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EXPERIMENTAL ARTICLES

Isolation and Investigation of Anaerobic Microorganisms Involved in Methanol Transformation in an Underground Gas Storage Facility

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Abstract—High methanol and acetate concentrations (up to 12 and 14 g l^{-1}, respectively) were found in water samples collected at different objects of the North Stavropol underground gas storage facility (UGSF), and significant seasonal variations in the content of these compounds were revealed. The dominant anaerobic microorganisms isolated from these samples during the study belonged to acetogens, methanogens, and sulfate reducers. The results of 16S rRNA gene sequencing and analysis of the physiological properties showed that the isolates were close to the species of *Eubacterium limosum, Sporomusa sphaeroides, Methanosarcina barkeri, Methanobacterium formicicum*, and *Desulfovibrio desulfuricans*. The isolated organisms, except for *Methanobacterium formicicum*, were capable of methylotrophic growth. All strains were characterized by resistance to high methanol concentrations (up to 40–50 g l^{-1}). Their other energy substrate was hydrogen. The combination of the growth characteristics of these strains (pH, temperature, and salinity ranges) was shown to correspond to the ecological situation observed in the UGSF. The results of investigation of the isolated strains suggest that organic acids (acetate, butyrate) found in high concentrations in the initial samples are metabolic products of the revealed acetogens. Based on the established biological peculiarities of the isolated strains of methanogens, acetogens, and sulfate-reducing bacteria, these microorganisms may be considered as the main agents of anaerobic transformation of methanol and some other organic and inorganic compounds in UGSFs.

Keywords: underground gas storage facility, methanol, acetogens, methanogens.

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Several microbiological investigations of underground gas storage facilities (UGSFs) have shown a large scale of biological processes and the necessity for deeper investigation of these giant anthropogenic ecosystems [1–3]. Exploitation of UGSFs, which are usually depleted gas deposits, is characterized by a number of peculiar features associated with periodic injection and extraction of the large volumes of gas of billions of cubic meters per year. Water, hydrogen, CO_2, and other substances may enter the stratum together with hydrocarbons in the course of gas injection. Among these substances, one of particular interest is methanol, which is extensively used in gas extraction to prevent the formation of gas hydrate plugs. The movement of the gas front is accompanied by the transfer of water with dissolved substances [4]. It is quite possible that microorganisms are transferred analogously.

The great influence of UGSFs on the strata and the presence of an ample quantity of nutrient substrates create conditions for active development of diverse microflora. Investigation of the water samples taken from the North Stavropol UGSF revealed high rates of sulfate reduction and methanogenesis in the presence of high quantities of acetogenic sulfate-reducing bacteria (SRBs) and iron-reducing bacteria (IRBs), as well as methanogenic archaea [1, 2]. In terms of diversity and quantity of anaerobes, the microflora of the North Stavropol UGSF and its technological features is comparable with the microflora of oil fields flooded with fresh water [5–7]. However, such a high rate of bacterial methanogenesis (2.8 ml CH_4 l^{-1} day^{-1}) at the UGSF under study has not been previously reported for underground systems. A sulfate reduction rate of about 0.5 mg S^{2-} l^{-1} day^{-1} is more typical of the well bottom zones of oil field injection wells upon additional activation of microbiological processes [8].

The unique nature of the North Stavropol UGSF as a subject of research is determined first of all by the high content of methanol and acetate in the stratum water (over 10 g l^{-1}) versus the low content of mineral components (general mineralization of 0.15–2.8 g l^{-1} with the average value of 0.59 g l^{-1}). We do not know any other natural objects with such a ratio of organic and inorganic compounds in stratum waters. Moreover, the carbon dioxide and hydrogen contained in the main gas are also the major substrates for acetogens, methanogens, and sulfate reducers. Thus, based on the set of potential substrates and products, these

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Table 1. Chemical composition of the water taken at different UGSF objects during the period of study (mg l^{-1})

	pH	SO_4^{2-}	HCO_3^-	NO_3^-	NH_4^+	Fe^{2+}	Acetate	Methanol
Inspection wells								
Average	7.63	31	1092	23	25	239	68	0
Minimum–maximum	6.30–9.30	1–130	156–2651	0–52	8–52	10–2000	3–390	0
Period of gas injection								
Average	5.84	236	13	19	57	3310	4070	**
Minimum–maximum	5.20–6.50	10–773	5–27	0–78	16–78	50–10000	220–14000	0–3000
Period of extraction								
Average	6.65	18	0	8	6	187	484	5940
Minimum–maximum	6.25–6.98	2–100	21–339*	0–49	1–12	50–500	125–1250	600–12000

Notes: * Detected in 2 samples out of 16.
** Detected in 2 samples (1000 and 3000 mg l^{-1}).

groups of microorganisms should be expected to hold a particular position in the UGSF ecosystem.

The main goals of the study were to isolate representatives of the dominant groups of anaerobic microorganisms from the objects of the North Stavropol UGSF, to study their biological peculiarities, and to determine their taxonomic positions.

MATERIALS AND METHODS

Subject of research. The North Stavropol underground gas storage facility (the largest one in the world) was created on the basis of the North Stavropol gas field. From late April to the middle of October, Urengoi gas is pumped through development wells in the central part of the field into reservoir beds (the Khadum horizon and the green suite horizon); from late October to the middle of April, the gas is extracted through the same wells for operating needs. In addition to methane, the makeup gas contains ethane (2.2%), propane (0.42%) and other hydrocarbons, nitrogen (1.78%), CO_2 (0.61%), and hydrogen (0.5%). No hydrogen was found in the gas taken from the UGSF. Reservoir beds are represented mainly by siltstones, aleurites, and clay variations. Glauconite is widespread among the minerals composing the rocks. The average depth of occurrence of reservoir beds is 800–1000 m, and the temperature within the beds varies from 30 to 50°C, depending on the mode of functioning. The initial composition of stratum water was determined in the water samples taken from inspection wells located along the periphery of the storage facility. This stratum water is characterized by low mineralization (1.4–24.2 g l^{-1}, or 8.5 g l^{-1} on average), high content of carbonates (up to 2651 mg l^{-1}, or 1092 mg l^{-1} on average), and pH ~7.6 (Table 1). The water taken from the gas preparation system was fresher (0.15–2.80 g l^{-1} by mineral components) but had a high content of organic compounds, especially methanol and acetate. The maximum methanol concentrations (0.6–12 g l^{-1}) were observed during gas extraction due to its application in this period as an inhibitor of formation of gas hydrate plugs. During the period of gas injection, methanol was found in single samples only (up to 3.0 g l^{-1}). Acetate was characterized by the opposite dynamics. The maximum concentrations were registered during gas injection (up to 14 g l^{-1}, or 4.07 g l^{-1} on average), while in the period of gas extraction its content dropped by an order of magnitude (to 1.25 g l^{-1}, or 0.484 g l^{-1} on average). In some samples, propionate (up to 0.6 g l^{-1}), butyrate (up to 1.12 g l^{-1}), and isobutyrate (up to 0.5 g l^{-1}) were found. Samples with high acetate concentrations showed very high concentrations of iron ions (up to 10 g l^{-1}) and nearly complete absence of carbonates. In the inspection wells, the acetate concentration was 68 mg l^{-1} on average.

Cultivation methods. The base medium used for obtaining enrichment cultures and isolating anaerobic microorganisms contained the following (g l^{-1}): NaCl, 2.0; KCl, 0.25; $MgCl_2 \times 6H_2O$, 0.2; $CaCl_2 \times 2H_2O$, 0.1; NH_4Cl, 0.3; K_2HPO_4, 0.025; MES, 0.5; MOPS, 1.5; TRIS, 0.6; yeast extract (Difco), 1.0; Balch's trace element solution [9], 10 ml; resazurin solution (0.2%), 1 ml; and vitamin solution [9], 5 ml. $NaHCO_3$ solution (to the final concentration of 10–25 mM) and cysteine and sulfide solution (to the final concentration of 0.5–2.0 mM) were added after sterilization (final pH 7.0).

The microbial community was obtained by inoculation of water samples taken from UGSF objects into the above medium with addition of methanol (60 mM) with the $CO_2 + H_2$ mixture (1:4) replacing nitrogen in the gas phase at an excess pressure of 1.5 atm. The cultures were incubated at 37°C.

Activity of the microbial community was determined by the increment of methane in the gas phase and accumulation of acetate in the medium. Stability of methanol conversion in the community was maintained during repeated transfers of the culture into fresh medium. After five transfers, the microbial culture was centrifuged for 10 min at 5000 *g* and the biomass was used to isolate total DNA.

Variants of the medium containing methanol (60 mM) and acetate (4 mM) under the nitrogen atmosphere were used for isolation and cultivation of methylotrophic methanogens. The $CO_2 + H_2$ mixture was used as a gas phase with acetate (4 mM) added to the medium for isolation and cultivation of hydrogenotrophic methanogens. Development of acetogenic and sulfate-reducing bacteria was suppressed by introduction of antibiotics: ampicillin (1 mg ml^{-1}) and vancomycin (100 μg ml^{-1}).

Acetogenic bacteria were isolated on the media containing methanol (2 g l^{-1}) or $CO_2 + H_2$ in the gas phase in the presence of bromoethane sulfonic acid (10 mg l^{-1}). Pure cultures of acetogenic and methanogenic microorganisms were obtained from the colonies cloned by reinoculation from the terminal dilutions onto agarized medium of the above composition (roll tube method) in $CO_2 + H_2$ atmosphere.

Enrichment cultures of sulfate-reducing bacteria were obtained in the mineral medium [10] with lactate (25 mM). Pure SRB cultures were obtained using hydrogen and CO_2 as the main substrates, similarly to isolation of other strains, in the base medium with sulfate (10 mM).

CH_4, CO_2, and H_2 concentrations were determined by gas chromatography on a Crystal 5000 chromatograph (Chromatec, Russia) using CaA 0.2-0.4 and Hayexp N 80-100 packed columns (2 m ± 3 mm) with argon as a carrier gas and a katharometer or a flame ionization detector. Methanol and volatile fatty acids were detected on a Crystal 5000.2 chromatograph (Chromatec, Russia) with a Sovpol B metal packed column (1 m ± 2 mm) with argon as a carrier gas and a flame ionization detector. The concentrations of sulfides, sulfates, and iron ions were determined using test kits (Merck, Germany) in the photometric variant.

Radioisotopic studies were carried out with ^{14}C-acetate labeled at the methyl group (specific activity of 1.6 GBq mol^{-1}), which was added as 0.2 MBq per test tube with 5 ml of the medium. The final content of acetate was 1 mM; the $H_2 + CO_2$ mixture, methanol (60 mM), or methanol with H_2 was introduced as the main substrate. Inclusion of acetate into the biomass was assessed as follows: after 7 days of incubation at 37°C, a 0.5-ml aliquot of the culture liquid was filtered through nitrocellulose membrane filters with a pore diameter of 0.22 μm. The biomass on the filters was washed twice with isotonic saline solution, dried out, and placed into vials with SL-106 scintillation liquid. The labeled methane in the gas phase was detected according to [11].

Determination of microbial community composition. The composition of the microbial community was determined using total DNA isolated from enrichment culture samples. DNA was isolated by the modified method of phenol–chloroform extraction [12]. The 16S rRNA gene fragments were amplified using a Tertzik multichannel DNA amplifier (DNK-Tekhnologiya, Russia) and two systems of primers: U515F 5'-GTGBCFGCVGCCGCGCTAA and Arch915R 5'-GTGCTCCCCCGCCAATTCCT for archaea and U515F and Bact907R 5'-CCGTCAATTCMTTT-GAGTTT for bacteria.

Amplification was carried out in 20 μl of a mixture containing normal-strength reaction buffer (Evrogen, Russia), 200 μM of each deoxyribonucleotide triphosphate, 0.5 μM of each primer, 1 unit of *Taq* DNA polymerase (Evrogen, Russia), and 1 μl (1–10 ng) of template DNA. Denaturing, annealing, and elongation were carried out at 95, 60 (50 for bacteria), and 72°C, respectively. Phase duration was adjusted experimentally. PCR products were visualized in 1% agarose gel using the standard protocol and the GeneRuler™ 1Kb DNA ladder (Fermentas, United States). The products were analyzed by denaturing gradient gel electrophoresis (DGGE). For this purpose, the obtained amplificates were reamplified with the same primers, using as a universal primer the same oligonucleotide but with a GC-enriched tail (GC-clamp 5'-CGCCCGCCGCGCCCCGCGCCCGTCCCG-CCGCCCCCGCCCGGTGBCAGCMGCCGCG-GTAA), which provided better separation of PCR products in the gradient electrophoresis. PCR products were separated under a 30–70% concentration gradient of denaturing agents (formamide and urea) using a SCIE-PLAS device (Yorkshire, United Kingdom) at 70 V and 60°C for 18 h. After electrophoresis, the gel was stained with SYBR^R Gold (Molecular Probes, Leiden, Netherlands) for 40 min in the dark and then visualized in a transilluminator. The bands were excised, placed into test tubes containing 20 μl of distilled water, and stored in a refrigerator overnight for DNA elution from the gel. The DNA was used for PCR with the above primer systems (without GC-clamp); the products were purified using Wizard^R SV Gel and PCR Clean-Up System (Promega, United States) and sequenced using a Big Dye Terminator v. 3.1 in an ABI 3730 automated sequencer (Applied Biosystems, Inc., United States) according to the manufacturer's instructions. The sequencing was carried out at the Bioengineering Center, Russian Academy of Sciences. The obtained nucleotide sequences were compared with sequences from the GenBank database using the BLAST software package (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were aligned using the online server Mutalin (http://multalin.tousouse.inra.fr/multalin/). The phylogenetic tree

Table 2. Chemical composition of the water samples used for isolation of pure cultures of microorganisms

Sample	pH	Mineralization, mg l^{-1}	SO_4^{2-} mg l^{-1}	HCO_3^- mg l^{-1}	NH_4^+ mg l^{-1}	Fe^{2+} mg l^{-1}	Acetate mg l^{-1}	Methanol mg l^{-1}	Sulfate reduction, ng S^{2-} l^{-1} day^{-1}	Methanogenesis, nl CH_4 l^{-1} day^{-1}
12 (Gas injection)	6.50	13400	83.0	27.0	50	10000	3300*	1000	489460	2847700
8 (Extraction)	6.83	455	18	0.0	7.5	80	225	12000	6620	109
3 (Extraction)	6.87	3442	3	339	12.0	50	300	600	66700	17470

* In addition to acetate, propionate (100 mg l^{-1}) and butyrate (1200 mg l^{-1}).

was constructed with the TREECONW software package using the neighbor-joining algorithm and bootstrap analysis.

Identification of pure cultures. The cultures were identified using polyphase taxonomic approaches. Generic affiliation of the microorganisms under study was established by determining partial 16S rRNA gene sequences (1000–1300 nucleotides) and comparing them with the data available in the database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The fragments of the genes encoding 16S rRNA were amplified and sequenced using universal primers: 11F 5'-GTTTGATCMTGGCTCAG-3'; 1492R 5'-TACGGYTACCTTGTTACGACTT. The sequencing was performed at the Bioengineering Center. Nucleotide sequences of the 16S rRNA genes were analyzed using the GenBank database and software (http://www.ncbi.nlm.nih.gov). The obtained sequences were deposited in GenBank under the following numbers: HM 590802, HQ 591417, HQ 591418, HQ 591419, and HQ 591420.

RESULTS AND DISCUSSION

The main gas contains up to 0.6% (vol/vol) CO_2 and up to 0.5% (vol/vol) H_2. Taking into account the volumes of injected gas, the inflow into the reservoir bed of considerable amounts of carbon dioxide and hydrogen, which are good metabolic substrates for various microorganisms, is apparent. Comparison of the number of parameters demonstrates a significant effect of microbiological processes on the chemical composition of water (Table 1). For example, the average bicarbonate concentration in most of the samples taken at the stage of gas injection was 13 mg l^{-1}, while no bicarbonate was found in 90% of the samples taken at the stage of extraction. At the same time, in the samples taken from inspection wells (outside the zone of influence of the gas bubble), its average content was 1092 mg l^{-1}.

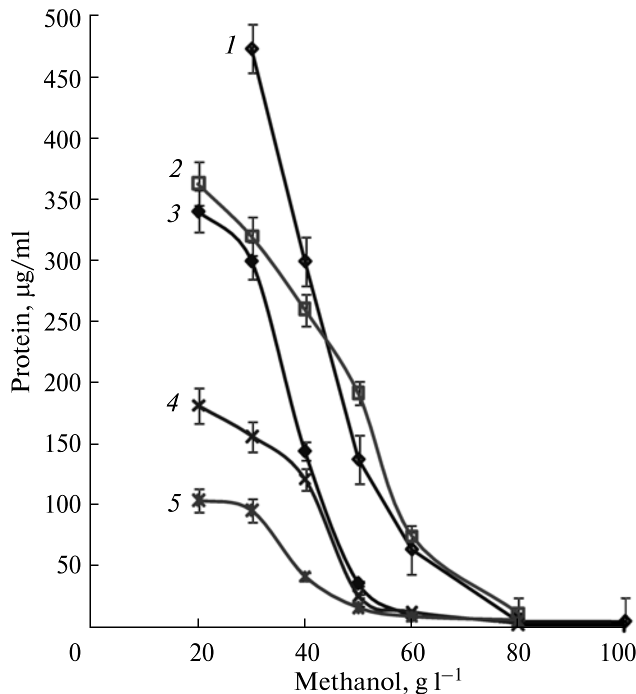
The considerable increase in the concentration of organic acids in the samples taken during the period of gas injection and the respective decrease in their pH values are probably associated with the high activity of acetogens (Table 1). The same samples were also shown to contain high concentrations of Fe^{2+} indicating certain involvement of acetogens in pipeline corrosion. It is apparently associated both with cathode depolarization as a result of bacterial uptake of hydrogen from the metal surface and with the formation of acetate complexes of iron. A significant role of acetogens in corrosion of gas-main pipelines was also mentioned by other authors [13], who have shown, by means of the methods of molecular biology, the presence of a considerable portion of acetogens in the microbial community developing in the gas pipeline condensate water.

At the North Stavropol UGSF, acetogens were found in more than 80% of the analyzed samples and their quantity reached 10^4 cells ml^{-1}. Methylotrophic acetogens were found in 65% of the samples taken during the period of gas injection.

The following strains were isolated from sample no. 12 (Table 2) taken in the end of gas injection: an acetogenic strain AG12, a strain of sulfate-reducing bacterium SR12, and a methanogenic strain MG134. The water of this sample was characterized by relatively low pH values (6.65), the presence of methanol (1.0 g l^{-1}) and organic acids (acetate, 3.3 g l^{-1}; propionate, 0.1 g l^{-1}; and butyrate, 1.2 g l^{-1}), and the high content of Fe^{2+} (10 g l^{-1}, Table 2). The microflora contained high quantities of acetogens (over 10^4 cells ml^{-1}) and SRBs and IRBs (10^6 cells ml^{-1}). The quantity of methanogens was estimated as 10_2 cells ml^{-1}; however, the rate of methanogenesis was extremely high (2.85 ml CH_4 l^{-1} day^{-1}). In addition, a very high rate of sulfate reduction was registered (489.4 μg S_2– l^{-1}day^{-1}; Table 2).

The above sample, from our point of view, was rather representative and adequately reflected microbial processes typical both of UGSF productive beds and of condensate waters inside the gas main.

Analysis of the total quantity of microorganisms in all samples at the stage of gas extraction showed a great number of large curved cells [2]. Banana-shaped cells were found in more than 80% of enrichment cultures of acetogens, including the sample from settler no. 8. Sample no. 8 was shown to have a very high content of methanol (12 g l^{-1}) and a moderate content of acetate (225 mg l^{-1}, Table 1) in the absence of bicarbonate. The quantity of acetogens (10_2 cells ml^{-1}) was higher



Inhibition of the growth of isolated strains by high methanol concentrations (incubation for 20 days). *Eubacterium limosum* AG12, growth on methanol (*1*); *Desulfovibrio desulfuricans* SR12, growth on lactate (*2*); *Sporomusa sphaeroides* AG8-2, growth on methanol (*3*); *Methanobacterium formicicum* MG134, growth on hydrogen (*4*); and *Methanosarcina barkeri* MGZ3, growth on methanol (*5*).

than that of methanogens and sulfate reducers (1–10 cells ml^{-1}).

In the acetogenic enrichment culture obtained from this sample, the major components revealed by DGGE were representatives of the genus *Sporomusa*; members of the genera *Clostridium, Bacillus, Peptococcus*, and *Syntrophomonas* were present in minor quantities. Subsequently, strain AG8-2 of the genus *Sporomusa* was isolated from this enrichment culture.

The UGSF water samples taken at the stage of gas extraction commonly contained *Sarcina*-like cells. Sarcina microcolonies were found also by microscopic examination of a number of enrichment cultures of methanogens, including sample no. 3. The presence of a methanogen from the genus *Methanosarcina* was confirmed by DGGE of 16S rRNA fragments. The methanosarcina strain MGZ3 was subsequently isolated from this sample. This water sample was one of two samples in which bicarbonate was found during this period (Table 2). The contents of methanol and acetate in this sample were 600 and 300 mg l^{-1}, respectively (Table 2). The sample was characterized by a high quantity of acetogens (10^4 cells ml^{-1}); IRBs and SRBs were present as well (10^2 cells ml^{-1}). The high rate of methanogenesis (17.470 nl CH_4 l^{-1} day^{-1}) at a low quantity of methanogens (single cells) was probably due to the growth of methanosarcinas as microcolonies.

Two acetogenic cultures, two methanogenic cultures, and one culture of a sulfate reducer were isolated from the obtained enrichment cultures.

Acetogenic strain AG12 was identified by the results of 16S RNA analysis and physiological characteristics as a strain close to *Eubacterium limosum* and represented by gram-positive, spore-forming, pleomorphic nonmotile rods. During growth on methanol and CO_2, or on hydrogen and CO_2, butyrate and acetate were major and minor products, respectively. Caproate was also formed in minor quantities.

Strain AG12 fermented glucose, fructose, lactate, and valine. Growth on glucose was accompanied by formation of acetate (1 g l^{-1}), butyrate (180 mg l^{-1}), and acetone (10 mg l^{-1}). No growth occurred on formate and lactose, while weak growth occurred on glycerol.

The studies of the effect of methanol content in the medium on culture growth showed that the strain *E. limosum* AG12 was sufficiently adapted to high methanol concentrations typical of the UGR under study. The methanol concentration optimal for its development was about 2 g l^{-1}, but growth was observed at up to 60 g l^{-1} of methanol l^{-1} (Fig. 1). Abundant spore formation was observed at methanol concentrations of 40–50 g l^{-1}, while no spore formation was observed under the optimal conditions. As regards other physiological characteristics (pH 7.0–7.5 optimal for growth, growth within pH 6.0–8.5, 37°C optimal for growth, growth at up to 42°C), no significant differences of the isolated strain from those described previously were revealed [14].

The acetogenic strain AG8-2 has gram-negative spore-forming banana-shaped cells. It was isolated and purified using penicillin antibiotics, because pasteurization (10 min at 80°C) did not remove the concomitant gram-positive clostridia forming heat-resistant spores. During isolation of a pure culture, the minimal amount of sulfide (0.5 μM) was used to obtain reduced media [15]. Apart from hydrogen and methanol, the strain also grew on formate, glycerol, and ethylene glycol, but not on fructose, lactose, glucose, succinate, or fumarate. Based on the analysis of the morphological and physiological properties and the data of 16S rRNA analysis, the isolated strain was classified as *Sporomusa sphaeroides* (DSM 2875) [15, 16]. The isolated strain *Sm. sphaeroides* AG8-2 was capable of intense growth at a methanol concentration of 30–35 g l^{-1} (figure). These methanol concentrations are extremely high for *Sporomusa*: for members of this genus, methanol concentrations not exceeding 160 mg l^{-1} are recommended [15]. Other characteristics of the isolated strain (temperature optimum of 35–39°C (growth at up to 43°C), pH optimum 7.1 (growth at pH 5.8–8.4), low NaCl requirement) were not different from those described in the literature.

Table 3. Inclusion of ^{14}C-acetate labeled at the methyl group into the biomass and methane by the cultures of *Methanosarcina* MG-Z3 and *Methanohalophilus euhalobius* when using various substrates

Experimental variant	Substrate	% CH_4 from acetate	% ^{14}C in biomass of the total ^{14}C in biomass and in CH_4	% ^{14}C of the total ^{14}C in biomass and in CH_4
1. *Methanosarcina* MGZ3	H_2/CO_2	2.24 ± 0.11	39.7 ± 2.0	40.3 ± 2.0
2. *Methanosarcina* MGZ3	Methanol + H_2	1.91 ± 0.10	89.8 ± 4.5	10.1 ± 0.5
3. *Methanosarcina* MGZ3	Methanol	0.12 ± 0.010	86.8 ± 6.5	13.1 ± 1.0
4. *Methanohalophilus euhalobius*	Methanol	0.0005 ± 0.00005	95.6 ± 7.2	6.44 ± 0.48

The methanogenic strain MG134 was similar in its physiological and biochemical characteristics to *Methanobacterium formicicum*, as was also confirmed by the 16S rRNA analysis (strains DSMZ1535 and OM15). The strain formed chains of small cells and grew on hydrogen in the presence of CO_2 and on formate. Methanol was not a growth substrate, but did not inhibit the growth at concentrations of up to 50 g l^{-1} (figure). Such resistance to methanol has not been shown previously for this species. Acetate (0.3–0.5 g l^{-1}) was necessary for growth, probably as a carbon source. Optimal growth conditions were observed at pH 6.8–7.6 and within the temperature range of 37–42°C.

Investigation of the physiological properties and the complete 16S RNA analysis of the methanosarcina strain MGZ3 made it possible to identify it as close to *Methanosarcina barkeri* strain Sar. Similarly to the strain Sar, the isolated strain *Ms. barkeri* MGZ3 had a slightly narrower growth range in the intervals of physicochemical factors under study (temperature, pH values, and salinity) compared to the type strain MS and the closely related strain 227 [17–19]. The maximum growth rate was observed at 39°C; growth was insignificant at 42°C and absent at 45°C. The pH optimum was within 6.5–7.1, with practically no growth at pH 6.0 and 7.6. The salinity optimum was 2.0–4.0 g l^{-1} NaCl; the growth rate slowed down with formation of sparse large colonies in the medium at 10 g l^{-1} and slowed down dramatically at 12 g l^{-1}. Methanol concentration of 10 g l^{-1} had no significant effect on the growth rate. The culture growth rate was half of the control (2 g l^{-1}) at 20 g l^{-1} methanol, and growth was insignificant at a methanol concentration of 40 g l^{-1}. According to the literature data, the toxic effect for the strain MS was noted at a methanol concentration of 4.8 g l^{-1} [20]. The strain *M. barkeri* MGZ3 isolated from UGSF samples grew on methanol or hydrogen ($H_2 + CO_2$) with formation of microcolonies. A significant difference between this strain and the isolates described previously was its inability to grow on acetate.

The isolated strain of a sulfate-reducing microorganism SR12, according to the results of 16S RNA analysis, was close to *Desulfovibrio desulfuricans.* The isolate was represented by gram-negative, non-spore-forming, motile, helically curved rods. The studies showed that the strain SR12 grew well on hydrogen in the presence of sulfate and bicarbonate, which was used for its isolation. The growth on methanol was slow; no growth was observed on acetate and butyrate. When grown on lactate, the culture was not inhibited by addition of methanol at the concentration of up to 7 g l^{-1} usual for the UGSF. Considerable inhibition was observed only at a methanol concentration of 50–80 g l^{-1} (figure). Similar results were obtained in experiments with hydrogen as an electron donor. The optimal growth conditions were observed at pH 7.0 (growth range 5.5–8.5) and 37°C (growth at up to 43°C).

The ability to grow at high methanol concentrations is untypical of the known strains *Sm. sphaeroides* and *Ms. barkeri* and has not been described previously for the strains of *E. limosum, Dv. desulfuricans*, and *Mb. formicicum.* The relationship between physiological properties of the isolated strains and the conditions of their habitat (the presence of methanol, temperature, etc.) demonstrates that their presence in the UGSF is not accidental; these microorganisms are important elements of a stable technogenic ecosystem.

The study of samples taken from the UGSF revealed no acetoclastic methanogens but showed high rates of methanogenesis on this substrate [1, 2]. Methanogenesis on acetate can, however, be performed syntrophically by other bacteria, which are suppressed on selective media. The existence of such associations with sulfate-reducing bacteria was suggested for an oil field [7]. Besides, acetate can be utilized by methanogens as a cosubstrate when using hydrogen or methanol.

Inclusion of carbon from the methyl group of acetate into methane and biomass was studied using $^{14}CH_3COO^-$ in order to estimate the possibility of participation of acetate in methanogenesis by the isolated strain *Ms. barkeri* MGZ3, which does not grow on

acetate. The maximal inclusion of acetate into methane was observed in the experimental variant with hydrogen and carbon dioxide as the main substrate. In this case, the contribution of acetate to methanogenesis was 2.24%. During this process, acetate was distributed as follows: about 40% in methane and 60% in biomass (Table 3). In the variants with other main substrates, the major portion of acetate was incorporated in the biomass (Table 3). Thus, strain MGZ3 in the presence of hydrogen and CO_2 or in the presence of methanol utilizes acetate not only as a carbon source, but also as a substrate for methanogenesis. For comparison, an experiment was performed with the methylotrophic methanogen *Methanohalophilus euhalobius*, which does not require acetate as a carbon source and cannot grow on $H_2 + CO_2$. In this case, the inclusion of acetate carbon into methane was practically absent (Table 3).

Thus, in the course of study, the dominant strains of methanogenic, sulfate-reducing, and acetogenic microorganisms were isolated from different objects of the North Stavropol UGSF. The isolated strains proved to be close to the species *Eubacterium limosum, Sporomusa sphaeroides, Methanosarcina barkeri, Methanobacterium formicicum*, and *Desulfovibrio desulfuricans*, but, in contrast to the known strains, could develop at high methanol concentrations (20 g l^{-1} and more). The study of the physiological properties of the isolates showed that all strains were well adapted to the habitat conditions, which indicated their active participation in transformation of methanol and other organic and inorganic compounds in the depths of the UGSF.

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