ISSN 0026-2617, Microbiology, 2014, Vol. 83, No. 3, pp. 205–210. © Pleiades Publishing, Ltd., 2014.
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EXPERIMENTAL ARTICLES

Functional Activity of the $modA_2$ Gene in *Methylobacterium dichloromethanicum* DM4

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Received November 14, 2013

Abstract—The putative METDI2644 ($modA_2$) gene of *Methylobacterium dichloromethanicum* DM4, present in the 126-kb chromosomal fragment associated with dichloromethane (DCM) degradation, was investigated. While this gene is presumed to encode the periplasmic substrate-binding subunit of a molybdate ABC transporter, its conceptual translation also exhibits similarity to proteins containing the *ostA* conservative domain and responsible for resistance of gram-negative bacteria to organic solvents. Reverse transcription polymerase chain reaction (RT-PCR) revealed RNA transcripts of this gene in the cells grown on either DCM or cells grown on methanol. The mobilizable suicide vector pK18mob was used to obtain a knockout mutant with the METDI2644 gene inactivated by insertion of the gentamycin cassette. The mutant pregrown on methanol exhibited lower growth rate on DCM than the wild-type strain DM4. The difference was not alleviated by the addition of sodium molybdate. Our results demonstrate that the METDI2644 gene product plays a role in cell adaptation to DCM degradation.

Keywords: aerobic methylobacteria, dichloromethane, $modA_2$

DOI: 10.1134/S0026261714030060

Dichloromethane (CH_2Cl_2, DCM) is a very toxic, carcinogenic, and mutagenic pollutant highly resistant to abiotic degradation. Aerobic methylobacteria can mineralize DCM due to dichloromethane dehalogenase DcmA, a cytoplasmic enzyme catalyzing CH_2Cl_2 transformation to formaldehyde and two HCl molecules in a reaction dependent on reduced glutathione [1].

Previously, the plasmid vector pCM62 was used to transfer the structural and regulatory genes of DCM dehalogenase, *dcmA* and *dcmR*, from the degrader *Methylobacterium dichloromethanicum* DM4 to *Methylobacterium extorquens* AM1 [2]. The latter methylotroph is genetically close to strain DM4 but does not grow on DCM. The resultant transformants expressed (inducibly or constitutively) DcmA at the level of strain DM4 but, nevertheless, could not grow on DCM as the only carbon and energy source. This fact demonstrates that CH_2Cl_2 mineralization involves, in addition to DcmA, other proteins and genes that may participate in DCM transport and in overcoming the toxic effect of the intermediate product *S*-chloromethyl glutathione and dehalogenation products CH_2O and HCl.

Genome sequencing has shown that the *dcmA* gene of the DCM degrader *M. dichloromethanicum* DM4 is located within a large (126-kb) chromosome fragment ("DCM island") which is not present in *M. extorquens* AM1 [3]. The DCM island has been little studied and comprises mainly hypothetical genes. The entity of particular interest within this DNA region is a cluster of three genes, $modA_2B_2C_2$ (METDI2644-2642), supposedly encoding the subunits of molybdate ABC transporter: a periplasmic substrate-binding protein, an integral membrane protein (permease), and a peripheral membrane protein (ATPase). These genes are present in the genome of strain DM4, along with the genes of a molybdate transporter typical of members of the genus *Methylobacterium* (METDI5514, METDI4626, METDI4629), but are substantially different: the identity of amino acid sequences is only 35%. Such a low identity level necessitates experimental confirmation of the function of the $modA_2B_2C_2$ gene cluster as a determinant of a molybdate ABC transporter. At the same time, the conceptual translation of the $modA_2$ gene was shown to exhibit similarity to proteins containing the conservative *ostA* domain and determining the resistance of gram-negative bacteria to organic solvents [4]. A corresponding gene (Hden_1702) was found in the genome of another DCM degrader, *Hyphomicrobium denitrificans* ATCC 5188. Hence, it has been hypothesized that the transporter of the DCM degrader DM4 encoded by the $modA_2B_2C_2$ gene cluster is involved in the adaptation of bacteria to degradation of this toxic compound.

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Bacterial strains and plasmids

Strain or plasmid	Characteristics	Reference or source
Methylobacterium dichloromethanicum DM4	DCM degrader, wild-type strain	VKM B-2191 = DSM 6343 [5]
$modA_2^-$	DM4 derivative, *modA*$_2$*::aacC1*, Gm^r	This work
Methylobacterium extorquens DM17	DCM degrader	[6]
Methylorhabdus multivorans DM13	DCM degrader, type strain	VKM B-2030 [6]
Methylorhabdus multivorans DM15	DCM degrader	DSM 21470 [6]
Methylopila helvetica DM6	DCM degrader, type strain	[5]
Albibacter methylovorans DM10	DCM degrader, type strain	DSM 22840 [6]
Ancylobacter dichloromethanicus DM16	DCM degrader, type strain	DSM 21507 [6]
Escherichia coli S17-1	F$^-$ *thi pro recA hsdR* [RP4-2Tc::Mu-Km::Tn*7*] Tp^r Sm^r	[7]
Escherichia coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX*74 *recA1 araD*139 Δ(*ara-leu*) 7697 *galU galK rpsL* (Str^r) *endA1 nupG* λ-	Invitrogen
Plasmids:		
pK18mob	Mobilizable multipurpose vector, Km^r	[8]
p34S-Gm	Source of Gm^r cassette, Ap^r, Gm^r	[9]
pmodA	pK18mob containing METDI2644 (*modA*$_2$) from *M. dichloromethanicum* DM4 (844 bp) inserted at the *Xba*I/*Hin*dIII sites	This work
pmodA-Gm	pmodA containing Gm^r cassette inserted in direct orientation at the *Sph*I sites from p34S-Gm	This work

The goal of the present work was to elucidate the role of the *modA*$_2$ gene (METDI2644) of *M. dichloromethanicum* DM4 in CH_2Cl_2 degradation. Thereto, the occurrence of this gene in DCM degraders with different C_1 assimilation pathways was studied, the transcription analysis of its expression was performed, a knockout mutant was obtained, and its ability to grow on DCM was characterized.

MATERIALS AND METHODS

Cultivation of bacteria and the vectors used. Bacterial strains and plasmids used in this work are listed in the table. Methylobacteria and *Escherichia coli* were cultivated in the K mineral medium with methanol and the Luria–Bertani (LB) medium, respectively; the mutant strains were cultivated with the addition of the respective antibiotics as described earlier [6, 10].

Phylogenetic analysis. The rooted phylogenetic tree was constructed based on analysis of the ModA amino acid sequences of *Proteobacteria* representatives accessible at the NCBI site in the GenBank/EMBL/DDBJ database, using the Neighbor-Joining method implemented in the TREECON software package [11].

PCR amplification and sequencing. The *modA*$_2$ genes of DCM degraders were amplified by PCR from genomic DNA using primers 2644for (5'-ggcctctagagtgcctaatagcgtcgaccgt-3') and 2644rev (5'- gccaagctttcaggagccttcgcccggtaa-3') specific to the nucleotide sequence of the METDI2644 gene of strain DM4. PCR was performed in a BIO-RAD MJ Mini Thermal Cycler (United States). PCR products were sequenced on an ABI PRISM capillary analyzer (Applied Biosystems, United States) using a Big-Dye® Terminator v1.1 kit.

Analysis of the METDI2644 and METDI2643 gene expression by RT-PCR. Total RNA from the cells of *M. dichloromethanicum* DM4 grown in MM mineral medium [6] containing CH_2Cl_2 or CH_3OH was isolated with TRIzol (Invitrogen, United States) and treated with DNase (Thermo Scientific, Lithuania) according to the manufacturers' instructions. The absence of DNA in the RNA preparation was controlled by PCR using primers DRTfor and DRTrev, targeting the *dcmA* gene [6]. The preparation was considered to be free from DNA admixture if amplicons of

the expected length were absent. The reaction of reverse transcription was performed with the use of reverse transcriptase RevertAid™ H Minus (Thermo Scientific, Lithuania) according to the manufacturer's protocol, using the primers modAfor (5'-gcagcgttg-tagttgcgagctt-3') and modArev (5'-gatcgaccttgaacg-gagcgtt-3') specific to the METDI2644 gene sequence, as well as modBfor (5'-gccggggatgctcccac-cta-3') and modBrev (5'-gcctcggtggcttcaccgtc-3') specific to the METDI2643 gene sequence. The conditions of RT-PCR were analogous to those reported previously [6].

Generation of a knockout mutant impaired in the $modA_2$ gene. Isolation of genomic and plasmid DNA, DNA cloning, and transformation of competent cells were performed by the standard methods [12]. The insertion mutant was obtained by the method of double homologous recombination [6, 13]. The $modA_2$ gene (825 bp) from *M. dichloromethanicum* DM4 was inserted into the suicidal vector pK18mob at the *Xba*I and *Hin*dIII restriction sites using primers 2644for and 2644rev. As a result, plasmid pmodA was obtained. Then, a gentamycin resistance gene was inserted into the sequence of the cloned gene at the *Sph*I restriction site (473 bp). The resultant vector p*modA*-Gm was used to transform the cells of *Escherichia coli* S17-1; the transformants were used for the conjugation with *M. dichloromethanicum* DM4. Transconjugants were selected based on their resistance to gentamycin and sensitivity to kanamycin. The insertion of the gentamycin cassette was additionally confirmed by PCR with primers specific to the sequences flanking the inactivated gene.

Cultivation of *M. dichloromethanicum* DM4 and the $modA_2^-$ mutant on DCM and methanol. Growth dynamics on DCM were studied as follows: the cells grown in MM medium with methanol were precipitated by centrifugation (6000 *g*, 30 min) and washed twice with sterile MM medium containing 0.1 μM sodium molybdate. Then, the cells were resuspended in this medium to $OD_{600} = 0.18$, and 50-mL portions of the suspension were transferred to 300-mL Erlenmeyer flasks. In the variants with increased concentrations of Na_2MoO_4 (0.8 and 1.6 μM), the necessary amount of molybdate was added into the medium from a 1 mM solution. The mutant strain was grown without the antibiotic. The flasks were closed with screw caps with a rubber membrane (Precision Sampling Corp., United States), and DCM was added through the membrane with a syringe to the final concentrations of 5, 10, and 15 mM. Upon pH shift to 5.0 in the course of culture growth, the hydrochloric acid formed was neutralized with 5 M NaOH after opening the flasks, after which the caps were screwed on, and a new portion of DCM was added with a syringe. The incubation was performed for 68 h. Culture samples (2 mL) were used to determine optical density and Cl^- ion concentration in the cultivation medium. All experiments were performed in triplicate.

Analytical methods. The concentration of chloride ions in the medium was determined with ferric (III) nitrate as described earlier [6]. Optical density of the cell suspension was measured at 600 nm with a Shimadzu UV-160 recording spectrophotometer (Japan).

RESULTS AND DISCUSSION

Molybdenum is a component of cofactors of various bacterial enzymes involved in the nitrogen, carbon, and sulfur metabolisms. In bacteria, the uptake of molybdate (the most stable molybdenum form widespread in nature) involves the primary high-affinity ABC transporter encoded by the *modABC* genes and two secondary systems (a low-affinity sulfate transporter and a nonspecific anion transporter) [14]. Molybdenum deficiency is critical for nitrogen-fixing and denitrifying bacteria because this trace element is necessary for the functioning of nitrogenase and nitrate reductase. It is known that strain *M. dichloromethanicum* DM4 does not fix nitrogen but can reduce nitrates to nitrites. Methylotrophic growth of *Methylobacterium extorguens* AM1 involves formate dehydrogenase, which is highly homologous to molybdopterin-dependent oxidoreductase-like proteins [15]; similar nucleotide sequences are also present in the genome of strain DM4.

BLAST search in the GenBank database (http://www.ncbi.nlm.nih.gov/) with the amino acid sequence of strain DM4 $ModA_2$ as a query showed that the levels of identity with the closest relevant sequences of *Pseudomonas stutzeri* TS44, *Parvibaculum lavamentivorans* DS-1, and *Methyloversatilis universalis* FAM5 were 65, 63 and 60%, respectively. The identity level with the amino acid sequence of $ModA_1$, which in phylogenetic trees forms a common cluster with ModA proteins of the genus *Methylobacterium*, was much lower (35%).

PCR with the primers specific to the $modA_2$ gene of *M. dichloromethanicum* DM4 allowed us to reveal respective sequences in DCM degraders with the serine (*Methylopila helvetica* DM6, *Methylobacterium extorquens* DM17) and ribulose bisphosphate (*Albibacter methylovorans* DM10) C_1 assimilation pathways. Sequencing of the resultant amplicons showed complete coincidence of the conceptual translations of these genes with the $ModA_2$ of strain DM4, which may be due to lateral gene transfer. Negative results of PCR were obtained for the DCM degraders *Ancylobacter dichloromethanicus* DM16 and *Methylorhabdus multivorans* DM13 and DM15. The phylogenetic position of the ModA proteins of DCM degraders among methylotrophic and nonmethylotrophic representatives of *Proteobacteria* is shown in Fig. 1.

RT-PCR showed the expression at the level of transcription of the adjacent genes of the putative ABC

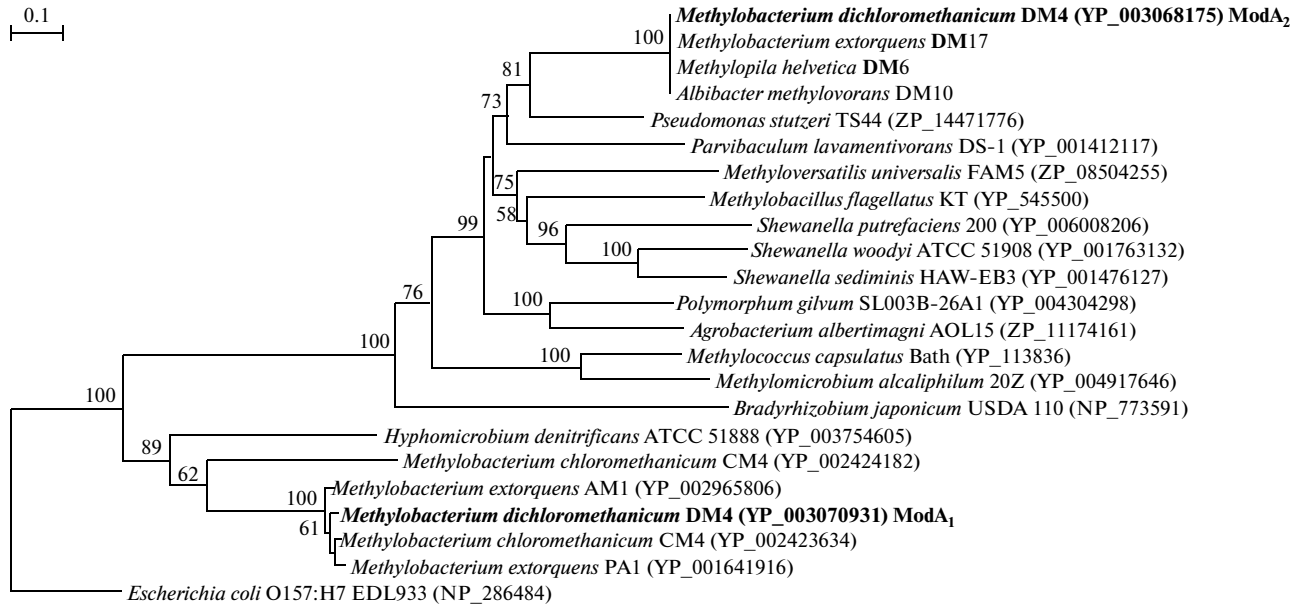

Fig. 1. Phylogram based on comparative analysis of amino acid sequences of ModA of methylotrophic and nonmethylotrophic representatives of *Proteobacteria*. The phylogram was rooted by including the sequence of ModA of *Escherichia coli* O157:H7 as an outgroup. The scale bar corresponds to 10 amino acid substitutions per 100 amino acids. Statistical significance of the branching order was assessed by bootstrap analysis of 100 alternative trees.

transporter, $modA_2$ (METDI2644) and $modB_2$ (METDI2643), in the cells of strain DM4 grown on DCM or methanol (Fig. 2).

The occurrence of the $modA_2$ gene among DCM-degrading methylobacteria, its conservativeness, and its transcription during cell growth on DCM indicated the probable role of this gene in aerobic degradation of CH_2Cl_2.

Then, the mobilizable suicidal vector pK18mob was used to obtain the knockout mutant $modA_2^-$ of *M. dichloromethanicum* DM4 with the METDI2644 gene switched off by interrupting its nucleotide sequence by the insertion of a Gm^r cassette. We

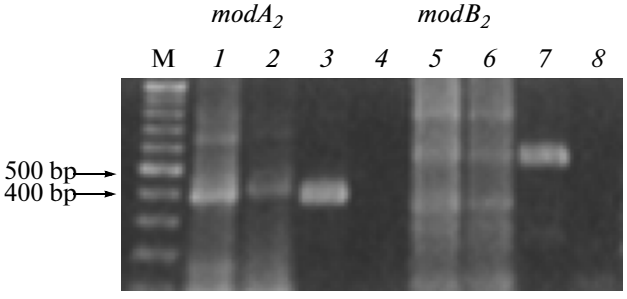

Fig. 2. RT-PCR analysis of the expression of the $modA_2$ and $modB_2$ genes in cells of strain DM4 grown on methanol (*1, 5*) and DCM (*2, 6*). PCR was run with cDNA (*1, 2, 5, 6*), with genomic DNA (positive control) (*3, 7*), and without template DNA (negative control) (*4, 8*). M is molecular weight marker.

revealed no differences in the growth dynamics of the $modA_2^-$ mutant on methanol compared to the wild-type strain, which is quite explicable given the presence in the genome of a paralog of the inactivated gene ($modA_1$, METDI5514), located beyond the DCM island. However, we found that the insertion mutant $modA_2^-$ pregrown on methanol was characterized by a lower growth rate on DCM compared to the wild-type strain DM4. Figure 3 shows the dynamics of cell growth and chlorine ion excretion during DCM degradation. The OD_{600} of the mutant in different sampling points was 56.6–77.9% relative to the control and the concentration of Cl^- ions was 36.2–76.5%. A statistically significant decrease in the growth of the mutant strain was observed at the optimal (10 mM) and increased (15 mM) DCM concentrations in the medium, but not at a reduced DCM concentration (5 mM). An increase in the sodium molybdate concentration in the medium from 0.1 to 0.8 and 1.6 μM (the concentration subinhibitory for culture growth) neither restored the phenotype of the mutant to the level of the wild- type strain DM4 nor had any substantial effect on the growth parameters of the wild-type on DCM and the on range of the DCM concentrations used. This indicates the importance of the $modA_2$ gene product for the growth of strain DM4 at the optimal and increased DCM concentrations, when, at a higher growth rate, the effects of unfavorable factors (oxidative, osmotic, and acidic stresses), coupled with dehalogenation of this toxic substrate, are more pronounced [16].

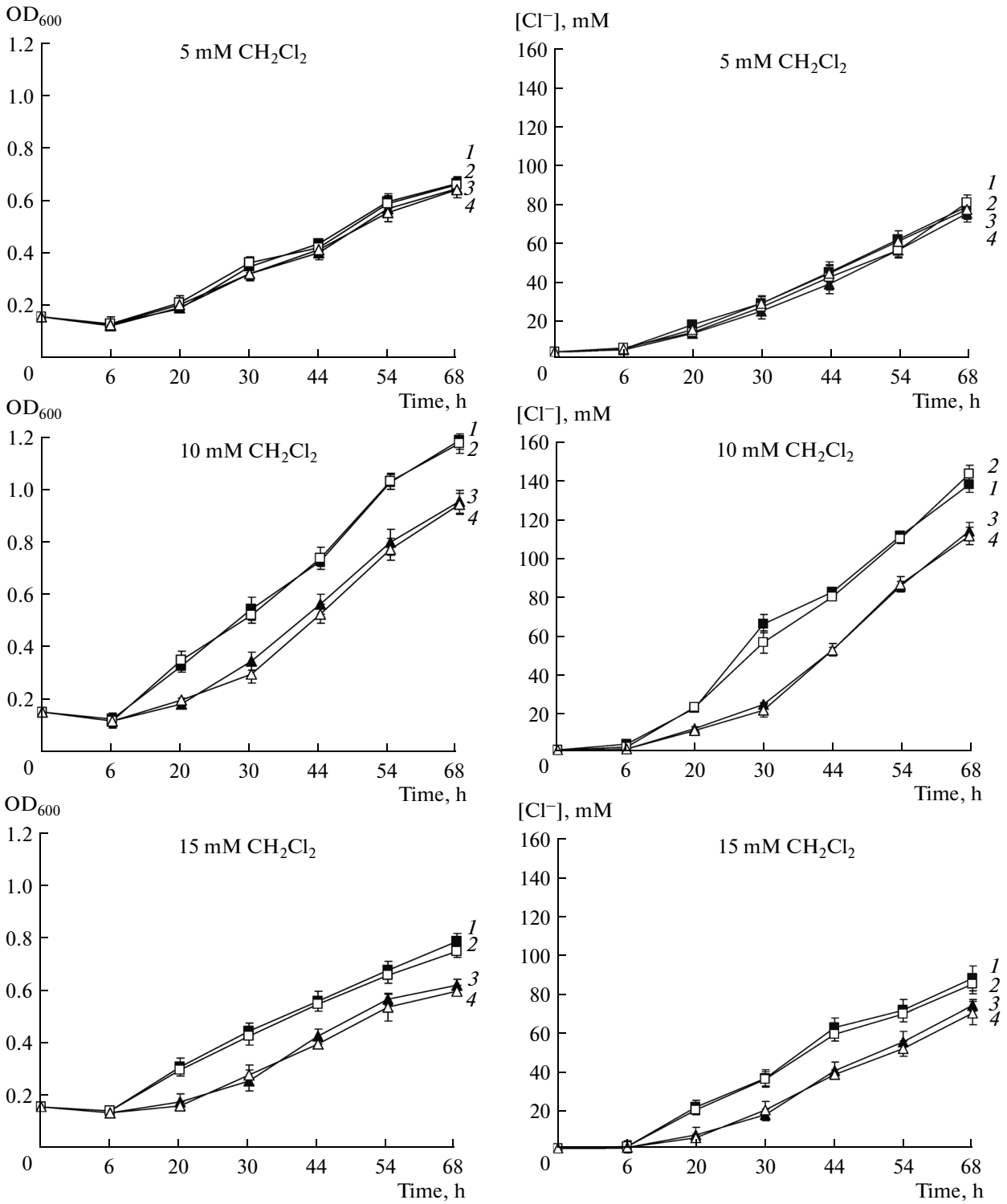

Fig. 3. Growth dynamics of strain DM4 and the $modA_2^-$ knockout mutant at various concentrations of CH_2Cl_2 and sodium molybdate in the medium. The graphs on the left show the optical density of the cultures at 600 nm, and the graphs on the right show the concentration of chloride ions in the medium. (*1*) DM4, 0.1 μM Na_2MoO_4; (*2*) DM4, 1.6 μM Na_2MoO_4; (*3*) $modA_2^-$, 0.1 μM Na_2MoO_4; (*4*) $modA_2^-$, 1.6 μM Na_2MoO_4.

Based on the assumption that the *modA*$_2$*B*$_2$*C*$_2$ gene cluster (METDI2644-2642) encodes subunits of a molybdate ABC transporter, we anticipated that an increase in the sodium molybdate concentration in the medium would restore the growth indices of the mutant to the values typical of the wild- type strain, because, in these conditions, the uptake of the necessary quantity of molybdate ions into bacterial cells can be provided for by the low-affinity and nonspecific transport systems. However, the observed decrease in the mutant growth rate on DCM does not seem to be due to impaired molybdate transport into the cells. As mentioned above, the level of identity of the amino acid sequences encoded by the genes of the *modA*$_2$*B*$_2$*C*$_2$ cluster (METDI2644-2642) to those encoded by the molybdate transporter genes (METDI5514, 4626, 4629) typical of representatives of the genus *Methylobacterium* is as low as 35%, which does not allow unambiguous annotation of the function of this cluster on the basis of amino acid sequences alone, without experimental testing. It is reasonable to assume that the function of the *modA*$_2$*B*$_2$*C*$_2$ cluster during DCM degradation is not directly related to molybdate transport into the cells.

Thus, our work is the first to demonstrate the involvement of the *modA*$_2$ (METDI2644) gene product, a component of the unusual ABC transporter of the strain DM4 DCM island, in the adaptation to DCM degradation. Further studies will be aimed at elucidation of the nature of the transported substrate. In particular, the hypothesis will be verified about the involvement of the putative transporter in active release from the cells of excess DCM molecules entering by diffusion. The existence of active release of excess toxic substrate is indirectly evidenced by the results of experiments on the fractionation of CH_2Cl_2 molecules differing in carbon and chlorine isotopes by intact cells and cell-free extracts of DCM degraders [17]. The answer to this question will be obtained in the forthcoming studies.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project no. 12-04-00760-a).

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Translated by E. Makeeva