DCM dehalogenase structural gene of *Methylophilus* sp. strain DM11 and compare the amino acid sequence of its product to that of the group A dehalogenase of *Methylobacterium* sp. strain DM4 as well as to the sequences of prokaryotic and eukaryotic GSH *S*-transferases.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Methylophilus* sp. strain DM11 is in the collection of the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, as DSM 6813. Solid and liquid media used for growth of this organism are described elsewhere (41). The *Escherichia coli* strain used in cloning and expression was DH5α [*supE44 ΔlacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (11). It was grown on LB agar or with vigorous aeration in LB (38), supplemented with ampicillin (200 μg/ml) when appropriate. pBluescript KS/SK(+) (42) and the expression vector pMS119EH were used for cloning. The latter is a derivative of pJF119EH (9) which was kindly supplied by E. Lanka. Relevant plasmids constructed in the course of the present work are shown in Fig. 1.

DNA techniques. Transformations and other cloning procedures were done by standard techniques (2, 38). Restriction enzymes were obtained from Boehringer GmbH (Mannheim, Germany) or New England Biolabs Inc. (Beverly, Mass.).

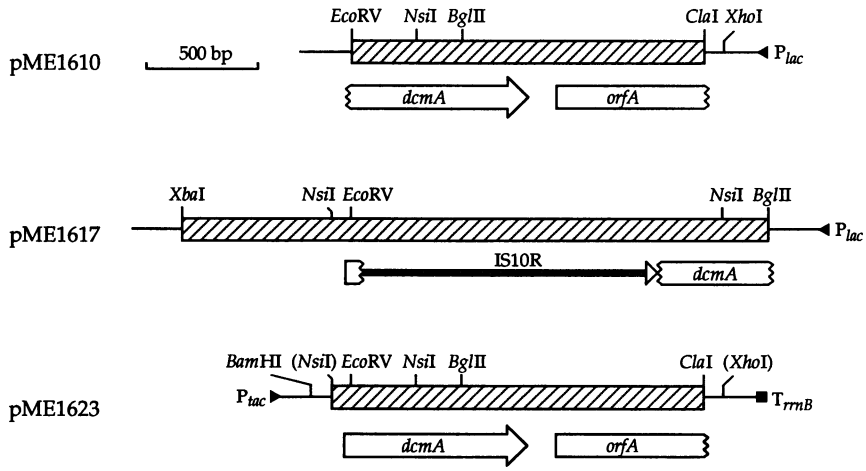

FIG. 1. Restriction map of the region encoding *dcmA*. Plasmid pME1610 is the clone detected by hybridization with oligonucleotide RB14. Plasmid pME1617 was then isolated by using the *Eco*RV-*Nsi*I fragment of pME1610 as a hybridization probe. Fragments of plasmids pME1610 and pME1617 were combined and introduced into the expression vector pMS119EH to yield plasmid pME1623. Cloned fragments of *Methylophilus* sp. strain DM11 DNA (hatched bars) are drawn to scale. Restriction sites in parentheses were lost on subcloning.

Digestions were performed as recommended by the manufacturers. Random priming of DNA was done by the method of Feinberg and Vogelstein (8) with [α-^{32}P]dCTP (3,000 Ci/mmol) or with digoxigenin-dUTP (Boehringer). Oligonucleotides were labelled at the 5′ end with [γ-^{32}P]ATP (5,000 Ci/mmol) as described by Sambrook et al. (38). Southern blots on Hybond-N nylon membranes (Amersham International, Amersham, United Kingdom) and DNA-DNA hybridizations were performed by Amersham protocol. Hybridization with nonradioactive probes was done by using the Boehringer digoxiglnin luminescent detection protocol. DNA sequences were determined by the dideoxy-chain termination method (39) from double-stranded DNA templates, using Sequenase (U.S. Biochemical Corp., Cleveland, Ohio).

DNA isolation. Genomic DNA from *Methylophilus* sp. strain DM11 grown with 24 mM DCM (41) was isolated from a 100-ml culture by the cetyltrimethylammonium bromide precipitation method (2). Additional purification on a cesium chloride gradient was omitted. Plasmid DNA from *E. coli* was isolated by the method of Del Sal et al. (6). Preparative amounts of plasmid DNA were obtained by using Qiagen columns (Qiagen Inc., Chatsworth, Calif.).

RNA isolation. RNA from *Methylophilus* sp. strain DM11 was obtained by the rapid isolation method for RNA from gram-negative bacteria (2), with the following modifications: (i) cells from 200 ml of a *Methylophilus* culture grown with 24 mM DCM were harvested and resuspended in 10 ml of protoplasting buffer, and (ii) the protoplasts were treated with a fourfold volume of lysis buffer. The RNA was stored as an ethanol precipitate.

Primer extension. Samples of at least 50 μg of total RNA were mixed with 1.5 μl of 10× hybridization buffer (2) and with 5×10^6 cpm of 5′-^{32}P-end-labelled oligonucleotides. The mixture was denatured for 3 min at 100°C, incubated for 2 h at 60°C, and then slowly cooled to room temperature. The sample volume was increased to a final volume of 45 μl by 10 μl of H_2O, 9 μl of 5× reaction buffer (GIBCO BRL, Gaithersburg, Md.), 4.5 μl of 0.1 M dithiothreitol, 4.5 μl of 5 mM deoxynucleoside triphosphate mixture, 1.0 μl of RNase inhibitor (Boehringer) and 1.0 μl of SuperScript RNase H^- reverse transcriptase (GIBCO BRL). Primer extension was carried out for 1 h at 42°C. Termination of the reaction and analysis of the product were done as described elsewhere (2).

Enzyme purification. Crude extract of *Methylophilus* cells grown with 24 mM DCM was prepared in 50 mM potassium phosphate (pH 8.0)–1 mM EDTA–2 mM dithiothreitol–25% (vol/vol) glycerol and treated with protamine sulfate at 4°C (41). The DCM dehalogenase was purified from the resulting supernatant by anion-exchange chromatography on a Mono Q column (Pharmacia, Uppsala, Sweden) as described elsewhere (41), using slightly different buffers. Buffer A contained 50 mM potassium phosphate (pH 7.0) and 2 mM dithiothreitol; buffer B contained 1 M Na_2SO_4 in buffer A.

Preparation of antiserum. A polyclonal antiserum against DCM dehalogenase was raised in a female New Zealand White rabbit. The rabbit was primed with 100 μg of purified enzyme mixed with 50% (vol/vol) Freund's complete adjuvant (Calbiochem, San Diego, Calif.). Every 10 days, the animal was boosted with 50 to 80 μg of enzyme mixed with 50% (vol/vol) Freund's incomplete adjuvant (Calbiochem). After 36 days, the rabbit was exsanguinated. The polyclonal serum was prepared, affinity purified, and stored as described by Harlow and Lane (13).

Immunoblot analysis. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19) in a Mini-Protean II dual-slab cell (Bio-Rad, Richmond, Calif.). SDS-PAGE was performed with 6% acrylamide stacking and 12% acrylamide separating gels by the Bio-Rad protocol. Low-range prestained SDS-PAGE standards (Bio-Rad) were used for molecular weight determinations. The proteins were transferred from the gel onto a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.), using a Mini Trans-Blot cell (Bio-Rad). Blotting and immunological detection with alkaline phosphatase were carried out as recommended by the supplier. The affinity-purified antiserum was diluted 1:10.

Enzyme assay. DCM dehalogenase activity was measured by colorimetrically assaying the rate of formaldehyde production. The previously published method (25) was modified as follows: (i) the incubation buffer was 0.1 M potassium phosphate (pH 8.0), and (ii) the reaction mixture contained 7.5 mM reduced

GSH. Protein was assayed by the method of Bradford (5), using the Bio-Rad protocol.

Sequence analysis. Nucleic acid and amino acid sequences were analyzed by using the Genetics Computer Group program package, version 7 (University of Wisconsin, Madison, Wis.).

Nucleotide sequence accession number. The nucleotide sequence presented in this report has been assigned GenBank/EMBL accession number L26544.

RESULTS

Cloning of the *dcmA* gene. The N-terminal amino acid sequence of the DCM dehalogenase from *Methylophilus* sp. strain DM11 has previously been found to differ from the N-terminal sequences of four group A enzymes which are identical over a 15-residue continuous stretch (16). Renewed determination of the N-terminal sequence of the strain DM11 DCM dehalogenase revealed one error at position 15 of the previously determined sequence (41). It now correctly reads STKLRYLHHPASQPCRAVHQFM. The degenerated oligonucleotide RB14 (GCI GTI CA[T/C] CA[G/A] TT[T/C] ATG) designed according to the six C-terminal amino acids of this sequence was used to probe restriction-digested genomic DNA of strain DM11 by Southern hybridization. A 1.5-kb *Cla*I-*Eco*RV fragment yielded a distinct signal, and the same signal was observed when the *dcmA* gene from *Methylobacterium* sp. strain DM4 was used as a probe under low-stringency hybridization conditions (not shown).

*Cla*I-*Eco*RV fragments between 0.7 and 2.0 kb in length of genomic strain DM11 DNA were cloned in *E. coli* DH5α, using the pBluescript vector. Screening of 200 recombinant plasmids by hybridization with oligonucleotide RB14 led to three hybridization-positive plasmids carrying an insert with the expected size of 1.5 kb. One of these, plasmid pME1610, was sequenced. Its nucleotide sequence revealed that the cloned fragment encoded all but the five N-terminal amino acids of the strain DM11 DCM dehalogenase as well as part of an open reading frame (*orfA*) located downstream of the dehalogenase structural gene (Fig. 1). A 0.3-kb *Eco*RV-*Nsi*I fragment of pME1610 was used as a homologous probe in the cloning of an overlapping DNA fragment from strain DM11 encoding the *dcmA* N terminus plus the *dcmA* upstream region. Southern hybridization experiments had indicated a 1.3-kb *Bgl*II-*Xba*I fragment as a target for this cloning step, which was performed as described above. The yield of recombinant plasmids was low, and the insert of plasmid pME1617, the only recombinant obtained, was 2.6 kb rather than the expected 1.3 kb in size (Fig. 1). Sequence analysis of this construct showed that it carried the expected DNA region of strain DM11, but that the *dcmA* gene was inactivated by insertion of the insertion sequence IS*10R* (15). This element was inserted at position 20 of the dehalogenase amino acid sequence, and it was flanked by 9-bp direct repeats of the *dcmA* target site. Insertion of IS*10R* resulted in two consecutive pairs of stop codons in the frame of *dcmA* at the 5′ end of the transposon sequence. Southern hybridization analysis of DM11 DNA with pME1617 as a probe demonstrated that IS*10R* was picked up in the course of cloning the *Methylophilus* DNA in *E. coli* DH5α, but the reasons leading to insertional inactivation of *dcmA* were not investigated.

In a series of cloning steps, the 0.1-kb *Nsi*I-*Eco*RV fragment of pME1617 was combined with pME1610 and introduced into the expression vector pMS119EH to yield plasmid pME1623 (Fig. 1). This construct contained the entire *dcmA* gene plus part of *orfA*, and it was stably maintained in *E. coli*.

DNA sequence of the *dcmA* gene. The complete sequence of the *Methylophilus* sp. *dcmA* gene and its flanking regions is shown in Fig. 2. *dcmA* starts with an ATG codon (base 722) and ends with a TAA codon (base 1523) 100 bp upstream of the putative translational start of the open reading frame *orfA*. *orfA* ends outside of the sequenced region and encodes an amino acid sequence with no homology to known proteins. The amino acid sequence deduced from the 5′ end of *dcmA* was in agreement with the N-terminal amino acid sequence determined chemically from purified DCM dehalogenase which is devoid of the terminal *N*-formylmethionine residue. *dcmA* thus encodes a 31.036-kDa protein of 266 amino acids whose molecular mass is comparable to the DCM dehalogenase molecular mass of 34.0 kDa determined by SDS-PAGE (41).

The open reading frame encoding DCM dehalogenase is preceded by a potential ribosome binding site (Fig. 2). At 25 bp downstream of the *dcmA* termination codon is a 35-bp sequence with almost perfect dyad symmetry that may be involved in transcription termination. The G+C contents of *dcmA* and *orfA* are 37.6 and 33.5 mol%, respectively. Considering that the overall G+C content of *Methylophilus* sp. strain DM11 amounts to 50.6 mol%, these are remarkably low values which suggest that *dcmA* and its neighboring gene were acquired by horizontal transfer from other bacteria. In line with this notion, it was recently observed that *dcmA* of strain DM11 is located on a 70-kb plasmid (3).

Transcriptional start site of *dcmA*. The 5′ end of the *dcmA* transcript was determined by primer extension using total RNA of DCM-grown *Methylophilus* sp. strain DM11 as a template. The 32-bp primer used was complementary to nucleotide positions 709 to 740 of the sequenced fragment, thus covering the translational start site of *dcmA*. As shown in Fig. 3, a single mRNA 5′ end was mapped at position 600, 122 bp upstream of the translation initiation codon (Fig. 2). The same signal, with extremely low intensity, was observed when the primer extension experiment was conducted with total RNA extracted from methanol-grown cells (not shown). This observation is taken to indicate that in *Methylophilus* sp. strain DM11, as in *Methylobacterium* sp. strain DM4 (21), *dcmA* expression is regulated predominantly at the level of transcription.

Elements of a putative *dcmA* promoter are located upstream of the transcription start site. Positions 565 to 570 and positions 588 to 593 encode hexamers which are homologous in five of six bases to the −35 and −10 regions, respectively, of the *E. coli* σ^{70} consensus promoter (Fig. 2). The bases of the *dcmA* promoter deviating from the *E. coli* consensus sequence are among those that are least conserved in a variety of *E. coli* promoters (12). No homology of the region upstream of the transcription start to the putative consensus promoter proposed for the *moxF* genes of several methylotrophs (27) was observed.

Expression of the *dcmA* gene from *Methylophilus* sp. strain DM11 in *E. coli*. To express DCM dehalogenase in *E. coli* DH5α, the *dcmA* coding region plus 70 bp of its upstream region were cloned into the expression vector pMS119EH (Fig. 1). *E. coli* DH5α carrying pME1623, the construct with *dcmA* under the control of the *tac* promoter, was grown at various temperatures under inducing (with isopropylthiogalactopyranoside [IPTG]) and noninducing (without IPTG) conditions. Cell extracts were used to determine DCM dehalogenase specific activity. Figure 4 shows that the formation of catalytically active DCM dehalogenase strongly depended on the growth temperature of the host. Low yields of active enzyme were observed upon growth of *E. coli* at 37°C. Growth at 30°C led to improved yields, and upon growth at 25°C, the dehalo-

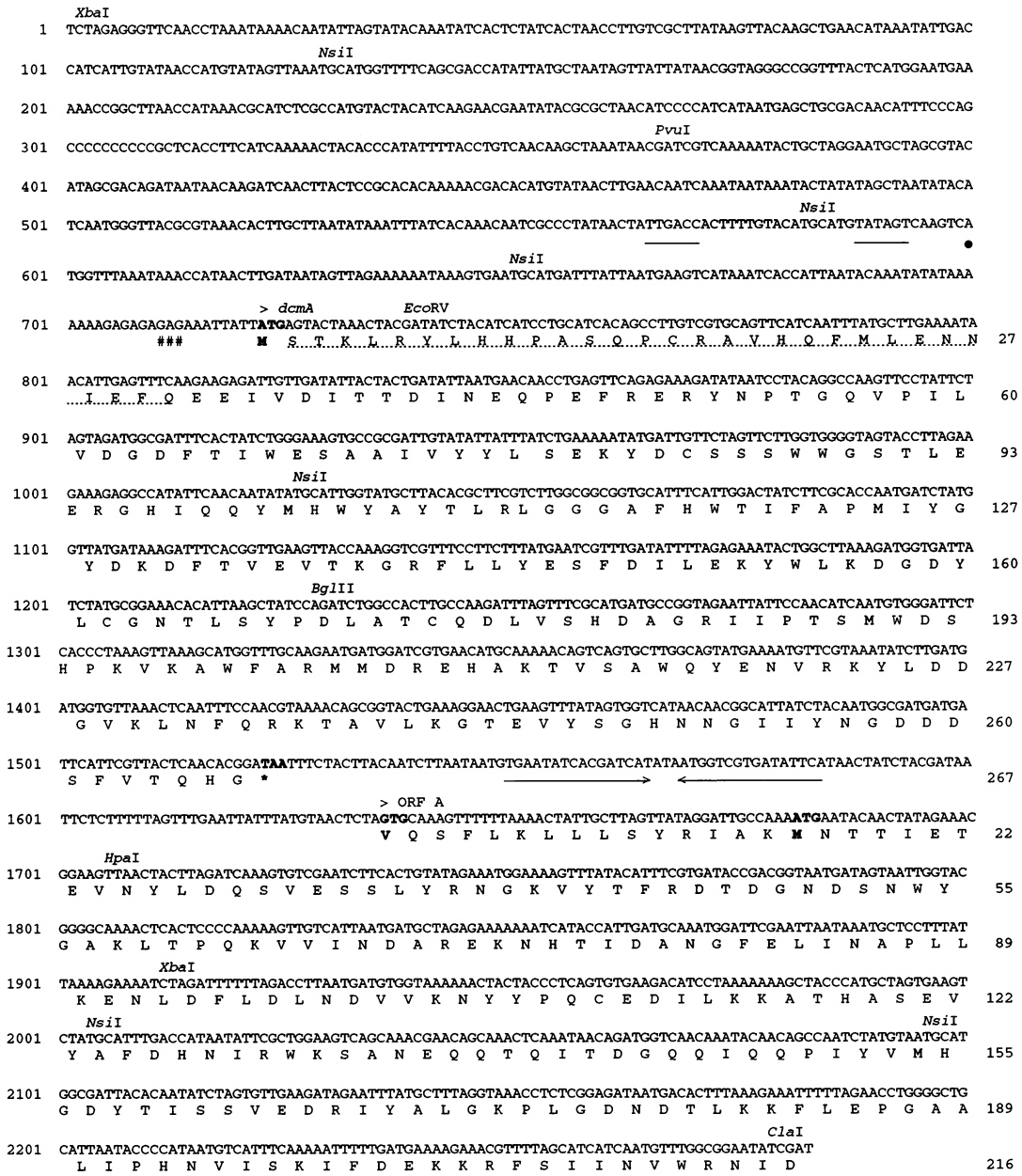

FIG. 2. Nucleotide sequence of the 2,283-bp *Xba*I-*Cla*I fragment containing the *dcmA* gene and its flanking regions of *Methylophilus* sp. strain DM11. The chemically determined N terminus is indicated by a dotted line. The transcription start point is marked by a black dot, and the sequences similar to the *E. coli* σ^{70} promoter sequence are underlined. The putative ribosome binding site for *dcmA* is marked by double crosses, and arrows indicate the inverted repeat downstream of *dcmA*.

genase specific activity amounted to 22 mkat/kg of protein, a value corresponding to a threefold increase over the specific activity measured in extracts from induced cells of *Methylophilus* sp. strain DM11. Accordingly, we estimate that active DCM dehalogenase makes up about 20% of the total soluble protein in induced recombinant *E. coli* cells grown at 25°C.

The strong effect of temperature on dehalogenase formation suggested that growth of *E. coli* DH5α(pME1623) at 37°C led to the formation of inclusion bodies (40). This notion was supported by the observation that cells grown at 37°C contained large amounts of insoluble material that sedimented upon centrifugation of crude extracts. SDS-PAGE of crude extracts from cells grown under a variety of conditions and immunostaining with polyclonal antibodies raised against purified DCM dehalogenase confirmed this notion. The electropherograms presented in Fig. 5 show that (i) the recombinant dehalogenase formed in *E. coli* had the same size as the enzyme produced in *Methylophilus* sp. strain DM11, (ii) *E. coli* DH5α(pME1623) produced low levels of dehalogenase under noninducing (without IPTG) conditions, and (iii) the major protein of the inclusion bodies formed by recombinant *E. coli* at 37°C consisted of DCM dehalogenase.

Formation of inclusion bodies was also observed upon expression of the DCM dehalogenase from *Methylobacterium*

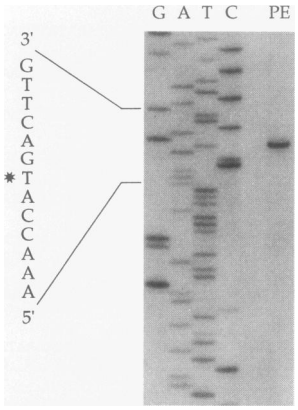

FIG. 3. Mapping of the *dcmA* transcriptional start site by primer extension. The cDNA product obtained by primer extension using *Methylophilus* sp. strain DM11 RNA (PE) and the corresponding sequencing ladder (GATC) are shown.

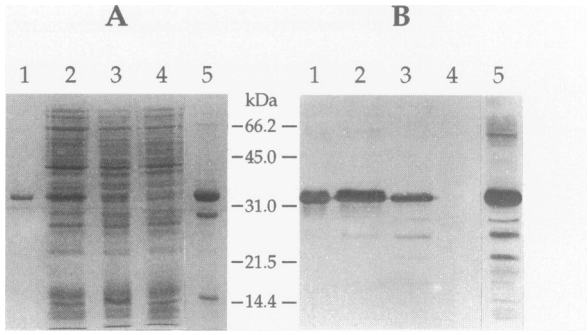

FIG. 5. SDS-PAGE (A) and the corresponding immunoblot with antibody raised against purified DCM dehalogenase of strain DM11 (B). The following preparations were separated and stained: lane 1, 0.5 μg of purified strain DM11 DCM dehalogenase; lane 2, 10 μg of crude extract of *E. coli* DH5α(pME1623) grown at 30°C plus IPTG; lane 3, 10 μg of crude extract of *E. coli* DH5α(pME1623) grown at 37°C minus IPTG; lane 4, 10 μg of *E. coli* DH5α(pMS119EH) grown at 30°C plus IPTG; lane 5, approximately 20 μg of inclusion bodies formed by *E. coli* DH5α(pME1623) upon growth at 37°C plus IPTG.

sp. strain DM4 in *E. coli* (46). In contrast, representatives of eukaryotic class alpha, mu, pi, and theta GSH *S*-transferases have been expressed in *E. coli* with no reported interference by the formation of inclusion bodies (18, 23, 43, 49). The insolubility of DCM dehalogenases in *E. coli* at high rates of expression thus may specifically be associated with the structure of these enzymes.

DISCUSSION

The nucleotide sequence of the *Methylophilus* sp. strain DM11 *dcmA* gene was primarily established for comparison with *dcmA* from *Methylobacterium* sp. strain DM4 (21). A high degree of homology between these genes would strongly suggest that *dcmA* from the *Methylophilus* strain, which specifies the catalytically more efficient group B DCM dehalogenase (41), has diverged recently from the version of *dcmA* present in the *Methylobacterium* strain, which encodes a less efficient group A enzyme. The driving force for such a divergence could possibly have been exerted by pollution of the environment with DCM and by the resulting selective pressure for fast growth with this xenobiotic. Our data show that this is not the case. The nucleotide sequence identity of the two genes is 59%, and the identity between the deduced amino acid sequences amounts to 56% (Table 1). Assuming an approximate nucleotide substitution rate of 10^{-9} nucleotide changes per site per year (26), the two *dcmA* genes are estimated to have diverged 4.1×10^8 years ago. To date, only dihalomethanes have been observed to be substrates of bacterial DCM dehalogenases. It is thus open to speculation whether the common ancestor of group A and group B dehalogenases exhibited activity with these substrates or whether it originally reacted with other substrates. If the latter were true, group A and group B enzymes would have acquired the ability to dehalogenate dihalomethanes, some of which are naturally occurring compounds (48), independently of each other.

Homology of the DNA fragments cloned and sequenced from the two DCM-utilizing methylotrophic bacteria was re-

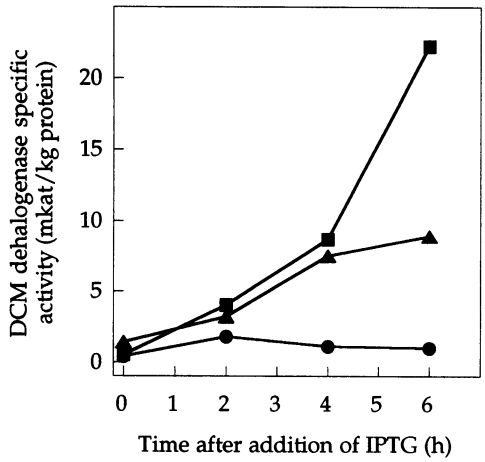

FIG. 4. Formation of DCM dehalogenase activity by *E. coli* DH5α(pME1623) at different growth temperatures. Cultures growing at 25°C (■), 30°C (▲), or 37°C (●) were induced at time zero by addition of IPTG to 0.1 mM and of glucose to 1% (wt/vol).

TABLE 1. Pairwise comparisons between *Methylophilus* sp. strain DM11 DCM dehalogenase and other GSH *S*-transferases, calculated by using the program GAP

Organism	GSH *S*-transferase enzyme designation[a]	% Identity	Reference
Methylobacterium sp. strain DM4	DCM dehalogenase	56	21
Proteus mirabilis	GSH *S*-transferase	25	32
Pseudomonas paucimobilis	β-Etherase	17	30
Flavobacterium sp.	Tetrachloro-*p*-hydroquinone reductive dehalogenase	23	34
Serratia marcescens	Fosfomycin: GSH *S*-transferase	14	1
Zea mays	GST III	25	10
Nicotiana tabacum	Par B	24	44
Rattus norvegicus	rGSTT1 (theta)	26	35
	rGSTT2 (theta)	27	33
	rGSTA2 (alpha)	23	45
	rGSTM1 (mu)	25	20
Sus scrofa domestica	pGSTP1 (pi)	23	37

[a] For the nomenclature of mammalian GSH *S*-transferases, see reference 29.

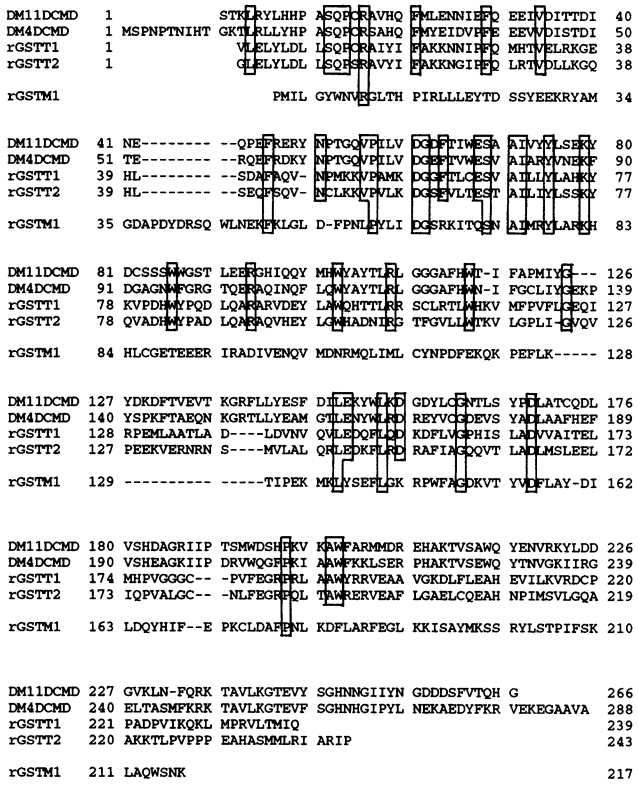

FIG. 6. Comparison of deduced amino acid sequences for four class theta and one class mu GSH *S*-transferases. Residues identical in all sequences and residues conserved among the class theta representatives are boxed. Abbreviations and references of the sequences used: DM11DCMD, DCM dehalogenase of *Methylophilus* sp. strain DM11 (this work); DM4DCMD, DCM dehalogenase of *Methylobacterium* sp. strain DM4 (21); rGSTT1, theta class rat GSH *S*-transferase (35); rGSTT2, theta class rat GSH *S*-transferase (33); rGSTM1, mu class rat GSH *S*-transferase (20).

stricted to the *dcmA* coding region, and there was no significant homology in the sequences flanking this gene. In both *Methylophilus* sp. strain DM11 (3) and *Methylobacterium* sp. strain DM4 (16), expression of *dcmA* is strongly induced by DCM. In the latter organism, inducibility of DCM dehalogenase was shown to be controlled by *dcmR*, the gene encoding a putative repressor (22). No signal was obtained when this gene was used to probe genomic DNA from *Methylophilus* sp. strain DM11 by hybridization at low stringency. This may indicate that the regulatory systems governing dehalogenase expression have not coevolved with *dcmA* but arose in the two organisms separately from *dcmA*.

Methylobacterium sp. strain DM4 DCM dehalogenase has been shown to belong to the GSH *S*-transferase supergene family (21), and within this family it exhibited greatest sequence homology with class theta representatives of mammalian GSH *S*-transferases (35, 47). The same holds true for DCM dehalogenase from *Methylophilus* sp. strain DM11 (Fig. 6; Table 1). The sequence homology between bacterial DCM dehalogenases and class theta GSH *S*-transferases is complemented by some shared properties of these enzymes such as their reactivity with DCM, their lack of activity with 1-chloro-2,4-dinitrobenzene, and their inability to bind to GSH affinity matrices (17, 31). Recently, sequences of a few bacterial GSH *S*-transferases have become available. We have compared them with respect to their overall amino acid identity (Table 1) and by sequence alignment (not shown) to eukaryotic GSH *S*-transferases and bacterial DCM dehalogenases. These comparisons show that DCM dehalogenases are more closely related to eukaryotic class theta enzymes than to the other bacterial GSH *S*-transferases described so far. Similarly, the *Proteus mirabilis* GSH *S*-transferase and the *Pseudomonas paucimobilis* β-etherase were more closely associated with plant or class theta GSH *S*-transferase enzymes, including the DCM dehalogenases, than with GSH *S*-transferases of the alpha, mu, and pi classes (not shown). As more sequences of prokaryotic GSH *S*-transferases become available, it will be interesting to see whether these are associated with class theta or with class theta-related enzymes rather than with GSH *S*-transferases of the other classes. A close relationship between prokaryotic GSH *S*-transferases and the class theta enzymes should be expected, according to Pemble and Taylor (35), who have suggested that class theta is representative of a progenitor GSH *S*-transferase whose descendants in eukaryotes have given rise to the other classes.

The alignment in Fig. 6 of two bacterial DCM dehalogenase amino acid sequences with the two class theta GSH *S*-transferases sequences available to date identifies regions common to the DCM dehalogenases as well as residues specifically conserved among representatives of class theta. The stretches conserved among the DCM dehalogenases allow the design of discovery primers to amplify by PCR *dcmA* sequences from genomic DNA of a range of DCM-utilizing methylotrophs. Assignment of functions in catalysis to amino acid residues conserved in the theta class remains speculative, since three-dimensional structures have been determined for GSH *S*-transferases of the alpha (42a), mu (14), and pi (36) classes but not for a theta class enzyme. For example, based on X-ray structures and on site-directed mutagenesis studies with mu and pi class GSH *S*-transferases, the conserved Tyr-6 (Fig. 6) of the rat liver GSH *S*-transferase of class mu is implicated in enhancing GSH nucleophilicity (28). Molecular modelling studies (4) suggest that the role of the thiol-activating tyrosine is played by a conserved serine (Ser-12 in DM11 DCM dehalogenase; Fig. 6) in the theta class enzymes. A number of other residues appear to be strongly conserved in theta class representatives but absent in members of other GSH *S*-transferase classes (Fig. 6). By using the expression system for DCM dehalogenase in *E. coli* reported here, their structural and functional importance can now be investigated by protein engineering techniques.

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