

## DNA Polymerase I Is Essential for Growth of *Methylobacterium dichloromethanicum* DM4 with Dichloromethane

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***Methylobacterium dichloromethanicum* DM4 grows with dichloromethane as the unique carbon and energy source by virtue of a single enzyme, dichloromethane dehalogenase–glutathione S-transferase. A mutant of the dichloromethane-degrading strain *M. dichloromethanicum* DM4, strain DM4-1445, was obtained by mini-Tn5 transposon mutagenesis that was no longer able to grow with dichloromethane. Dichloromethane dehalogenase activity in this mutant was comparable to that of the wild-type strain. The site of mini-Tn5 insertion in this mutant was located in the *polA* gene encoding DNA polymerase I, an enzyme with a well-known role in DNA repair. DNA polymerase activity was not detected in cell extracts of the *polA* mutant. Conjugation of a plasmid containing the intact DNA polymerase I gene into the *polA* mutant restored growth with dichloromethane, indicating that the *polA* gene defect was responsible for the observed lack of growth of this mutant with dichloromethane. Viability of the DM4-1445 mutant was strongly reduced upon exposure to both UV light and dichloromethane. The *polA*'-lacZ transcriptional fusion resulting from mini-Tn5 insertion was constitutively expressed at high levels and induced about twofold after addition of 10 mM dichloromethane. Taken together, these data indicate that DNA polymerase I is essential for growth of *M. dichloromethanicum* DM4 with dichloromethane and further suggest an important role of the DNA repair machinery in the degradation of halogenated, DNA-alkylating compounds by bacteria.**

Dichloromethane (DCM) is an organic solvent produced industrially in large amounts for a wide range of technical applications (Halogen Solvents Industry Alliance [http://www.hsia.org/white\_papers/methchlor.htm]). Its low boiling point and high solubility in water make it a frequently encountered environmental contaminant (36, 46). The toxicity of DCM to mammals continues to be investigated intensively (9, 14, 21, 40, 45), but its causes are not yet fully characterized at the molecular level. Many specialized aerobic methylotrophic bacteria have been isolated from soil and groundwater environments contaminated with DCM for their ability to grow with DCM as the sole source of carbon and energy (49). Such bacteria rely on a single enzyme, DCM dehalogenase, for this purpose. DCM dehalogenase, which can make up to 20% of the soluble protein during bacterial growth with DCM, was purified and shown to catalyze the glutathione-dependent transformation of DCM to formaldehyde, used in both biomass and energy production, and to two molecules of hydrochloric acid (31). The corresponding gene *dcmA* was cloned (33) from *Methylobacterium dichloromethanicum* DM4 (15) (formerly *Methylobacterium* sp. strain DM4), *Methylophilus* sp. strain DM11 (3), and, more recently, from several other DCM-degrading strains (49, 50). Sequence analysis indicates that DCM dehalogenases belong to the glutathione S-transferase (GST) enzyme family (27, 47). DCM dehalogenases were the first bacterial GSTs to be characterized, but it is becoming clear that the genomes of some gram-negative bacteria may contain more than a dozen GST genes (48). In all higher organisms with an aerobic lifestyle, GSTs serve as versatile, relatively nonspecific catalysts for the conjugation and subsequent detoxification of reactive

electrophilic compounds (27). In bacteria, however, GST often represent specialized catabolic enzymes with an essential role in the mineralization of toxic chemicals (47, 48).

Although the conversion of DCM to formaldehyde by DCM-degrading bacteria only requires the presence of DCM dehalogenase, growth-supporting GST-mediated dehalogenation of DCM may impose quite drastic requirements upon bacterial metabolism. On the one hand, DCM-converting GSTs from mammals and methylotrophic bacteria have toxic and mutagenic effects in *Salmonella enterica* serovar Typhimurium (18, 44) and *Methylobacterium* (18). On the other hand, the massive production of hydrochloric acid by cytosolic DCM dehalogenase during growth with DCM suggests that DCM-degrading methylotrophic bacteria may have evolved efficient systems for the maintenance of intracellular pH and for the excretion of chloride ions. These aspects of bacterial dehalogenation metabolism have been rather neglected until now and, in large part, remain to be explored. In the present work, we have used minitransposon insertion mutagenesis to identify genes associated with DCM metabolism in the DCM-degrading strain *M. dichloromethanicum* DM4. We report that a mutant of this strain disrupted in the gene encoding DNA polymerase I, an enzyme with a well-known role in DNA repair (16), is no longer able to grow with DCM as the sole carbon source. This suggests an important role for the DNA repair machinery during bacterial mineralization of DCM.

### MATERIALS AND METHODS

**Materials.** Restriction and DNA modifying enzymes used in cloning were from Fermentas. Oligonucleotides were purchased from Microsynth (Balgach, Switzerland). *Escherichia coli* DNA polymerase I and Klenow fragment were from New England Biolabs. All other chemicals were analytical grade or better and were purchased from Fluka except where noted.

**Bacterial strains, media, and growth conditions.** *E. coli* strains DH5 $\alpha$  (GIBCO/BRL Life Technologies) and XL1-Blue (Stratagene) were used for cloning, and *E. coli* strains S17-1 (41) and S17- $\lambda$ pir (38) were used as donor strains in biparental mating experiments. *E. coli* strains were grown under shaking at 37°C in Luria-Bertani medium (2), with kanamycin (25 mg/liter), ampicillin (100 mg/liter), and tetracycline (25 mg/liter) antibiotics as required. *M. dichlo-*

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*romethanicum* DM4 wild type (17) and derivatives of the mini-Tn5 insertion mutant strain DM4-1445 were grown at 30°C in liquid minimal medium (MM) on a rotary shaker at 150 rpm in glass flasks with gastight mininert caps (Supelco), with methanol (40 mM) and/or DCM (10 mM) as described (19). Solid media contained (per liter) 15 g of agar and 50 mg of cycloheximide. Bacterial growth in liquid cultures was determined by monitoring optical density at 600 nm (OD<sub>600</sub>). MM agar plates were incubated in 3-liter gastight glass jars to which 960 µl of methanol (MeOH) (yielding 40 mM final concentration) and/or 380 µl of DCM (10 mM final concentration) was added.

**Mini-Tn5 mutagenesis.** Mini-Tn5 transposon mutagenesis (13) of *M. dichloromethanicum* DM4 was performed by biparental plate conjugation of *E. coli* S17-1 $\lambda$ pir containing plasmid pUT/mini-Tn5lacZ1 (12) with wild-type strain DM4. A mixture of 50 µl of resuspended and 20-fold-concentrated cultures of donor (OD<sub>600</sub> = 0.5) and recipient (OD<sub>600</sub> = 1.0) strains was spotted on nutrient broth agar (Difco) at 30°C for 24 h. Kanamycin-resistant transconjugants were obtained by spreading the mating mixture on MM agar plates containing kanamycin (5 mg/liter) and incubation for 7 to 10 days in 3-liter gastight jars with 40 mM methanol as the carbon source. Colonies were patched on agar plates of the same medium and screened for impairment of growth with DCM.

**DNA isolation and manipulation.** Preparation of total DNA, restriction enzyme digestions, cloning, and Southern blot analysis were performed by standard procedures (2). Southern blot analysis of total DNA from DM4-1445, restricted with several enzymes, was performed with a 795-bp digoxigenin (DIG)-labeled fragment of the kanamycin gene generated by PCR with primers 5'-GAGCAT CAAATGAACTGC-3' and 5'-CATATTCAACGGGAAACG-3' using PCR DIG labeling mix (Boehringer Mannheim). The hybridizing 8.5-kb *Pst*I fragment was cloned into pBluescript KSII(+) (Stratagene) from total *Pst*I-digested DNA of the DM4-1445 mutant by selection for kanamycin resistance. Detection of the *polA* gene by Southern blot analysis was performed with a 530-bp DIG-labeled DNA probe generated by PCR with the primers 5'-GACCGAAGAGACGCA ACC-3' and 5'-CCTGACTCGAAATCGTAGAAC-3' selected from sequence analysis of the cloned *Pst*I fragment from mutant DM4-1445. The wild-type *polA* gene was cloned by ligation of a 7.5-kb *Sma*I/*Sal*I fragment of *M. dichloromethanicum* DM4 with pBluescript KSII(+) vector cut with *Sma*I/*Sal*I. A 4.7-kb *Sma*I/*Hind*III subfragment containing the entire *polA* gene was then ligated to broad-host-range cloning vector pVK100 (30) cut with *Bgl*II, blunted with T4 DNA polymerase, and then digested with *Hind*III. The resulting plasmid pME8112 was transformed into *E. coli* S17-1 and conjugated into mutant strain DM4-1445 by biparental mating as described above.

**Sequence analysis.** The 4.7-kb *Sma*I/*Hind*III fragment containing the entire *polA* gene was sequenced by primer walking on both strands with the Dye-Terminator kit using an ABI377 automated sequencer (Perkin-Elmer). Sequences were analyzed with the Genetics Computer Group sequence analysis package (version 10), and the assembled sequence was deposited in the EMBL database (accession no. AJ242630). Similarity searches were performed by using gapped BLAST and PSI-BLAST programs (1) for comparisons against public protein and gene databases.

**Preparation of cell extracts.** Bacterial cultures (100 ml) were harvested, resuspended in 3 ml of cold extraction buffer (50 mM Tris-HCl [pH 7.5], 2 mM dithiothreitol, 0.5 mM EDTA, 25% [wt/vol] glycerol), and disrupted in a French pressure cell (three times at 55 MPa). Cell debris were removed by centrifugation (30,000 × g at 4°C for 45 min), and the protein concentration was determined in the supernatant using a commercial Bradford reagent (Bio-Rad) and bovine serum albumin (Sigma) as the standard. Cell extracts were either analyzed directly or flash frozen in liquid nitrogen and stored at -80°C.

**DCM dehalogenase assay.** Specific activity of DCM dehalogenase was measured in triplicate in cell extracts (2 to 20 µg protein) using a previously described coupled enzyme assay with formaldehyde dehalogenase (51).

**DNA polymerase assay.** Nicked DNA was prepared from calf thymus DNA (0.8 mg/ml) with DNase I (0.26 µg/ml) in a solution containing 8 mM Tris-HCl (pH 8), 16 mM NaCl, 4 mM MgCl<sub>2</sub>, 0.8 mM EDTA, and bovine serum albumin (200 µg/ml) for 10 min at 37°C and isolated by ethanol precipitation after extraction. DNA polymerase activity was detected in cell extracts separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (10% acrylamide) containing nicked calf thymus DNA (125 µg/ml) and fibrinogen (40 µg/ml) using previously described methods (5, 22) with minor modifications. A prestained broad-range protein marker (New England Biolabs) was run to facilitate subsequent analysis. After electrophoresis, gels were washed for 24 h in 10 mM Tris buffer (pH 7.6) and further incubated overnight in the same buffer containing 2 mM MgCl<sub>2</sub>; a 15 µM concentration (each) of dATP, dGTP, and dTTP; and 100 µCi of [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham). Gels were then washed three times in 5% trichloroacetic acid containing 1% sodium pyrophosphate, dried, and autoradiographed for 48 h.

**$\beta$ -Galactosidase assay.**  $\beta$ -Galactosidase activity was measured in cell extracts by standard methods (37) and expressed as *o*-nitrophenyl- $\beta$ -D-galactoside hydrolyzed (in nanomoles/minute/milligram of protein), using a value of 4.5 mM<sup>-1</sup> cm<sup>-1</sup> for the extinction coefficient at 420 nm (8).

**Chloride determination.** Chloride was determined colorimetrically as its ferric thiocyanate complex as described previously (4). Samples of growing cultures were centrifuged at 4°C (13,000 rpm, 10 min, Heraeus biofuge) and cell supernatants were first diluted 1:1 with 30% H<sub>2</sub>O<sub>2</sub> and heated at 80°C for 2 min. Six hundred microliters of this solution was then mixed with 200 µl of 0.25 M ferric

ammonium sulfate, dissolved in 9 N nitric acid and then with 200 µl of saturated mercuric thiocyanate in ethanol. The absorbance at 460 nm was determined after 10 min of incubation at room temperature, and the chloride concentration was determined with a standard curve of 0 to 500 µM NaCl.

**Viability assessment.** Bacterial survival after various treatments was determined using a spot plating technique. Serially diluted cultures (10- to 10<sup>9</sup>-fold) were spotted (5 µl) in triplicate onto MM agar plates. Plates were then incubated in 3-liter gastight containers containing 40 mM MeOH, 10 mM DCM, or both carbon sources for 7 days at 30°C. Dilutions with spots containing 5 to 30 colonies were counted, and the resulting average was expressed as the ratio of viable cells in treated cultures to those in untreated cultures. In the case of UV exposure, cells were grown in MM with 40 mM MeOH, washed, serially diluted, and spotted on plates as described above. Plates were irradiated for different times with UV light (254 nm) from a germicidal lamp (Stratalinker UV model 1800 cross-linker; Stratagene) at a rate of 5 J m<sup>-2</sup> s<sup>-1</sup> and then incubated in the dark as described above. For determination of survival after DCM treatment, cultures were grown in MM with 40 mM MeOH and treated with 10 mM DCM at an OD<sub>600</sub> of 0.05, and viability of the cultures was determined at different time points after addition of 10 mM DCM as described above.

## RESULTS

**Isolation of strain DM4-1445, a mutant of *M. dichloromethanicum* DM4 unable to grow with DCM as the sole carbon source.** Random transposition of the mini-Tn5lacZ1 element into the chromosome of *M. dichloromethanicum* DM4 was obtained by biparental conjugation with *E. coli* S17-1 $\lambda$ pir (38) harboring plasmid pUT/mini-Tn5lacZ1 (12). Mini-Tn5 mutants of strain DM4 were obtained on solid MM with MeOH as the carbon source by selecting for the kanamycin resistance afforded by the minitransposon element. Screening of about 1,000 kanamycin-resistant clones for the loss of the ability to grow on plates with DCM as the carbon source yielded 10 independent mutants of strain DM4. However, this mutagenesis experiment was not performed to saturation, since no mutant was recovered that featured a mini-Tn5 insertion in the DCM dehalogenase gene itself. One of the mutants obtained, DM4-1445 was also unable to grow with DCM in liquid culture (Table 1) and was therefore investigated in detail.

**Cloning and sequence analysis of the gene disrupted by minitransposon insertion in mutant DM4-1445.** Hybridization with a DIG-labeled probe specific for the kanamycin resistance gene of the mini-Tn5 element confirmed that only one minitransposon insertion had occurred in mutant DM4-1445 (data not shown). The insertion was located on an 8.5-kb *Pst*I DNA fragment (Fig. 1A) which was rescued from total digested DNA by selection for kanamycin resistance. Sequencing of the cloned *Pst*I DNA fragment revealed that the gene disrupted by minitransposon insertion in mutant DM4-1445 displayed strong similarity to the DNA polymerase I gene (*polA*) of *E. coli* (28). Hybridization with a DIG-labeled probe to the rescued *M. dichloromethanicum* DM4 *polA* gene fragment confirmed that *M. dichloromethanicum* DM4 only contained one copy of the *polA* gene (Fig. 1A) and led to the cloning of a 7.5-kb *Sma*I-*Sal*I fragment from total DNA of wild-type DM4

TABLE 1. Growth rates of shaken batch cultures of *M. dichloromethanicum* DM4 and its *polA* derivatives with different carbon sources<sup>a</sup>

Strain	Growth rate (h <sup>-1</sup> ) on carbon source			
	MeOH	DCM	MeOH-DCM	MeOH-CH <sub>2</sub> O
DM4 wild-type	0.19	0.08	0.12	0.19
DM4-1445	0.17	— <sup>b</sup>	0.08	0.17
DM4-1445(pME8112)	0.17	0.08	0.11	0.19

<sup>a</sup> The concentration of MeOH was 40 mM, that of DCM was 10 mM, and that of formaldehyde (CH<sub>2</sub>O) was 1 mM. Standard errors are <0.01 h<sup>-1</sup> in all cases.

<sup>b</sup> —, no growth.

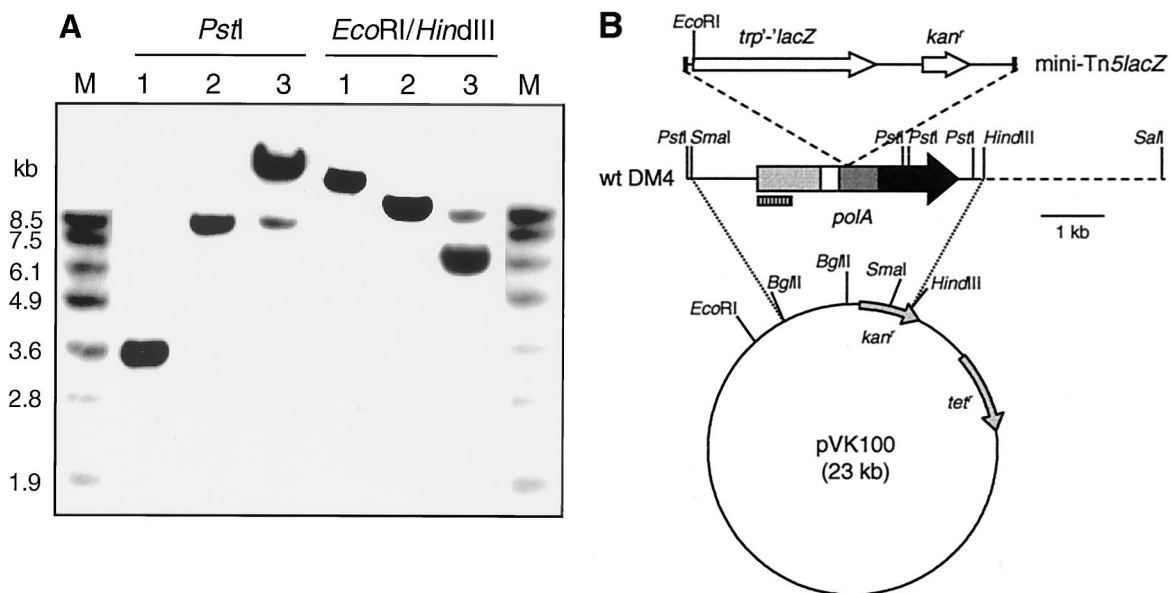


FIG. 1. Southern blot analysis of the *polA* gene of *M. dichloromethanicum* DM4. (A) Chromosomal DNA (5  $\mu$ g) from wild-type *M. dichloromethanicum* DM4 (lanes 1), mutant *M. dichloromethanicum* DM4-1445 (lanes 2), and mutant DM4-1445 conjugated with complementing plasmid pME8112 and digested with *Pst*I or with *Hind*III/*Eco*RI (lanes 3) was hybridized with a *polA*-specific DIG-labeled probe after agarose gel electrophoresis. Lanes M, molecular size markers. (B) Corresponding schematic map of cloned DNA fragments with the DM4 *polA* gene, indicating the point of insertion of the mini-Tn5 element (12) in mutant DM4-1445 (top), and vector pVK100 (30) used for mutant complementation with the wild-type (wt) *polA* gene. Restriction sites relevant for Southern analysis and the location of the DIG-labeled probe (striped box) are shown. Segments corresponding to 5'-3' exonuclease, 3'-5' exonuclease, and DNA polymerase domains of the *polA* gene product are indicated as light grey, dark grey and black boxes, respectively.

containing the *polA* gene in its entirety (Fig. 1B). Sequence analysis of the *polA* gene from *M. dichloromethanicum* DM4 showed that the corresponding gene product was most closely related to DNA polymerase I from *Rhizobium leguminosarum* (24) and *E. coli* (28) (54 and 42% pairwise identity at the protein level, respectively). Unlike some homologs lacking the sequence region encoding the 3'-5' exonuclease activity (see, e.g., reference 34), the PolA protein sequence from *M. dichloromethanicum* DM4 featured the three sequence domains corresponding to the 5'-3' exonuclease, proofreading 3'-5' exonuclease, and DNA polymerase activities of DNA polymerase I from *E. coli*.

**Complementation of the growth defect of DM4-1445 on DCM with the intact *polA* gene.** The growth rate of wild-type *M. dichloromethanicum* DM4 is 0.19 h<sup>-1</sup> with methanol and 0.08 h<sup>-1</sup> with DCM under the conditions used (Table 1; Fig. 2). In addition to its lack of growth with DCM as the sole carbon source, mutant DM4-1445 showed delayed and poorer growth with 40 mM MeOH in the presence of 10 mM DCM (Table 1; Fig. 2). Plasmid pME8112, a derivative of the IncP shuttle plasmid pVK100 (30) containing a *Sma*I-*Hind*III fragment with the intact *polA* gene, restored growth of the mutant with DCM as the unique carbon source (Table 1), and relieved the growth defect observed for growth with mixtures of MeOH and DCM (Table 1; Fig. 2). Some plasmids, such as ColE1 in *E. coli* (29) and an IncQ plasmid in *R. leguminosarum* (10), were previously shown to require DNA polymerase I for replication. Both plasmid pME8112 and its parent vector pVK100 were stably replicated in the *polA* mutant as well as in wild-type strain DM4 (data not shown). The DM4 *polA* mutant contained about three copies of plasmid pME8112 per cell (Fig. 1A).

**Biochemical characterization of the growth defect of mutant DM4-1445 with DCM as the carbon source.** Absence of growth of mutant DM4-1445 with DCM (Table 1) was not due to lack

of active DCM dehalogenase: mutant DM4-1445 contained the DCM dehalogenase gene and expressed the corresponding protein, as verified by Southern and Western analysis, respectively (data not shown). In addition, intracellular levels of reduced glutathione in the mutant were similar to that of the wild-type strain (about 200 nmol/mg of protein [data not shown]). Measurements of DCM dehalogenase activity in cell extracts of cells grown with 40 mM MeOH and 10 mM DCM

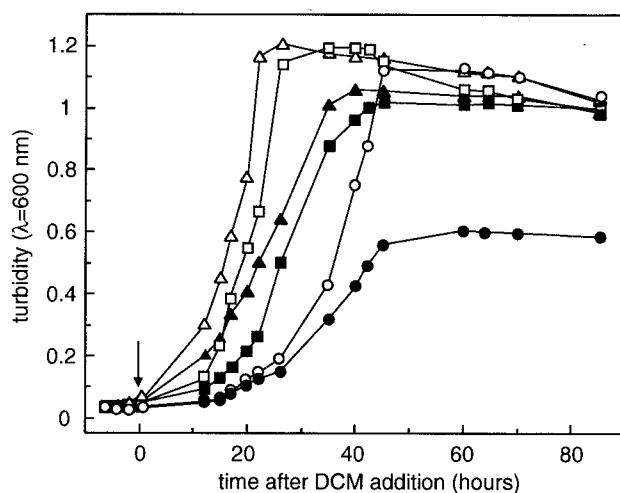


FIG. 2. Effect of DCM on growth of wild-type *M. dichloromethanicum* DM4 and of the *polA* mutant. Strains DM4 wild-type (squares), mutant DM4-1445 (circles), and complemented mutant DM4-1445(pME8112) (triangles) were grown at 30°C in minimal medium with 40 mM MeOH as the unique carbon source (open symbols). The growth behavior of cultures additionally treated with 10 mM DCM at an OD<sub>600</sub> of 0.05 (arrow) is depicted with filled symbols. Data from one representative experiment are shown.



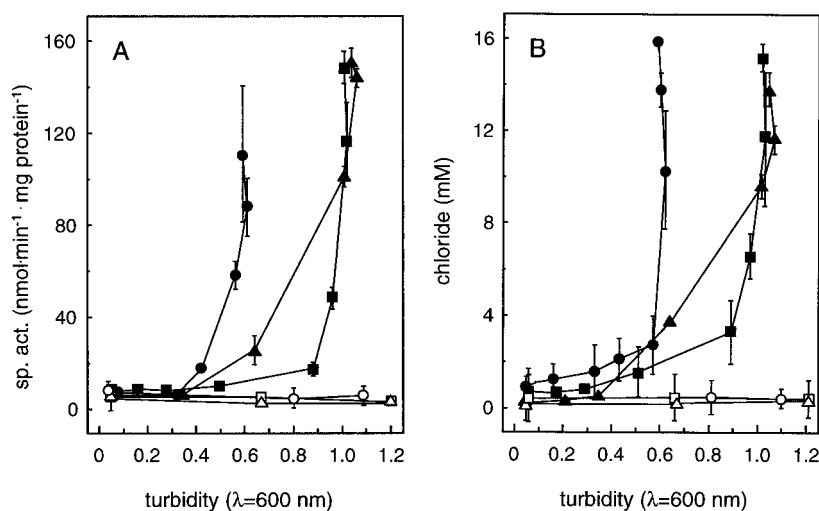


FIG. 3. Induction of DCM dehalogenase after addition of DCM. DCM dehalogenase induction during growth is given as a function of culture turbidity. (A) Specific activity in crude extracts harvested at various stages of growth; (B) concentration of chloride ions released in the growth medium (symbols as defined in the legend to Fig. 2). Error bars, standard errors of triplicate measurements.

confirmed the normal onset of DCM dehalogenase expression at about 20 h after DCM addition in mutant DM4-1445 (Fig. 3A; compare Fig. 2). Levels of DCM dehalogenase activity in mutant DM4-1445 also appeared normal *in vivo*, as evidenced by the amount of chloride ions excreted into the growth medium (Fig. 3B). DNA polymerase activity was then investigated in SDS-PAGE gels of cell extracts containing nicked DNA that were incubated with deoxynucleotides including  $\alpha$ - $^{32}\text{P}$ -labeled dCTP. No DNA polymerase activity could be detected in the resulting autoradiogram with cell extracts of mutant DM4-1445 (Fig. 4). In contrast, extracts of wild-type DM4 and of the mutant strain complemented with plasmid pME8112 containing an intact *polA* gene showed bands of very similar sizes to DNA polymerase I and Klenow fragment of *E. coli* run as controls (Fig. 4). These results suggest that the lack of a functional *polA* gene product is responsible for the observed growth defect of mutant DM4-1445 with DCM as the carbon source.

**Expression of the *polA* gene upon DCM addition.** Insertion of the mini-Tn5*lacZ*1 element in the *polA* gene resulted in a transcriptional *polA*'-lacZ fusion in mutant DM4-1445 (Fig. 1B). Expression of the gene fusion was investigated in cell extracts of mutant DM4-1445 and of complemented mutant DM4-1445(pME8112) harvested at various stages of growth with and without addition of DCM (Fig. 5). Only negligible  $\beta$ -galactosidase activity was detected in protein extracts of the wild-type strain (<20 nmol/min/mg of protein [data not shown]). The *polA* gene was constitutively expressed at a high level during growth with MeOH as the carbon source in both *polA* mutant and complemented strains, in agreement with the reported estimate of 400 copies of DNA polymerase I for an *E. coli* cell (16). Addition of DCM resulted in an approximately twofold increase of  $\beta$ -galactosidase activity which accompanied the induction of DCM dehalogenase expression (Fig. 5; compare Fig. 3).

**Sensitivity of *M. dichloromethanicum* DM4 to toxic insults.** Mutant DM4-1445 showed a markedly lower resistance to the DNA cross-linking agents UV light (Fig. 6A) and mitomycin (data not shown) typical of that observed previously with *polA* mutants of various bacteria (e.g., see references 22 and 42) (Fig. 6A). In contrast, the degrees of resistance of wild-type

and complemented mutant strains to UV and mitomycin were undistinguishable and comparable to those previously reported for *E. coli* (42), taking the low growth rate of *M. dichloromethanicum* into account (doubling time of about 3.6 h with MeOH at 30°C [Table 1]). Most notably, the *polA* mutant from strain DM4 was also sensitive to treatment with DCM (Fig. 6B). The viability of bacteria plated on MM after the addition of 10 mM DCM markedly decreased upon induction of the DCM dehalogenase (after about 20 h) (compare Fig. 2 and 3), while that

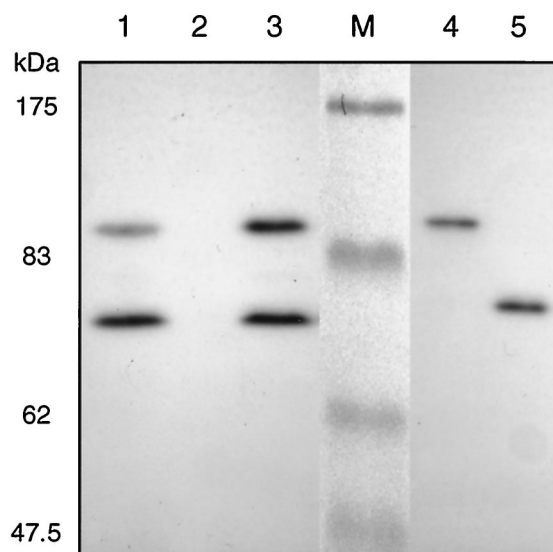


FIG. 4. DNA polymerase I activity of wild-type *M. dichloromethanicum* DM4 and of the *polA* mutant. Shown is an autoradiogram of cell extract protein (100  $\mu\text{g}$ ) of wild-type strain DM4 (lane 1), mutant DM4-1445 (lane 2), and complemented mutant DM4-1445(pME8112) (lane 3), separated by SDS-PAGE in a gel containing nicked DNA, after incubation with dideoxynucleotides and  $\alpha$ - $^{32}\text{P}$ -labeled dCTP (see Materials and Methods). Lane M, prestained marker from the scanned gel; lanes 4 and 5, *E. coli* DNA polymerase I and Klenow fragment, respectively (0.02 U [ $\sim$ 0.1 ng] each).

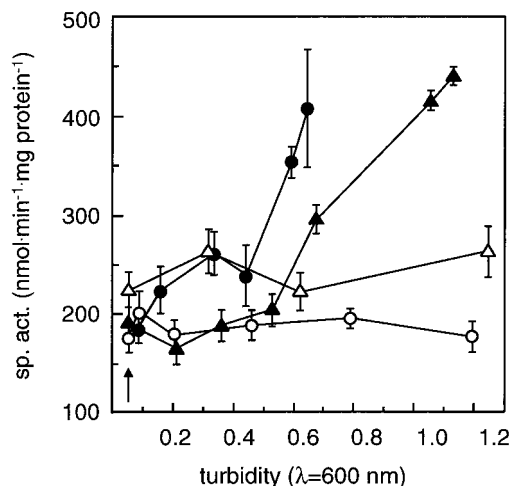


FIG. 5. Effect of DCM on the expression of the *polA'*-*lacZ* fusion in the DM4-1445 *polA* mutant background. The  $\beta$ -galactosidase specific activity (sp. act.) arising from the *polA'*-*lacZ* transcriptional fusion was determined in cell extracts of *M. dichloromethanicum* DM4-1445 (circles) and DM4-1445(pME8112) (triangles) grown with MeOH as the carbon source with (filled symbols) and without (open symbols) prior treatment with 10 mM DCM at an  $OD_{600}$  of 0.05 and expressed as a function of the optical density of the cultures at the time of cell harvest. Error bars, standard errors of triplicate measurements.

of wild-type and complemented mutant strains remained unaffected.

## DISCUSSION

The data reported here show that disruption of the *polA* gene in *M. dichloromethanicum* DM4 is responsible for the observed growth defect of mutant DM4-1445 with DCM. This provides a clear indication that repair of DNA damage is important for DCM metabolism by *Methylobacterium* expressing DCM dehalogenase-GST. DNA polymerase I is a multi-domain protein with 5'-3' exonuclease, 3'-5' exonuclease, and DNA polymerase activity involved in DNA repair processes (16). The DM4-1445 *polA* strain may still express the 5'-3'

exonuclease domain of DNA polymerase I in active form, since insertion of the minitransposon element occurred downstream of the section encoding this activity (Fig. 1A). In any case, the disruption of the 3'-5' exonuclease domain by minitransposon insertion led to loss of DNA polymerase function (Fig. 4) and was sufficient to prevent growth of *M. dichloromethanicum* DM4 with DCM.

Although the toxic agent in DCM metabolism remains to be characterized in detail, several observations, summarized below, provide suggestive clues as to its identity. Clearly, a role for DCM itself can be excluded under the experimental conditions used: wild-type strain DM4 grows with 10 mM DCM without its viability being detectably affected (49), and DCM is nontoxic to a mutant of *M. dichloromethanicum* DM4 lacking the DCM dehalogenase gene (49).

Thus, GST-mediated DCM turnover is likely to be responsible for poor growth of the *polA* mutant in the presence of DCM (Fig. 2). Formaldehyde, the product of DCM conversion by DCM dehalogenase-GST, can give rise to DNA cross-links and is a known cytotoxic agent and mutagen (9, 20, 35). Indeed, mutations in the *polA* gene are known to be detrimental for viability of *E. coli* upon formaldehyde exposure (43). Several lines of evidence indicate, however, that formaldehyde is unlikely to be the main toxic agent associated with DCM conversion. First, the growth rate of *Methylobacterium* with MeOH, which involves formaldehyde as a central metabolic intermediate, was not affected in the DM4-1445 *polA* mutant (Table 1). Second, the presence of 1 mM formaldehyde did not affect growth and viability of both the wild type and the *polA* mutant (Table 1 and data not shown [2 mM formaldehyde led to a prolonged lag phase prior to growth in both wild-type and *polA* mutant strains]). Finally, constitutive expression from plasmids of the DCM-active GST theta 1-1 from rat in the presence of DCM was more toxic and mutagenic to *Methylobacterium* and *S. enterica* serovar Typhimurium TA1535 than that of bacterial DCM dehalogenases, despite a lower conversion rate of DCM to formaldehyde by the rat enzyme under the conditions used (18). In addition, formaldehyde requires a proficient nucleotide excision repair machinery to unfold its mutagenic effects (52), but *S. enterica* serovar Typhimurium TA1535 is excision repair deficient (26).

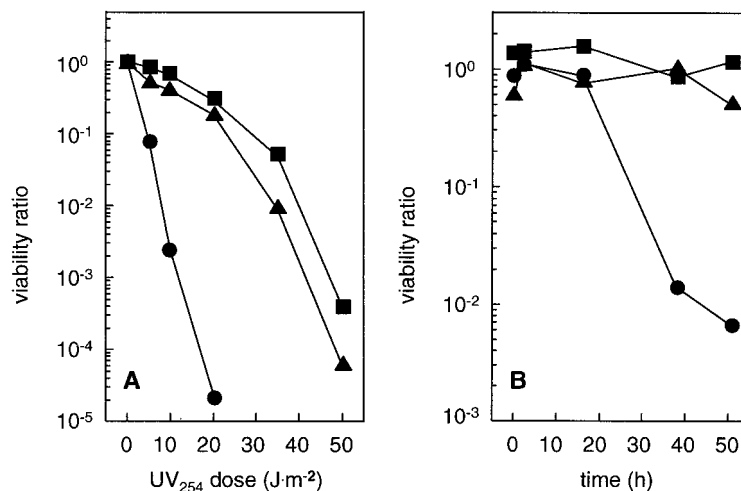


FIG. 6. Effects of UV light and DCM on the viability of wild-type *M. dichloromethanicum* DM4 and of the *polA* mutant. Methanol-grown cultures of *M. dichloromethanicum* DM4 wild-type (squares), DM4-1445 (circles), and DM4-1445(pME8112) (triangles) were exposed to UV light at 254 nm ( $5 \text{ J} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ) (A) or treated with 10 mM DCM at an  $OD_{600}$  of 0.05 (B) for various times before plating on MM with 40 mM MeOH. Bacterial viability is expressed relative to that of untreated cultures.

Inability of the *polA* mutant to cope with intracellular acid production would constitute another possible explanation for the toxicity of GST-mediated DCM conversion. Expression of the apparently monocistronic *polA* gene is upregulated twofold upon acidification of the culture medium in *E. coli* (23), and a twofold increase in  $\beta$ -galactosidase activity arising from the *polA* transcriptional fusion in the DM4-1445 mutant was also observed after addition of DCM (Fig. 5). The exact nature of the inducer(s) of *polA* expression during growth of strain DM4 with DCM remain to be determined. However, the possibility that the intracellular pH was lowered to a detrimental level in the mutant can be excluded since DCM dehalogenase, which is inactive below pH 6 (S.V., unpublished data), was as active in the *polA* mutant as in the wild-type strain (Fig. 3B).

Nevertheless, the finding that DNA polymerase I is essential for DCM metabolism in *Methylobacterium* suggests that the main toxic agent of GST-mediated conversion of DCM is able to cause extensive DNA damage. In addition, available experimental data indicate that this agent is not DCM, formaldehyde, or hydrochloric acid, but rather an intermediate in the reaction catalyzed by DCM dehalogenase. *S*-Chloromethylglutathione is generally believed to be formed during the reaction of DCM to formaldehyde catalyzed by DCM dehalogenase-GSTs (11, 21) but remains poorly characterized due to its high reactivity: *S*-fluoromethylglutathione, the less-reactive fluorinated homolog of *S*-chloromethylglutathione, is hydrolyzed with a half-life of 5.8 min at room temperature in D<sub>2</sub>O (11). Transient formation of a compound with a <sup>19</sup>F-nuclear magnetic resonance signal compatible with *S*-fluoromethylglutathione was observed with DCM dehalogenase from *Methylophilus* sp. DM11 incubated with glutathione and chlorofluoromethane (6). Chemically synthesized *S*-chloromethylglutathione alkylated deoxyguanosine in vitro (11), and the main product of this reaction was characterized as *S*-[1-*N*<sup>2</sup>-deoxyguanosinylmethyl]glutathione (11, 44). Nevertheless, the formation of DNA lesions as a consequence of *S*-chloromethylglutathione formation during conversion of DCM by DCM dehalogenase-GST remains to be demonstrated. Certainly, an obvious role for DNA polymerase I in DCM metabolism would be in participating in nucleotide excision repair of bulky base adducts formed upon reaction of DNA with *S*-chloromethylglutathione and in preventing stalling of the UvrABC excision nuclease protein complex (7, 25, 32, 39), which would presumably recognize such lesions. Whether DNA adducts are formed during DCM conversion by DCM dehalogenase is currently being investigated and should contribute to clarifying the role of DNA repair processes in DCM metabolism. More generally, the *polA* mutant of *M. dichloromethanicum* DM4 provides a first indication that genes other than that involved in the dehalogenation reaction itself are needed for bacterial growth with halogenated methanes. It is expected that the characterization of other minitransposon mutants showing impaired growth with DCM will shed light on the nature of these accessory requirements.

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