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EXPERIMENTAL ARTICLES

Physiological and Biochemical Analysis of the Transformants of Aerobic Methylobacteria Expressing the *dcmA* Gene of Dichloromethane Dehalogenase

Yu. E. Firsova, N. V. Doronina, and Yu. A. Trotsenko[1]

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia

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Abstract—Transformants of *Methylobacterium dichloromethanicum* DM4 (DM4-2cr$^-$/pME 8220 and DM4-2cr$^-$/pME8221) and of *Methylobacterium extorquens* AM1 (AM1/pME8220 and AM1/pME8221) that express the *dcm A* gene of dichloromethane dehalogenase undergo lysis when incubated in the presence of dichloromethane and are sensitive to acidic shock. The lysis of the transformants was found to be related neither to the accumulation of Cl^- ions, CH_2O, or HCOOH, nor to the impairment of glutathione synthesis or to the disturbance of intracellular pH homeostasis. The (exo$^-$) Klenow fragment–mediated incorporation of [α-^{32}P]dATP into the DNA of the transformants DM4-2cr$^-$/pME8220 and AM1/pME8220 was considerably greater when the transformed cells were incubated with CH_2Cl_2 than when they were incubated with CH_3OH, indicating the occurrence of a significant increase in the total length of gaps. At the same time, the strain AM1 (which lacks dichloromethane dehalogenase) and the dichloromethane-degrading strain DM4 incubated with CH_2Cl_2 showed an insignificant increase in the total length of the gaps. The transformed cells are likely to lyse due to the relatively inefficient repair of DNA lesions that are induced in response to the alkylating action of S-chloromethylglutathione, an intermediate product of CH_2Cl_2 degradation. The data obtained suggest that the bacterial mineralization of dichloromethane requires an efficient DNA repair system.

Key words: aerobic methylobacteria, dichloromethane, dehalogenation, transformants, *dcmA* gene.

Dichloromethane (DCM, CH_2Cl_2) is a severely toxic, carcinogenic compound, which is widely used in industry as a solvent and refrigerant. Because of its high toxicity, carcinogenicity, volatility, solubility in water, and persistence, DCM is included on the list of priority pollutants [1]. This has attracted the interest of researchers to the study of the microbial degradation of DCM [2–4]. The analysis of 12 isolates of aerobic methylobacteria that are able to utilize DCM as a source of carbon and energy made it possible to describe three new genera and six new species of methylobacteria, which implement different C_1-assimilation pathways [4]. The primary dehalogenation of DCM by aerobic methylobacteria was found to occur in the cytoplasm and to be catalyzed by DCM dehalogenase, which belongs to the theta class of glutathione S-transferases [5]. The aerobic methylobacteria are likely to bring about the dehalogenation of DCM by a reaction that is similar to that which occurs in the rat liver:

$$CH_2Cl_2 + GSH \xrightarrow[HCl]{} [GS\text{–}CH_2Cl] \xrightarrow[HCl]{H_2O} GS\text{–}CH_2OH \rightleftharpoons GSH + CH_2O.$$

The unstable intermediate S-chloromethylglutathione (GS–CH_2Cl) is spontaneously hydrolyzed to S-hydroxymethylglutathione (GS–CH_2OH), which breaks down to formaldehyde and reduced glutathione (GSH) [6]. Recent studies showed that S-chloromethylglutathione acts as a mutagen and impairs DNA replication through the alkylation of DNA bases [7].

The DCM dehalogenase of *M. dichloromethanicum* DM4 is a cytoplasmic protein that is encoded by the *dcm A* gene [5]. This gene was cloned and sequenced, and the relevant region of the bacterial genome was partially mapped [8]. The structural *dcm A* gene is nega-

[1] Corresponding author. E-mail: trotsenko@ibpm.pushchino.ru

tively regulated at the transcriptional level by the closely located regulatory gene *dcm R* [9]. In the absence of DCM, the *dcm R* gene acts as a repressor of the *dcm A* gene. When the regulatory gene is impaired, DCM dehalogenase is synthesized constitutively [9].

It should be noted that the occurrence of DCM dehalogenase in a methylobacterium is not sufficient for its ability to utilize DCM. Some transformants of methylobacteria which have inducible or constitutive DCM dehalogenase are unable to grow on DCM. This suggests that DCM metabolism is governed not only by the *dcm A* gene but also by other genes [3, 10, 11].

The DM4-2cr mutant of *M. dichloromethanicum* DM4 lacks the *dcmA* gene because of a deletion in the chromosome or a megaplasmid [10]. The deletion contains no other genes necessary for the growth of *M. dichloromethanicum* on DCM, as is evident from the fact that the transformants DM4-2cr/pME8220 and DM4-2cr/pME8221, which bear, respectively, plasmid pME8220 with the *dcmA* gene and plasmid pME8221 with the *dcmA* and *dcmR* genes, are able to grow on DCM [12]. Two other transformants, DM4-2cr$^-$/pME 8220 and DM4-2cr$^-$/pME 8221, with the active *dcm A* gene are unable to grow on CH_2Cl_2, which is likely to be due to the occurrence of new mutations that impair the normal functioning of genes necessary for bacterial growth on DCM. The transformants AM1/pME8220 and AM1/pME8221 of the *Methylobacterium extorquens* strain AM1, which is unable to utilize CH_2Cl_2, show a normal expression of the *dcm A* gene but remain unable to utilize CH_2Cl_2.

These transformants are suitable for a comparative study with the DCM-degrading strain *M. dichloromethanicum* DM4 aimed at determining genes (other than *dcmA*) that are necessary for bacterial growth on CH_2Cl_2. The inability of the transformants to grow on CH_2Cl_2 may be associated with a diminished level of glutathione synthesis, a disturbance of intracellular pH homeostasis because of the production of HCl in cells, an accumulation of the toxic products of CH_2Cl_2 dehalogenation (CH_2O and Cl^-) in the medium, and an induction of DNA lesions under the action of S-chloromethylglutathione.

This work was aimed at studying the extent to which these factors are actually responsible for the inability of the transformants to utilize DCM.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. Experiments were carried out with the methylobacteria that accomplish the serine pathway of C_1-metabolism (*Methylobacterium dichloromethanicum* DM4 [13] and *Methylobacterium extorquens* AM1) and their transformants. *M. extorquens* AM1 does not have DCM dehalogenase and hence is unable to grow on CH_2Cl_2, although it is able to grow on CH_3OH. The transformants DM4-2cr$^-$/pME8220, DM4-2cr$^-$/pME8221, AM1/pME8220, and AM1/pME8221 express the *dcmA* gene but are unable to grow on CH_2Cl_2. Plasmid pME8220 bears the *dcmA* gene from *M. dichloromethanicum* DM4 and provides for a constitutive expression of this gene in the transformants. The plasmid was obtained by cloning a 1500-bp *Hin*dIII–*Pst*I fragment of plasmid pME1540 [12] in the broad-host-range vector pCM62 [14]. The other plasmid pME8221 carries not only the structural *dcmA* gene from strain DM4 but also the regulatory *dcmR* gene, which is responsible for a 15- to 20-h lag phase in the expression of the *dcmA* gene. This plasmid was obtained by cloning a 2000-bp *Bam*HI fragment of plasmid pME1518 [5] in the same vector pCM62. Plasmids pME8220 and pME 8221 were transferred to *E. coli* S17-1 and then to *M. dichloromethanicum* DM4-2cr and *M. extorquens* AM1 by means of biparental crossing [12]. The transformant DM4-2cr lacks native DCM dehalogenase because of a large (>21 kb) deletion in the chromosome or a megaplasmid [10].

The strains were grown at 29°C in shaken (180 rpm) glass flasks with liquid mineral K medium [13], which contained either CH_3OH (120 mM) or CH_2Cl_2 (10 mM) as a source of carbon and energy. For the cultivation of the transformants, the medium was supplemented with 20 mg/l tetracycline. For cultivation on CH_2Cl_2, 300-ml Erlenmeyer flasks with 50 ml of the nutrient medium were sealed with screw caps furnished with rubber septa (Precision Sampling Corp., Baton Rouge, United States). CH_2Cl_2 was added to a final concentration of 10 mM when the pH of the medium in the course of cultivation fell down to 5.0. At this moment, the pH of the medium was adjusted to 7.2 with 3 M NaOH, and dichloromethane was introduced into the flask through the septum by using a syringe with a needle. Then the cultivation was continued until the pH of the medium again fell down to 5.0. At that moment, the cultivation was either stopped or the aforementioned procedure of pH adjustment and dichloromethane addition was repeated, and the cultivation was continued until the third pH downshift to 5.0.

The intracellular level of glutathione. To determine the total intracellular pool of glutathione (i.e., the total amount of reduced (GSH) and oxidized (GSSG) forms of glutathione) in cells of the parent strains DM4 and AM1 and their transformants, the cells grown on CH_3OH to the late logarithmic growth phase (72 h of cultivation) were harvested by centrifugation at 10000 *g* for 20 min, washed in 0.05 M Tris–HCl buffer (pH 7.4), and resuspended in 0.01 N HCl in a proportion of 0.5 g wet cells per 2.5 ml of the HCl solution. Cells were disrupted at 4°C with an MSE ultrasonic disintegrator (150 W; 20 kHz) for a total of 3 min in 30-s bursts. Unbroken cells and cell debris were removed by centrifugation at 20000 *g* for 40 min, and the supernatant was analyzed for glutathione by measuring the reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) in the presence of glutathione reductase (Sigma, United States) [15]. The content of glutathione was calculated for mg

of soluble protein, which was quantified by the method of Lowry *et al.*

The viability of cells incubated with DCM. Cells of the parent strains DM4 and AM1 and their transformants grown on an agar medium with methanol were transferred to a liquid K medium with DCM and incubated with shaking to the third pH downshift to 5.0 as described above, but no longer than for 96 h. (During cultivation, the culture liquid was analyzed for the content of Cl^- ions, formaldehyde, and formate.) Then the pH of the culture was again adjusted to 7.2, the culture was supplemented with 120 mM CH_3OH and an equal volume of fresh nutrient medium, and the cultivation was resumed with a regular measurement of culture turbidity.

The viability of cells exposed to acidic shock. The parent strains DM4 and AM1 and their transformants were grown in a medium with methanol for 24 h. The cultures were exposed to acidic shock by adding 3 M HCl to the medium until its pH dropped to 4.0 or by adding 3 M HCOOH until the pH of the medium dropped to 5.0. Then the cultivation was resumed for 16 h. The culture was neutralized with 3 M NaOH, supplemented with 50 mM CH_3OH, and incubated for the next 6 days. The increase in the culture turbidity was expressed as a percent of the control (the culture that was not exposed to acidic shock).

Intracellular pH was determined from the partition pattern of 9.5 μM [^{14}C]benzoic acid (0.1 μCi/ml; Isotop, Russia) between cells and the culture liquid [16] with 1 μM [^{3}H]inulin (1 μCi/ml; Chemapol, Czech Republic) as the extracellular marker. Cells of strain DM4 grown on CH_2Cl_2 for 24 h to induce DCM dehalogenase and of the transformant DM4-2cr$^-$/pME8220 grown on CH_3OH were washed twice with K medium without any carbon source and resuspended in the same medium with pH 7.2 to an optical density of 1.4. The suspension was dispensed in 3.5-ml aliquots into 20-ml screw-cap flasks and incubated at 29°C with the aforementioned radiolabeled compounds with or without DCM for 45 min on a shaker. The incubation time (45 min) was chosen empirically, by taking into account the results of the determination of intracellular pH in the transformants of *E. coli* expressing the *dcmA* gene [6]. Cells were separated from the culture liquid by centrifugation at 15000 *g* for 15 min, and the radioactivities of the cell pellet and the supernatant were measured with an Intertechnique SL-30 scintillation spectrometer (France).

The degree of DNA damage in the parent strains DM4 and AM1 and their transformants DM4-2cr$^-$/pME8220 and AM1/pME8220 was determined by comparatively analyzing the total DNA of these strains cultivated in the presence of 120 mM CH_3OH or 10 mM CH_2Cl_2 for 72 h. The total DNA was isolated by the conventional method with the use of cetyltrimethylammonium bromide [17] and analyzed by electrophoresis in 0.8% agarose gel. The reaction mixture for gap filling contained 2.5 μg DNA, 1.25 U of the (exo$^-$) Klenow fragment (Fermentas, Lithuania), 15 μCi [α-^{32}P]dATP (5000 Ci/mmol; Nuklidtrans, Russia), and 0.2 mM of each of the other dNTPs in 50 μl of the reaction buffer recommended by Fermentas. The mixture was incubated at 30°C for 15 min, and the reaction was stopped by adding 2 μl of 0.5 M EDTA. Aliquots of the reaction mixture were placed onto Whatman GF/A filters (United Kingdom). The filters were dried and washed six times with 5 ml of 10% trichloroacetic acid and one time with 5 ml of 96% ethanol to remove the unincorporated radiolabeled dATP, and the radioactivity of the filters was measured with an Intertechnique SL-30 scintillation spectrometer.

Analytical methods. The concentration of chloride ions in the supernatant was measured spectrophotometrically with Hg(II) thiocyanate [18], the concentration of formaldehyde was measured with acetylacetone [19], and the concentration of formate was measured by recording the reduction of NAD^+ at 340 nm in the presence of NAD^+-dependent formate dehydrogenase (Boehringer, Germany). Spectrophotometric measurements were conducted by using recording spectrophotometers Specord UV-VIS (Germany) and Shimadzu UV-160 (Japan).

RESULTS AND DISCUSSION

The measurement of DCM dehalogenase activity from the rate of formation of chloride ions showed that this activity is approximately the same in the parent strain DM4 and the transformants.

Further analysis showed that the total glutathione pool (GSH + GSSG) in the *M. dichloromethanicum* strain DM4 grown on CH_2Cl_2 is 10% higher than in the same strain grown on CH_3OH. The total glutathione pools in the transformants grown on CH_3OH were no less than 91 and 95% of the respective pools in the parent strains DM4 and AM1. These data show that glutathione synthesis in the transformants was not impaired. The intracellular level of glutathione in the transformants grown on DCM was not measured because, as mentioned above, the transformed cells undergo lysis in the presence of DCM.

Unlike the cultivation of *M. dichloromethanicum* DM4, the long-term cultivation of all the transformants on DCM until the third pH downshift to 5.0 (72 to 96 h of incubation) irreversibly affected their viability, so that the transformants remained unable to grow after the neutralization of the culture with HCl and the addition of fresh nutrient medium and CH_3OH.

To verify the suggestion that the death of the transformants in the presence of DCM was due to the accumulation of toxic metabolites, we analyzed the content of chloride ions, formaldehyde, and formate in the medium and found that the concentration of chloride ions in the culture liquids of all the transformants by the end of the cultivation period varied from 17 to 32 mM,

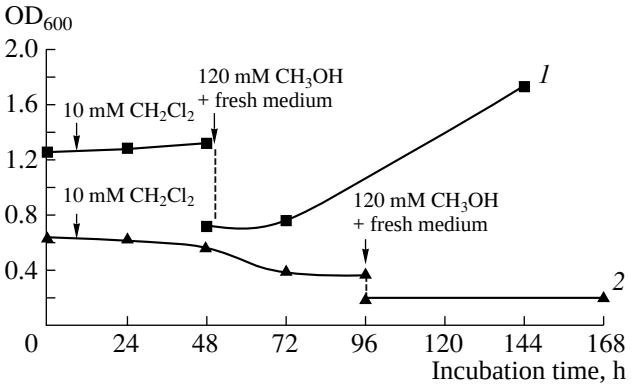

Fig. 1. The survival of (*1*) *M. dichloromethanicum* DM4 and (*2*) the transformant DM4-2cr⁻/pME8220 after incubation with dichloromethane

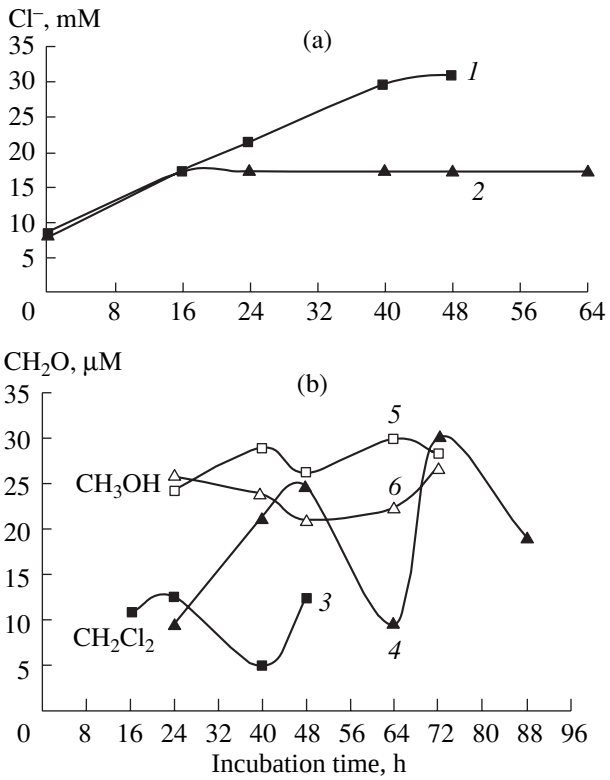

Fig. 2. The production of (a) Cl^- ions and (b) formaldehyde during the incubation of (*1*, *3*, *5*) *M. dichloromethanicum* DM4 and (*2*, *4*, *6*) the transformant DM4-2cr⁻/pME8220 in the presence of (*1–4*) dichloromethane or (*5*, *6*) methanol

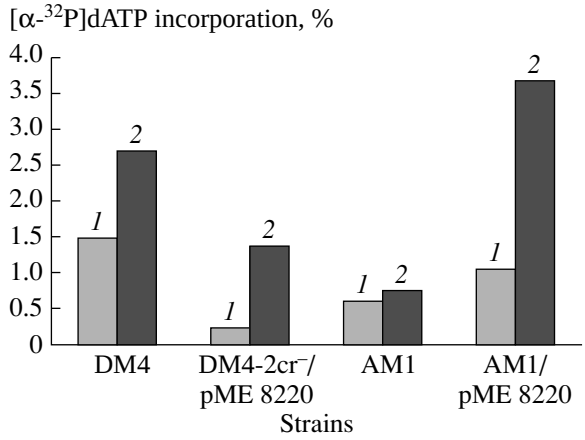

Fig. 3. The (exo⁻) Klenow fragment–mediated incorporation of [α-^{32}P]dATP into the DNA of *M. dichloromethanicum* DM4, *M. extorquens* AM1, and their transformants DM4-2cr⁻/pME8220 and AM1/pME8220 incubated in the presence of (*1*) methanol or (*2*) dichloromethane.

whereas was equal to 31 mM in the culture liquid of the parent strain DM4. The maximum concentration of chloride ions (32 mM), which was observed for the transformant AM1/pME8220, corresponded to the concentration of NaCl in the culture liquid (0.2%). Consequently, chloride ions at the concentrations observed (less than or equal to 32 mM) could not cause the death of the transformants, since, according to our earlier data [13], the growth of the parent strains DM4 and AM1 is only partially inhibited by 1% NaCl and is completely inhibited by NaCl at a concentration as high as 3%. Nor could formaldehyde produced from DCM and not utilized by cells for energetic and biosynthetic purposes cause the death of the transformants cultivated in the presence of DCM, because the concentration of formaldehyde in the cultivation media of the transformants grown on DCM did not considerably exceed its concentration in the cultivation media with methanol. As for formate, this compound was not detected in the culture liquids of either the transformant or the parent strains cultivated in the presence of DCM. Consequently, the accumulation of chloride ions, formaldehyde, or formate in the cultivation medium could not induce the lysis of the transformants cultivated in the presence of DCM. Changes in the viability of the transformant DM4-2cr⁻/pME8220 and the parent strain DM4 after incubating them in the presence of DCM, as well as the accumulation of the products of DCM dehalogenation in the cultivation media, are shown in Figs. 1 and 2.

The high concentration of protons, which are produced in the cytosol during the primary dehalogenation of DCM and are then extruded outside the cells, can presumably cause the death of the transformants too. It was found that the pH of the medium decreased in this case to levels as low as 3.9. The results of experiments on the effect of artificial acidic shocks on the viability of the methanol-grown parent and transformant strains are presented in Table 1. The shocks were created by exposing cells either to hydrochloric or to formic acid. As can be seen from Table 1, the parent strains DM4 and AM1 and the transformant DM4-2cr⁻/pME8221 exhibited high viability under the acidic shocks. The viability of the other transformants under the shocks was considerably lower, which suggested that the physiological mechanisms maintaining pH homeostasis in

Table 1. The effect of acidic shocks on the survival rate of the parent and transformed strains of methylobacteria on methanol

Methylobacterium strain	Increase in the biomass (as % of the control) after:	
	exposure to HCl (pH 4.0) for 16 h	exposure to HCOOH (pH 5.0) for 16 h
M. dichloromethanicum DM4	100.0	41.3
DM4-2cr$^-$/pME 8220	8.9	5.7
DM-2cr$^-$/pME 8221	95.0	62.6
M. extorquens AM1	74.3	40.5
AM1/pME 8220	44.8	22.8
AM1/pME 8221	9.4	2.1

Table 2. Intracellular pH values for *M. dichloromethanicum* DM4 and its transformant DM4-2cr$^-$/pME8220 incubated in the presence of dichloromethane. Cells were suspended in a medium with pH 7.2

Experimental variant	Intracellular pH	
	DM4	DM4-2cr$^-$/pME8220
Incubation without substrate	7.64	7.69
Incubation with 10 mM CH_2Cl_2 for 45 min	7.55	7.37

these transformants may be impaired. In our opinion, the small difference in the intracellular pH values of the DCM-grown transformant DM4-2cr$^-$/pME8220 and the parent strain DM4 (Table 2) cannot explain the lysis of the transformant in the presence of DCM, although a slight detrimental effect of the low intracellular pH is likely to have taken place.

It should be noted that recent studies showed a direct alkylating action of S-chloromethylglutathione (an intermediate of DCM dehalogenation) on DNA bases with the formation of DNA adducts [7]. The related inefficient repair of DNA could be responsible for the inability of the transformants to grow on DCM.

To evaluate the degree of DNA damage induced by cultivation in the presence of DCM, we isolated and comparatively analyzed the total DNA of cells that were grown on CH_2Cl_2 and CH_3OH. The analysis of the total DNA by electrophoresis in agarose gel did not reveal notable differences in the electrophoretic pattern of DNA samples. At the same time, the filling of equal DNA aliquots with the (exo$^-$) Klenow fragment in the presence of [α-^{32}P]dATP and the unlabeled dNTPs did reveal considerable changes in the incorporation rates of the radiolabeled probe into DNA (Fig. 3). Namely, the levels of the ^{32}P incorporation into the DNA of the transformants DM4-2cr$^-$/pME8220 and AM1/pME8220 incubated with DCM were, respectively, 6.2 and 3.5 times higher than when the transformants were incubated with methanol. This finding suggests the occurrence of a considerable increase in the total length of gaps in the DNA of the DCM-grown transformants. The increase in the total lengths of gaps in the DCM-grown DM4 and AM1 strains was 1.8- and 1.3-fold, respectively (compared to the methanol-grown strains). Consequently, the filling of gaps in the DNA of the transformants cultivated in the presence of DCM occurs more slowly than in the case of the DCM-degrading DM4 strain. The fact that the total lengths of the gaps in the DNA of the parent strain AM1 incubated with CH_2Cl_2 and CH_3OH are almost the same is likely to be due to the absence of DCM dehalogenase in this strain.

Thus, the lysis of the DCM-grown cells of the transformants DM4-2cr$^-$/pME8220 and AM1/pME8220 seems to be due to their relatively inefficient DNA repair system, compared to the DCM-degrading strain *M. dichloromethanicum* DM4. The data obtained suggest that the bacterial mineralization of dichloromethane requires an efficient DNA repair system.

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