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EXPERIMENTAL ARTICLES

Analysis of the Key Functional Genes in New Aerobic Degraders of Dichloromethane

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Abstract—The genes of dichloromethane (CH_2Cl_2, DCM) degradation have been characterized in the aerobic degraders *"Gottschalkia methylica"* DM15, *"Ancylobacter dichloromethanicus"* DM16, and *Methylobacterium extorquens* DM17, isolated from different regions of Russia. The sequencing of the structural gene *dcmA* of DCM dehalogenase, followed by phylogenetic analysis, showed that the new degraders possess A-type dehalogenases. The DcmAs of the strains DM15 and DM17 were identical to the known orthologous proteins of *Methylorhabdus multivorans* DM13 and *Methylobacterium dichloromethanicum* DM4, respectively. DcmA of the degrader DM16 differed by three amino acid substitutions from DcmA of strain DM4. In agreement with the organization of the cluster of DCM degradation genes in *M. dichloromethanicum* DM4, the regulatory gene *dcmR* and the open reading frame *orf353,* flanking *dcmA*, were identified in the new degraders. The similarity of DCM degradation genes in aerobic degraders of different taxonomic position and geographical origin suggests their distribution among methylotrophic bacteria by means of horizontal transfer.

Key words: aerobic methylotrophic bacteria, dichloromethane, dehalogenation, *dcmA* and *dcmR* genes.

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Dichloromethane (CH_2Cl_2, DCM) is a toxic carcinogenic compound included in the priority list of air and water pollutants because of its volatility, good solubility in water, persistence, and large volume of annual commercial production (3×10^5 t per year) [1]. The problem of biodegradation of this compound is of great scientific and practical interest.

It has been shown that the primary dehalogenation of CH_2Cl_2 in most of aerobic methylotrophic bacteria is catalyzed by cytoplasmic dichloromethane dehalogenase (glutathione-S-transferase) encoded by the *dcmA* gene [2]. The *dcmR* gene, found next to *dcmA* in *Methylobacterium dichloromethanicum* DM4, performs negative regulation of the *dcmA* gene expression [3]. The loss of ability for DCM degradation during cultivation of degrader strains in the absence of selective pressure of the pollutant is an important problem conditioned by localization of specific catabolic genes on complex transposons of class I. The analysis of the fragment carrying the *dcmA* and *dcmR* genes in some of the DCM degraders resulted in identification of three types of insertion sequences (IS1354, IS1355, and IS1357) and two open reading frames (*orf353* and *orf192*) of unknown function (Fig. 1) [4].

The previously created collection of aerobic DCM degraders includes only 13 strains of methylotrophic bacteria (DM1–DM13) [5] isolated from water treatment plants in Switzerland and Germany. However, the biodiversity of DCM degraders in other climatic zones and biotopes polluted with this dangerous compound has not been studied, although it is important for effective bioremediation. Recently, three novel aerobic DCM degraders, *"Gottschalkia methylica"* $DM15^T$, *"Ancylobacter dichloromethanicus"* $DM16^T$, and *Methylobacterium extorquens* DM17, have been isolated from different regions of Russia and identified [6, 7].

The goal of this work was to analyze the key genes associated with methylotrophic growth of the new DCM-degrading strains.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. The subjects of research were three new aerobic DCM degraders: *"Gottschalkia methylica"* $DM15^T$, *"Ancylobacter dichloromethanicus"* $DM16^T$, and *Methylobacterium extorquens* DM17. Strains DM16 and DM17 were isolated from polluted ground of chemical plants of Volgograd and Sergiev Posad (Moscow oblast); strain DM15 was isolated from garden soil in the town of Pushchino (Moscow oblast) that had been treated with CH_2Cl_2 for two years.

The bacteria were grown in 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). The agarized medium contained 1.5% agar. CH_3OH (120 mM) or

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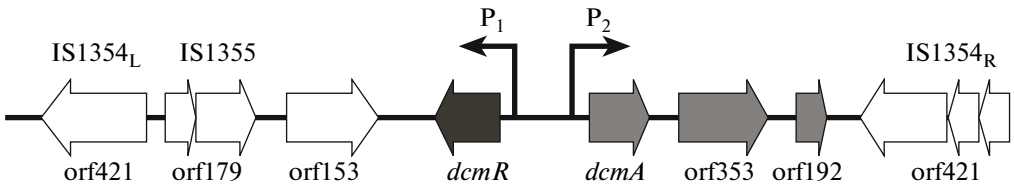

Fig. 1. The DCM-operon of *M. dichloromethanicum* DM4, bearing the structural (*dcmA*) and regulatory (*dcmR*) genes of dichloromethane dehalogenase [4].

CH_2Cl_2 (10 mM) was added as the carbon and energy source. During cultivation on CH_2Cl_2, 300-ml Erlenmeyer flasks with 50 ml of the medium were closed with screwed caps with a rubber membrane (Precision Sampling Corp., Baton Rouge, United States). DCM was introduced into the medium through the membrane with a syringe up to a final concentration of 10 mM. As pH was shifted to 5.0, 3M NaOH was added until pH 7.0. For the cultivation of *"A. dichloromethanicus"* DM16, biotin and pantothenate (20 μg/l) were added to sterile K medium.

Isolation and purification of DNA preparations. Genomic DNA was isolated by the modified method [8]. Cells (50 mg) were suspended in 1 ml of TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA), followed by the addition of lysozyme (1 mg/ml), and incubated for 1 h at 37°C. Then, proteinase K (100 μg/ml) was added and the lysate was kept at the same temperature for 30 min, followed by introduction of 10% sodium dodecyl sulfate solution to a concentration of 0.5%. After the incubation for 1 h at 37°C, NaCl was introduced to a final concentration of 0.7 M, and 10% cetyl trimethyl ammonium bromide, to a concentration of 1% (w/v), and the mixture was incubated for 20 min at 65°C. Then, successive extractions with equal volumes of chloroform and a phenol–chloroform mixture (1 : 1) and two–four extractions with chloroform were performed. DNA was precipitated from the water phase by adding one volume of isopropanol and centrifugation (10000 *g*, 10 min), after which DNA was twice washed with 70% ethanol and then with 96% ethanol and dissolved in 20–100 μl of H_2O.

PCR amplification and sequencing. The structural gene of DCM-dehalogenase, *dcmA,* was amplified by the PCR method [9] from genomic DNA using primers DMfor and DMrev [10]. PCR amplification was carried out in a Hybaid DNA thermocycler (England) in the following mode: initial denaturation, 90 s at 95°C; 27 amplification cycles of 30 s at 95°C, 20 s at 55°C, and 90 s at 72°C; and final extension, 6 min at 72°C.

For amplification of the DNA regions flanking the *dcmA* gene, two pairs of specific primers, J1f-J1r and J2f-J2r, were used that we developed on the basis of the known sequence of *Methylobacterium dichloromethanicum* DM4. The nucleotide sequence of the primers is presented in Table 2, and their positioning is shown in Figure 4. Amplification conditions were as follows: initial denaturation, 90 s at 95°C; 25 cycles of 30 s at 95°C, 30 s at 58°C (J1) or 60°C (J2) at 30 s, and 90 s at 72°C; and final extension, 6 min at 72°C.

The 550-bp fragment of the methanol dehydrogenase gene, *mxaF*, was amplified by the PCR method from genomic DNA using primers f1003 and r1561 [11].

The reaction mixture (30 μl) contained *Taq*-polymerase buffer; respective primers, 100 pmol; DNA, 100 ng; dNTP, 0.2 mM; and *Taq*-polymerase/*Pfu*-polymerase mixture (10 : 1), 1 μl (Fermentas, Lithuania).

Amplicons were purified on Quantum Prep^R PCR Kleen Spin Column (Biorad, United States). PCR fragments were sequenced using Big-Dye^R Terminator v1.1 kits and an ABI PRISM capillary analyzer (Applied Biosystems, United States).

Phylogenetic analysis. Phylogenetic screening in the GenBank database for nucleotide and amino acid sequences similar to those of the *dcmA, dcmR*, and *mxaF* genes of the strains under study and to the proteins encoded by them was carried out using the BLAST software package (http://ncbi.nlm.nih.gov). For more exact determination of their phylogenetic position, the amino acid sequences of DcmA and MxaF were aligned manually with the corresponding sequences available in the NCBI Database Project. The rooted phylogenetic tree was constructed by the neighbor-joining method implemented in the TREECON software package [12]. Evolutionary distances were calculated as the number of substitutions per 100 amino acids. Statistical reliability of the branching order was assessed by bootstrap analysis of 100 alternative trees, using the corresponding function of TREECON.

Plasmids were detected in methylotrophic bacteria by the method of Kado and Liu [13].

Depositing nucleotide sequences. The de novo determined nucleotide sequences of the *dcmA* genes of DCM degraders (strains DM15 and DM16) were deposited in GenBank under accession numbers FJ235914 and EU596479; the nucleotide sequences of the fragments of the *mxaF* genes of these strains were deposited under accession numbers FJ235915 and EU589387.

Table 1. Characteristics of the new aerobic DCM degraders

Property		"*Gottschalkia methylica*" DM15^T	"*Ancylobacter dichloromethanicus*" DM16^T	*Methylobacterium extorquens* DM17
Source of isolation		DCM-polluted soil		
Cell morphology:				
Size (μm)		0.5–0.7 × 1.2–1.5	1.0–1.3 × 1.2–1.5	0.8–1.1 × 1.5–2.5
Shape		ovoids	bean-shaped rods	ovoids
Cell wall type		gram-negative	gram-negative	gram-negative
Reproduction		binary fission	binary fission	binary fission
Motility		+	–	+
Colony color		white	white	pink
C_1 substrates used as C and energy sources		methanol, formate, DCM	methanol, formate, DCM, formaldehyde	methanol, formate, DCM, methylamine
Type of methylotrophy		facultative	facultative	facultative
C_1 metabolic pathway		ribulose-bisphosphate		serine
Demand for vitamins		biotin and pantothenate		–
Temperature: growth at	4°C	–	+	–
	37°C	+	+	+
	43°C	–	–	–
	optimum, °C	28–30	28–30	28–30
pH	growth range	5.5–9.0	6.0–9.0	6.0–9.0
	optimum	7.0–7.5	7.0–7.5	6.5–7.5
NaCl, %	growth range	2.0	2.0	2.5
	optimum	0.05	0.5	0.5
Class		*Alphaproteobacteria*		

Table 2. Oligonucleotide primers for amplification and sequencing of the DNA region bearing the *dcmA* gene

Primer name	Nucleotide sequence	Gene/fragment	PCR product length, bp
DMfor*	aaaaaaaaca**tctaga**gaatgacaaccgtgcgc	*dcmA*	1225
DMrev*	aaaaaaaaaa**ggatcc**ggtcatcgaaggaatgc		
J1f	ggatcctccaaggcttgaa	*dcmR*	1461
J1r	ttattggcttgtcgcgcg		
J2f	taacttgcgtgcacggcct	*orf353*	1237
J2r	gccatcgtgttgtgccatt		
dcmRfor	ttatcgggaagcgatggcc	*dcmR*	423
dcmRrev	ctcaagtcgccggaatggac	inner fragment	

* Developed by S. Vuilleumier [10].

RESULTS AND DISCUSSION

The aerobic DCM degraders being studied have been isolated from places distant geographically (Central Russia, Povolzhye) and different in the duration of selective pressure of the pollutant. Previously, we identified the isolates by the methods of polyphasic taxonomy as representatives of different taxa of the class *Alphaproteobacteria*: *"Gottschalkia methylica"* DM15^T, *"Ancylobacter dichloromethanicus"* DM16^T, and *Methylobacterium extorquens* DM17 [6, 7] (the former two taxa are new genera and species). As shown in Table 1, the cells of new degraders are gram-negative, mesophilic, and neutrophilic ovoids or bean-shaped rods. They utilize C_1 compounds such as methanol, formate, dichloromethane, and a wide range of mono- and polycarbon substrates (monosaccharides, organic acids, amino acids, alcohols) as carbon and energy sources; i.e., they are facultative methylotrophs. They implement different routes of C_1

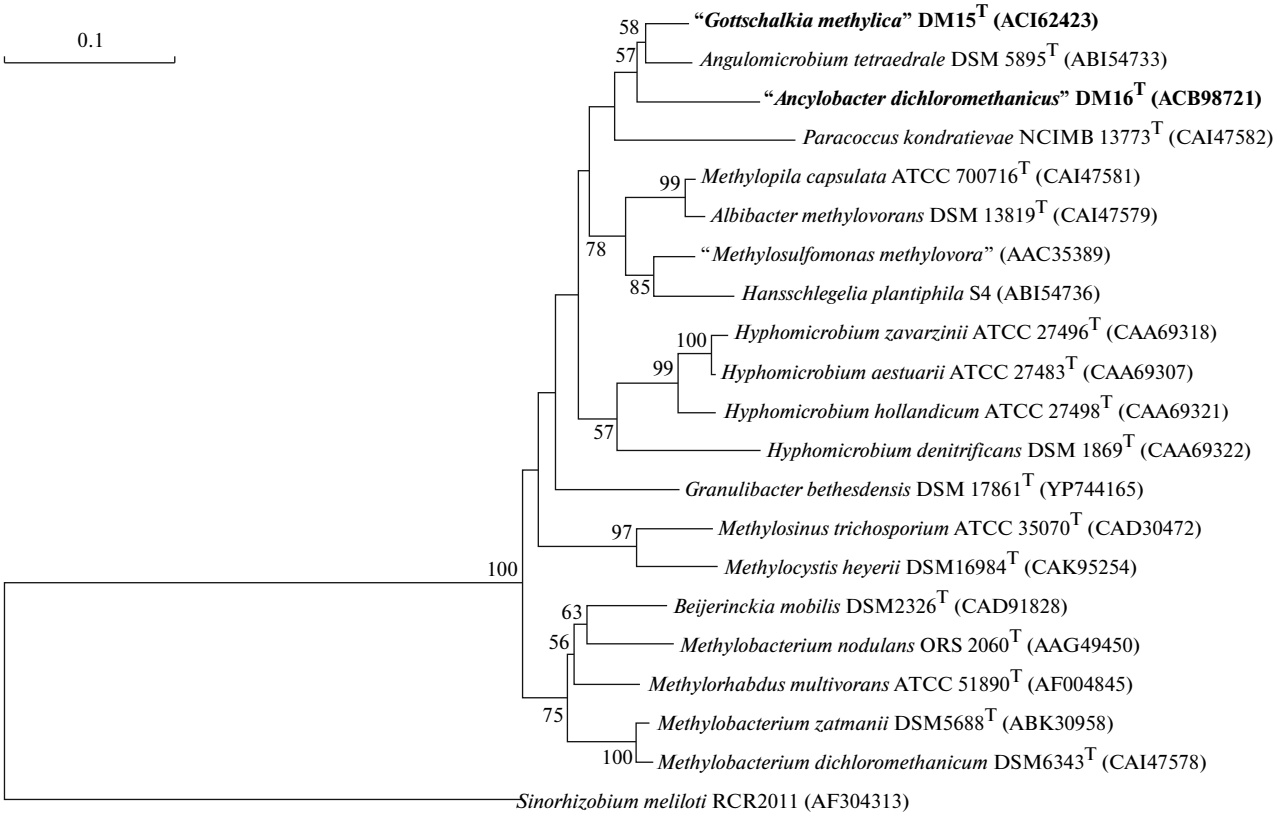

Fig. 2. Phylogenetic tree constructed on the basis of comparative analysis of the fragments of MxaF amino acid sequences of *"Gottschalkia methylica"* DM15T, *"Ancylobacter dichloromethanicus"* DM16T, and other representatives of the class *Alphaproteobacteria*. Scale bar, 10 amino acid substitutions per each 100 amino acids. Statistical reliability of the branching order was assessed by bootstrap analysis of 100 alternative trees.

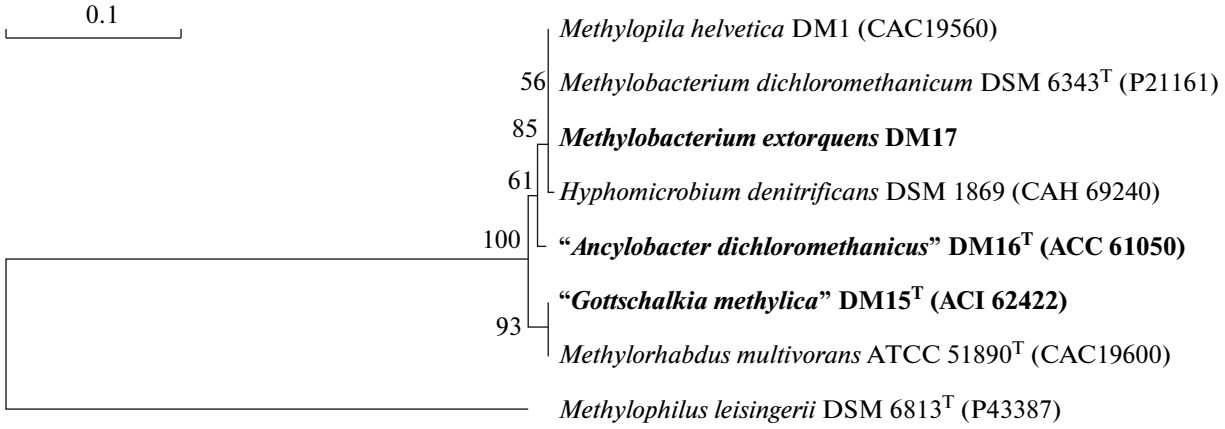

Fig. 3. Phylogenetic tree constructed on the basis of comparative analysis of DcmA amino acid sequences of DCM-degrading methylotrophic bacteria. The root was determined by inclusion of the DcmB sequence of *Methylophilus leisingeri* DM11 as an outgroup. Scale bar, 10 amino acid substitutions per each 100 amino acids. Statistical reliability of the branching order was assessed by bootstrap analysis of 100 alternative trees.

metabolism: the ribulose bisphosphate (DM15 and DM16) or serine (DM17) pathways.

We performed phylogenetic analysis of the 550-bp fragment of the functional *mxaF* gene, encoding the large subunit of PQQ-dependent methanol dehydrogenase (MxaF), which catalyzes methanol oxidation to formaldehyde in most of gram-negative methylotrophic bacteria. It has been established that the MxaF amino acid sequences of the degrader strains DM15 and DM16 are most closely associated phylo-

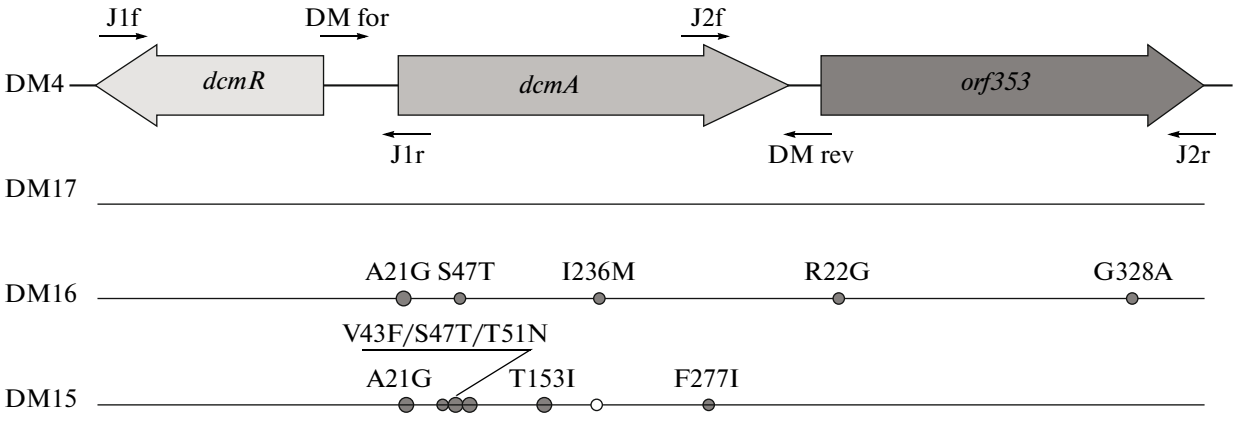

Fig. 4. Variability of nucleotide sequences of the *dcmA* cluster in the new DCM degraders *"Gottschalkia methylica"* DM15, *"Ancylobacter dichloromethanicus"* DM16, and *Methylobacterium extorquens* DM17 as compared with *Methylobacterium dichloromethanicum* DM4. ○, a silent nucleotide substitution; ● , a nucleotide substitution leading to an amino acid substitution.

genetically with the analogous MxaF regions of other methylotrophs belonging to the class *Alphaproteobacteria*, which is in agreement with the previous data of 16S rDNA sequencing (Fig. 2). The highest level of similarity was found for MxaF of the strains DM15 and DM16 themselves (90.9%) and with the respective protein of *Angulomicrobium tetraedrale* DSM 5895^T (95.3 and 90.8%).

Amplification of the structural genes of DCM dehalogenases of the strains DM15, 16, and 17 from genomic DNA was performed with the primers DMfor and DMrev developed for the cloning of A-type dehalogenases [10]. In all cases, it was possible to obtain the corresponding PCR products, sequencing of which yielded the complete nucleotide sequences of *dcmA*. It was found that the *dcmA* gene of strain DM16 has the highest similarity to the gene of DCM dehalogenase of *Methylobacterium dichloromethanicum* DM4 (=DSM 6343^T). The deduced DcmA amino acid sequence of the degrader DM16, consisting of 288 amino acids, contained three amino acid substitutions compared to the ortholog protein of strain DM4, and had not been found previously [10, 14]. Therefore, we are planning to characterize this enzyme in more detail. It was also found that the DCM dehalogenase of strain DM15 is identical at the nucleotide and amino acid levels to the protein of *Methylorhabdus multivorans* DM13 (ATCC 51890^T) and that the dehalogenase of strain DM17 is identical to the corresponding protein of *M. dichloromethanicum* DM4. As a result of phylogenetic analysis, the complete amino acid DcmA sequences of strains DM15, 16, and 17 were positioned into a cluster formed by A-type DCM dehalogenases from *M. dichloromethanicum* DM4, *Methylopila helvetica* DM1, *Hyphomicrobium denitrificans* DSM 1869, and *Methylorhabdus multivorans* DM13, with the level of homology between them of 97.6–100% (Fig. 3). On the contrary, a low level of homology (58.4%) was shown between the DcmA amino acid sequences of the new strains and the most divergent DCM dehalogenase of the B type from *Methylophilus leisingeri* DM11 (=DSM 6813^T), which suggests the involvement of other mechanisms in DcmB genesis.

Taking into account the substantial similarity of the *dcmA* genes in most of aerobic DCM degraders, it was logical to suppose similar organization of the clusters of CH_2Cl_2 degradation genes in these methylotrophs. Therefore, DNA sequences flanking *dcmA* in the new degraders were amplified and sequenced using specific primers designed on the basis of the known sequence of *M. dichloromethanicum* DM4. Two pairs of primers were designed, J1f-J1r and J2f-J2r (Table 2). Using these primers, amplicons of appropriate length were obtained and sequenced. It was ascertained that the new degraders have a similar organization of this DNA region (Fig. 4). The *dcmR* gene found upstream of *dcmA* (5') is transcribed in the opposite direction and is identical to the corresponding regulatory gene of the strain DM4. A 20-h lag period in DCM degradation, judged by the absence of HCl from the culture liquid, was observed in all of the strains under study; this confirms the presence of the *dcmR* gene in them. The open reading frame *orf353* found downstream *dcmA* (3') encodes a hypothetical protein (40 kDa) that apparently does not participate in DCM degradation [4]. However, the presence of an ortholog of *orf353* downstream of *dcmA* in the studied degraders evidences the evolutionary conservatism of this feature. The size of the sequenced DNA fragments, including *dcmA*, was 3.5 kb. Nucleotide substitutions were found only in protein-coding DNA regions, while the intergenic and regulatory regions were entirely coincident with the homologous nucleotide sequence of strain DM4.

For the detection of the regulatory gene of DCM dehalogenase, *dcmR*, we designed, based on the corresponding nucleotide sequences of the strains *M. dichloromethanicum* DM4, *"Gottschalkia methyl-*

ica" DM15, *"Ancylobacter dichloromethanicus"* DM16, and *Methylobacterium extorquens* DM17, primers dcmRfor and dcmRrev (Table 2) for the amplification of an internal fragment of this gene (423 bp). In this case, the expected PCR product was obtained for each of the three strains: DM4 (control), DM15, and DM16. These primers can be used for detection of the regulatory gene of DCM-dehalogenase, *dcmR*, in DCM degraders.

It was established that the stable ability to grow on DCM in the degraders under study correlates with the duration of the selective pressure of the pollutant in the sites of isolation. Strain DM16, isolated from the territory of a large-scale plant for halomethanes production, retained the ability to grow on DCM after three years of cultivation in the absence of CH_2Cl_2, as well as after lyophilization. This suggests localization of the *dcmA* and *dcmR* genes on the chromosome or on a megaplasmid and the presence of more than one copy of these genes. A plasmid of a size of >100 kb has been found in the strain DM16; its role in DCM degradation is to be elucidated.

The degrader strain DM15, obtained by means of two-year artificial treatment of soil with CH_2Cl_2, lost its ability to grow on DCM after lyophilization. The quick adaptation of the microbial community to CH_2Cl_2 pollution, observed in the course of the isolation of this strain, and the similarity of DCM degradation genes in aerobic degraders of different taxonomic positions and geographical origins suggest lateral transfer of these genes.

It is believed that the contribution of natural sources (volcanic gases, excretions of marine algae) to CH_2Cl_2 emission is extremely low [14, 15]. Consequently, in the absence of anthropogenic pollution with dihalomethanes, the putative precursors of DCM-dehalogenase could participate in degradation of other, structurally close substrates of natural origin: CH_2Br_2 or CH_3Cl [5, 16, 17]. This hypothesis is favored by the high activity of DCM dehalogenases of strains DM13 and GJ21 with dibromomethane (in spite of the extreme toxicity of the catabolic products of this compound) and by the high similarity of the gene clusters responsible for the degradation of chloro- and bromoalkanes (*dhaA, dhaAf*) [18, 19]. It is logical to anticipate that comparative genomics and proteomics will provide an answer to the intriguing questions concerning the origin and evolution of DCM dehalogenases.

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