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EXPERIMENTAL ARTICLES

Analysis of the 3'-Region of the *dcmA* Gene of Dichloromethane Dehalogenase of *Methylobacterium dichloromethanicum* DM4

Yu. E. Firsova, D. N. Fedorov, 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—Two hypothetical genes of a dichloromethane (DCM) degrader *Methylobacterium dichloromethanicum* DM4, METDI2657 and METDI2658, located in the 3'-region of the *dcmA* gene of DCM dehalogenase, have been studied. The method of reverse transcription polymerase chain reaction (RT-PCR) was used to show that the cells of *M. dichloromethanicum* DM4 grown on both DCM and methanol contained the transcripts of all three of the above genes. RT-PCR amplification of the intergenic regions showed that the genes *dcmA,* METDI2657, and METDI2658 formed an operon. Orthologs of the METDI2657 and METDI2658 genes were also found in the DCM destructors *Methylopila helvetica* DM9, *Methylorhabdus multivorans* DM13 and DM15, *Ancylobacter dichloromethanicus* DM16, and *Methylobacterium extorquens* DM17. A mobilized suicide vector pK18mob was used to obtain a knock-out mutant of *M. dichloromethanicum* DM4 (NOK353) with the "turned-off" METDI2657 gene, the nucleotide sequence of which was interrupted by insertion of a gentamycin cassette. After cultivation on methanol, the NOK353 mutant had a lower rate of growth on DCM than the wild type strain DM4.

Keywords: aerobic methylobacteria, dichloromethane, *dcmA,* METDI2657, METDI2658.

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Dichloromethane (CH_2Cl_2, DCM) is a highly toxic volatile compound, which has a carcinogenic and mutagenic effect on living organisms and participates in the degradation of the ozone layer of the Earth [1]. Commercial production of this compound is as high as 3×10^5 tons/yr [2]. DCM is used as a cooling agent (Freon-30, Khladon-30), as a solvent for chemical synthesis in pharmaceutical industry, for production of aerosols, thermoplastics, synthetic fibers, and photographic films. DCM is degraded under the action of abiotic environmental factors only with great difficulty: the half-life of DCM is 70 days in the atmosphere and 700 years in water systems [3, 4]. Hence, the interest in microorganisms capable of degrading this pollutant is quite clear.

Aerobic DCM degraders belonging to the phyla *Alpha-* and *Betaproteobacteria* actively mineralize DCM, utilizing it as a carbon and energy source [5, 6]. It was established that the primary dehalogenation of DCM to formaldehyde and HCl in aerobic methylobacteria is catalyzed by cytoplasmic glutathione transferase with formation of a toxic, short-lived intermediate: S-chloromethyl glutathione [7]. This enzyme is encoded by the structural *dcmA* gene and is negatively controlled by the regulatory *dcmR* gene [8]. It was shown, however, that DCM degradation by aerobic methylobacteria is a complex process involving, apart from DCM dehalogenase, a complex of additional unknown enzymes and genes [9, 10]. The new prospects for their study are opened due to the recently accomplished sequencing of the genome of *Methylobacterium dichloromethanicum* DM4.

A 126-kbp region of the bacterial chromosome ("DCM island"), which is unique for the degrader strain DM4 and is associated with DCM degradation was revealed [11]. Functional analysis of this DNA fragment will make it possible to reveal additional genetic determinants necessary for the growth of methylobacteria on DCM. The two hypothetical genes, METDI2657 (ORF353) and METDI2658 (ORF192), located downstream *dcmA* and supposed to form an operon together with the latter, are of particular interest. These genes have been found in DCM degraders *Hyphomicrobium denitrificans* ATCC 51888, *Ancylobacter dichloromethanicus* DM16, and *Methylobacterium extorquens* DM17 [6, 12]. The genes are rather conservative and can encode proteins of 353 and 192 amino acid residues, respectively. The DNA fragment carrying the *dcmR* and *dcmA* genes, together with the hypothetical METDI2657 and METDI2658 genes, has a structure typical of transposons (Fig. 1) and seems to be able to spread among prokaryotes by way of horizontal transfer [13]. However, up to now it has not been ascertained whether the METDI2657 and METDI2658 genes are transcribed in *M. dichloromethanicum* DM4 and what their role in DCM deg-

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

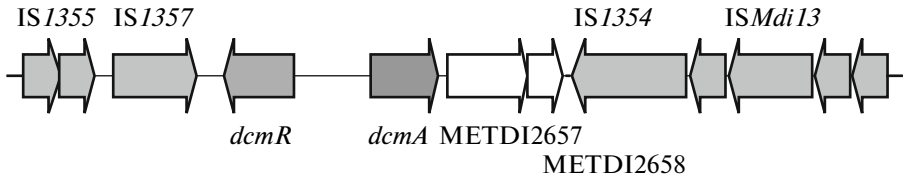

Fig. 1. The region of the chromosomal DNA of *M. dichloromethanicum* DM4 flanked by insertion (IS) elements, containing the structural (*dcmA*) and regulatory (*dcmR*) DCM dehalogenase genes, and the hypothetical genes METDI2657 and METDI2658 [11].

radation is. The goal of the present work was to elucidate the above questions.

MATERIALS AND METHODS

Cultivation of the bacteria and the vectors used. Bacterial strains and plasmids are listed in Table 1. The initial strain *M. dichloromethanicum* DM4 VKM B-2191 (= DSM 6343) and the mutant NOK353 based on the latter, as well as other DCM-degrading methylobacteria, were grown on the minimal K medium containing (g/l): KH_2PO_4, 2; $(NH_4)_2SO_4$, 2; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.025; $FeSO_4 \cdot 7H_2O$, 0.002; pH 7.2, at 29°C in 0.75-l flasks on a shaker (180 rpm). Methanol (120 mM) was added as a carbon and energy source. Agarized K medium contained 1.5% agar. Under cultivation on DCM, the liquid minimal MM medium [21] contained (g/l): KH_2PO_4, 6.8; $(NH_4)_2SO_4$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; pH 7.2, and trace elements (mg/l): $Ca(NO_3)_2$, 25; $FeSO_4 \cdot 7H_2O$, 0.1; $MnSO_4 \cdot 5H_2O$, 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.025; H_3BO_3, 0.01; $CuCl_2 \cdot 2H_2O$, 0.025; $ZnSO_4$, 0.03; $Na_3VO_4 \cdot 12H_2O$, 0.03; $CoCl_2 \cdot 6H_2O$, 0.02; and $NiCl_2 \cdot 6H_2O$, 0.009. For cultivation on CH_2Cl_2, 300-ml Erlenmeyer flasks with 50 ml of the medium were closed with screw caps with a rubber membrane (Precision Sampling Corp., Baton Rouge, United States). DCM was added to the medium through a membrane in portions with a syringe up to a final concentration of 10 mM. As pH value shifted to 5.0, 3 M

Table 1. Bacterial strains and plasmids

Strain or plasmid	Characteristic	Reference or source
Methylobacterium dichloromethanicum DM4	DCM degrader, wild type strain	VKM B-2191 = DSM 6343 [14]
NOK353	DM4 derivative, METDI2657::*aacC1*, Gm^r	The present work
Methylobacterium extorquens DM17	DCM degrader	[12]
Methylorhabdus multivorans DM13	DCM degrader, type strain	VKM B-2030 [15]
Methylorhabdus multivorans DM15	DCM degrader	DSM 21470 [12]
Methylopila helvetica DM9	DCM degrader, type strain	VKM B-2189 [14]
Albibacter methylovorans DM10	DCM degrader, type strain	DSM 22840 [16]
Ancylobacter dichloromethanicus DM16	DCM degrader, type strain	DSM 21507 [17]
Escherichia coli S17-1	F^- *thi pro recA hsdR* [RP4-2Tc::Mu-Km::Tn*7*] Tp^r Sm^r	[18]
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	Mobilized multipurpose vector, Km^r	[19]
p34S-Gm	Gm^r cassette source, Ap^r, Gm^r	[20]
p353f-01	pK18mob containing a 695-bp *Xba*I/*Hind*III fragment of the hypothetical gene METDI2657 (ORF 353) from *M. dichloromethanicum* DM4	The present work
p353f-02	p353f-01 containing a Gm^r cassette cloned at the PstI sites from p34S-Gm, in direct orientation	The present work

Table 2. Primers used in the work

Primer	Nucleotide sequence (5-3') and restriction site	Target gene	Purpose
NOKfor	AGGTCTCTAGAGTGTCCGTCCGTTCTGT *Xba*I	METDI2657	Cloning of the 695-bp gene fragment in pK18mob
NOKrev	TGCCAAGCTTATAAGGGCAAACCGCTTGC *Hind*III		
DRTfor	TGAGATTGACGTTCCCTTCG	*dcmA*	Analysis of expression by the method of RT-PCR, product – 418 bp
DRTrev	ATATTCGCGGTCCCTCAGC		
353for	TGTGATCAATGACGCGCG	METDI2657	Analysis of expression by the method of RT-PCR, product – 408 bp
353rev	ATTTCGGGCGTGAGCA		
192for	GATGGTAATGGCACAACACG	METDI2658	Analysis of expression by the method of RT-PCR, product – 391 bp
192rev	CGAGCTGGATACCGTAGTCG		
OP1for	TGTGTTGAAGGGGACGGAGGTC	intergenic region *dcmA*/METDI2657	RT-PCR, product – 433 bp
OP1rev	ATGTTCCATCACCGCCCCGT		
OP2for	GCTTATGACGCGGGACGAGGTA	intergenic region METDI2657/METDI2658	RT-PCR, productт – 431 bp
OP2rev	GCCGTCCGAGTCGATAAACCTG		

NaOH was added to pH 7.0. *Escherichia coli* strains presented in Table 1 were cultivated at 37°C in LB medium [22] with addition of the relevant antibiotics (μg/ml): ampicillin, 100; kanamycin, 50; and gentamycin, 2.5. For *M. dichloromethanicum*, the antibiotics were added in the following concentrations (μg/ml): gentamicin, 20; and kanamycin, 50.

The study of expression of the genes of the supposed DCM operon by RT-PCR. Total RNA from the cells of *M. dichloromethanicum* DM4 grown on CH_2Cl_2 and CH_3OH was isolated with the TRIzol reagent (Invitrogen, United States) and treated with DNase (Fermentas, Lithuania) according to the manufacturers' instructions. The absence of DNA in the RNA preparation was controlled by PCR, using the primers DRTfor and DRTrev for the *dcmA* gene (Table 2). PCR was carried out on a BIO-RAD MJ Mini amplifier (United States). In the absence of amplification of the fragments of expected length, the preparation was considered free from DNA admixtures. The reverse transcription reaction was carried out with the RevertAid™ M-Mulv reverse transcriptase (Fermentas, Lithuania) using specific primers for the *dcmA*, METDI2657, and METDI2658 genes and the intergenic regions (Table 2) according to the manufacturer's instruction. The reaction mixture was incubated for 1 h at 42°C. The subsequent PCR was carried out with 3 μl of the resultant cDNA preparation and the same primers as for the reverse transcription reaction. The mode of amplification for all pairs of the primers was as follows: initial denaturing, 2 min, 95°C; denaturing, 45 s, 95°C; annealing of the primers, 45 s, 58°C; elongation, 50 s, 72°C; 30 cycles of amplification; final polymerization, 6 min, 72°C. PCR was performed with *Taq* polymerase (SibEnzyme, Russia).

Obtaining of the knock-out mutant of *M. dichloromethanicum* DM4 with the turned-off gene METDI2657. Isolation of genomic and plasmid DNA, DNA cloning, and transformation of competent cells were carried out by the standard methods [22]. To obtain an insertion variant of the METDI2657 gene by homologous recombination, a 674-bp fragment bearing the 5'-terminal region of the METDI2657 gene sequence was amplified using the NOKfor and NOKrev primers carrying the endonuclease sites *Xba*I and *Hind*III, respectively (Table 2). This fragment was amplified using Pfu-DNA polymerase (Fermentas, Lithuania) in the following mode: initial denaturing, 2 min, 95°C; denaturing, 30 sec, 95°C; annealing of the primers, 40 sec, 61°C; elongation, 2 min, 72°C; 30 cycles of amplification; final polymerization, 4 min, 72°C. The PCR fragment was purified in Quantum *Prep*® PCR Kleen Spin Columns (Biorad, United States) according to the manufacturer's instructions.

The obtained fragment treated with the *Xba*I and *Hind*III restriction endonucleases was cloned in a mobilized suicide vector pK18mob at the same sites; as a result, a plasmid *p353f-01* was obtained (Table 1). Then the *Pst*I fragment (895 bp) from the vector p34S-Gm carrying the gentamycin resistance gene was

cloned in the vector p353f-01 bearing the only *Pst*I restriction site located approximately in the middle of the cloned 674-bp METDI2657 gene fragment. The resulting vector p353f-02 contained a METDI2657 gene fragment, with the directly oriented Gm^r cassette in its center. The METDI2657 gene regions flanking the Gm^r cassette were 312 and 362 bp, respectively.

The plasmid p353f-02 was mobilized by two-parental crossing of *M. dichloromethanicum* DM4 and *E. coli* S17-1 (p353f-02); the latter was used as a plasmid donor. *M. dichloromethanicum* DM4 cells in the early exponential growth phase (OD_{600} 0.2–0.3) were precipitated by centrifugation and mixed with the overnight culture of *E. coli* S17-1 (p353f-02) cells prewashed with the K medium (the ratio of donor and recipient cells was 1:5). The cell suspension was applied to the membrane filters (0.45 μm, Millipore, United States), placed onto the surface of agarized K medium containing 0.02% casamino acids and 0.5% methanol, and incubated at 28°C for 24 h. Then the cells were washed off the filter with sterile K medium and transferred to the agar plates with selective K medium containing 2% methanol and 20 μg/ml gentamycin. Potential transconjugants were purified by twofold transfers from single colonies on the selective medium with antibiotics, and only double recombinants (Gm^r, Km^s) were selected for further work. All recombinants were additionally checked by PCR for the presence of a mutant allele with insertion of the Gm^r cassette.

Cultivation of *M. dichloromethanicum* DM4 and the NOK353 mutant on DCM and methanol. For plotting the curves of growth on DCM, the cells grown in the K medium with methanol were precipitated by centrifugation (6000 *g*, 30 min) and washed from growth substrate residues with a fresh MM medium. The pellet was resuspended in sterile MM medium up to $OD_{600} = 0.27$; the suspension (50 μl) was transferred to 300-ml Erlenmeyer flasks and closed with screw caps with a rubber membrane (Precision Sampling Corp., United States). The mutant strain was grown without the antibiotic. DCM was added to the medium through the membrane in portions with a syringe: initially 2.6 mM (50 μl) and then 1.7 mM (30 μl), up to a final concentration of 10 mM. Incubation was carried out for 92 h. All experiments were repeated three times. Bacterial suspension samples (2 ml) were used for determination of optical density at 600 nm and measurement of the concentration of Cl^- ions in the cultivation medium formed by degrader cells during DCM dechlorination. Cl^- concentration in the supernatant after centrifugation (8000 *g*, 5 min) of the cell suspension was determined by the method of Jörg and Bertau [23]. The sample (200 μl) was supplemented with 475 μl of 12 M $HClO_4$, 100 μl of 0.2 M $Fe(NO_3)_3 \cdot 9H_2O$, and 225 μl of water. Optical density was measured at 340 nm on a Shimadzu UV-160 registering spectrophotometer (Japan).

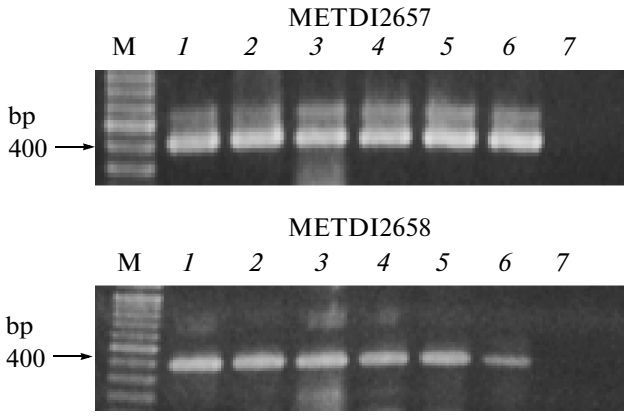

Fig. 2. Detection of the METDI2657 and METDI2658 genes in the genomes of aerobic DCM-degrading methylobacteria. Molecular weight marker, M; PCR with the genomic DNA of the strains *M. dichloromethanicum* DM4 (*1*), *M. extorquens* DM17 (*2*), *Methylorhabdus multivorans* DM13 and DM15 (*3, 4*), *Ancylobacter dichloromethanicus* DM16 (*5*), *Methylopila helvetica* DM9 (*6*), and *Albibacter methylovorans* DM10 (*7*).

RESULTS AND DISCUSSION

At the first stage of the study we looked for the orthologs of the hypothetical genes METDI2657 and METDI2658 of *M. dichloromethanicum* DM4 in the genomes of other DCM-degrading methylobacteria. The screening for the similarity between the respective amino acid sequences from the GenBank database using the BLAST software package (http:ncbi.nlm.nih.gov) showed their identity in *M. dichloromethanicum* DM4 and *Hyphomicrobium denitrificans* ATCC51888. Previously we have shown the complete coincidence of amino acid sequences corresponding to the METDI2657 gene in the reference strain *M. dichloromethanicum* DM4 and DCM degraders *Methylorhabdus multivorans* DM15 and *Methylobacterium extorquens* DM 17, whereas the ortholog protein of the strain *Ancylobacter dichloromethanicus* DM16 differed only in two amino acid substitutions [12]. It is notable that the 3'-region of the *dcmA* gene of the DCM degrader *Methylophilus leisingeri* DM11 was also shown to have an ORF homologous to METDI2657 of *M. dichloromethanicum* DM4, with the 45% similarity between the encoded amino acid sequences [24].

PCR with specific primers revealed the METDI2657 and METDI2658 genes also in *Methylopila helvetica* DM9 and *Methylorhabdus multivorans* DM13 and DM15 (Fig. 2). No PCR products corresponding to these genes were found when using the genomic DNA of *Albibacter methylovorans* DM10, which may be due both to the absence of the homologous genes and to their noticeable difference from the sequences of the strain DM4. Thus, the orthologs of the hypothetical genes METDI2657 and METDI2658 are very conservative, present in many DCM degrad-

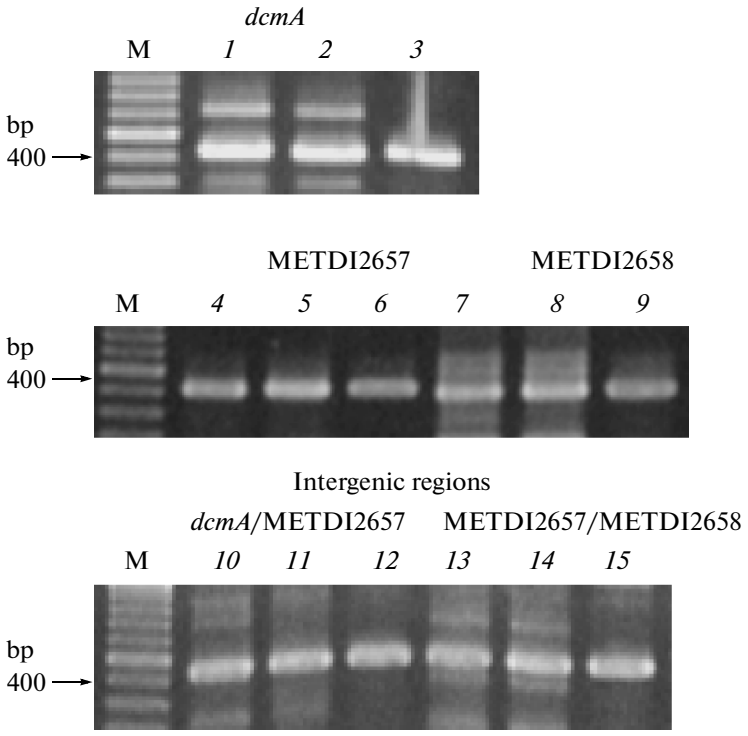

Fig. 3. RT-PCR analysis of expression of the *dcmA*, METDI2657 and METDI2658 genes and RT-PCR amplification of the intergenic regions *dcmA/METDI2657* and METDI2657/METDI2658 in the cells of *M. dichloromethanicum* DM4 grown on methanol (*1, 4, 7, 10, 13*) and DCM (*2, 5, 8, 11, 14*). Molecular weight marker, M; PCR with cDNA of *dcmA* (*1, 2*), METDI2657 (*4, 5*), METDI2658 (*7, 8*), *dcmA/METDI2657* (*10, 11*), and METDI2657/METDI2658 (*13, 14*); PCR with the genomic DNA of the strain DM4 (control) with the primers for the above genes and intergenic regions, respectively (*3, 6, 9, 12, 15*).

ers, and their location in the genome relative to the structural *dcmA* gene of DCM dehalogenase resembles an operon. All the above suggests that these genes encode the proteins functionally associated with DCM degradation. Accordingly, it was necessary to find out whether these genes were expressed during the growth of methylobacteria on DCM. Transcription analysis of expression of the *dcmA*, METDI2657 and METDI2658 genes showed that the RNA transcripts of all three genes were present simultaneously in the cells grown on both DCM and methanol (Fig. 3). This is an indication of incomplete switching-off of the inducible promoter of DCM dehalogenase and the supposed DCM operon (promoter "leaking"). Previously, La Roche and Leisinger [25] showed by the Northern blot method the presence of RNA hybridized with the *dcmA* probe in the cells of *M. dichloromethanicum* DM4 grown on either DCM or methanol. In the 3'-region of the *dcmA* gene (40 bp downstream the stop codon), these authors found a GC-rich imperfect inverted repeat (34 bp), probably resulting in formation of a termination loop in mRNA. However, it seems that transcription may continue under certain conditions. Indeed, the results of RT-PCR with the primers located at the termini of the coding sequences (which made it possible to amplify the intergenic regions) demonstrated that the *dcmA*, METDI2657 and METDI2658 genes formed an operon (Fig. 3).

Then, using the mobilized suicide vector pK18mob, we obtained the knock-out mutant NOK353 with the switched-off METDI2657 gene, the nucleotide sequence of which was interrupted by insertion of the Gm^r cassette. The NOK353 mutant was shown to preserve the ability to grow on DCM both in liquid and on agarized nutrient media. However, transfer from cultivation on methanol to cultivation on DCM revealed a statistically reliable decrease in growth rate of the mutant compared to the initial strain. This decrease was visible on the graphs of optical density of the bacterial suspension and concentration of chlorinde ions excreted into the medium by the cells during DCM degradation (Fig. 4). The growth of the mutant and initial strains was stopped upon reaching the same final concentration of Cl^- ions: 97 mM. The results obtained are in agreement with the data of Kayser et al. [9], who used in their experiments the Dcm^- mutant of *M. dichloromethanicum* DM4-2cr with a very large deletion (21 kbp) [26], which was complemented by way of mobilization of plasmids with the cloned DNA fragment carrying the *dcmA* gene. It was shown that transconjugants of the strain *M. dichloromethanicum* DM4-2cr, constitutively expressing the *dcmA* gene on the plasmid pME8220,

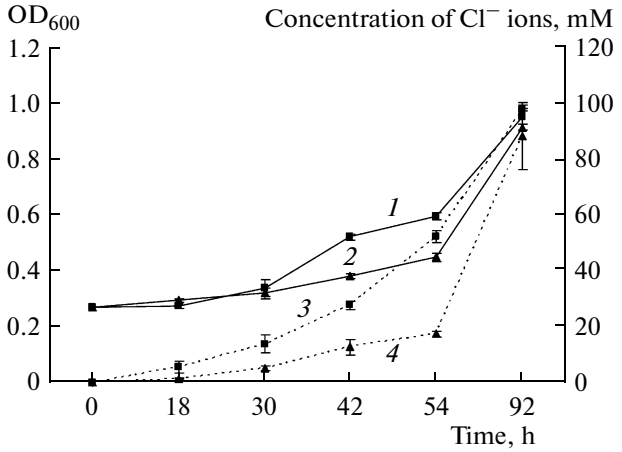

Fig. 4. Optical density of the cell suspension (*1, 2*) and the concentration of chloride ions in the medium (*3, 4*) during the growth on DCM of the strain *M. dichloromethanicum* DM4 (*1, 3*) and the knock-out mutant NOK353 (*2, 4*).

were able to grow on DCM, although at a somewhat lower rate than the wild type strain *M. dichloromethanicum* DM4. The authors, however, could not find out its association with the loss of any particular genes of the large deletion in the mutant DM4-2cr.

The performed knocking-out of the METDI2657 gene suggests that the observed phenotypic effect is determined by switching-off of the hypothetical METDI2657 gene, which is not critical for the ability of this strain to grow on DCM but is of a certain functional significance. It should, however, be taken into account that the "switching-off" of METDI2657 results in the simultaneous "switching-off" of the hypothetical gene METDI2658 following it in the DCM operon. Since the respective proteins have no conservative domains, it is difficult to say what function they perform. Amann et al. (John Craig Venter Institute, United States) suggested that the ortholog of the METDI2657 gene found in the gamma-proteobacterial strain NOR51-B encoded the CmcJ methyl transferase (34% similarity of the respective amino acid sequences; NCBI ZP_04956622). Similar proteins of some ascomycetous fungi are also annotated in the NCBI protein database as methyl transferases; however, they show a lower homology (28–31%).

Thus, the METDI2657 and METDI2658 genes can encode the new enzymes probably involved in detoxification of the intermediate or products of DCM degradation; however, the answer to this intriguing question can be obtained only in the course of further studies.

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