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EXPERIMENTAL ARTICLES

Investigation of the Trophic Relations between Anaerobic Microorganisms from an Underground Gas Repository during Methanol Utilization

A. L. Tarasov[1], I. A. Borzenkov, and S. S. Belyayev

Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, Moscow, 117312 Russia

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Abstract—Joint cultivation of the dominant strains of acetogenic, sulfate-reducing and methanogenic microorganisms isolated from water samples of the North Stavropol underground gas storage facility (UGSF) was carried out for revealing their probable trophic relationships. It was shown that acetogenic strains *Eubacterium limosum* AG12 and *Sporomusa sphaeroides* AG8-2 growing on methanol could form a considerable pool of hydrogen, which may support development of hydrogenotrophic cultures, the methanogen *Methanobacterium formicicum* MG134, or the sulfate reducer *Desulfovibrio desulfuricans* SR12. Growth of this sulfate-reducing strain was not stimulated under joint cultivation with *Methanosarcina barkeri* MGZ3 on methanol, probably due to its inability to take up low hydrogen concentrations observed during methanosarcina development. The results show that acetogens in the UGSF system are the most important consumers of methanol and hydrogen and after exhaustion of the latter and switching over to methanol utilization they can supply hydrogen to other microorganisms, including methanogens and sulfate reducers. The role of methanosarcina in the UGSF increases as the hydrogen and CO_2 reserves are exhausted, and methanogenesis on methanol becomes the main way of its destruction.

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

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Periodic flooding and extraction of natural gas at the North Stavropol underground gas storage facility (UGSF) has been shown to be accompanied by intense development of a diverse anaerobic microflora in the aqueous phase, including iron-reducing, sulfate-reducing, methanogenic, and acetogenic microorganisms [1, 2]. The intensity of microbial processes is confirmed by exceptionally high rates of sulfate reduction and methanogenesis, as well as formation of high amounts of acetate (up to 14 g/l) in the water. The changes in the chemical composition of the water samples taken at the stages of gas flooding and extraction and the quantity of different groups of microorganisms demonstrate that hydrogen, methanol, and carbon dioxide are the main metabolic substrates. The content of hydrogen in the makeup gas was up to 0.6%. Hydrogen was not found in the gas extracted from the UGSF, probably due to its uptake by the microbial community functioning in the depths of the UGSF. A decrease in the content of CO_2 and its derivatives in the UGSF system was also observed. Bicarbonate was not found in most of the water samples analyzed at the end of the period of gas extraction.

During the period of gas extraction, methanol is the only renewable substrate and its concentration in the stratum waters may reach 10 g/l, while the concentrations of other substrates gradually decrease as they are utilized by microorganisms. However, methanol metabolism in acetogens is known to be accompanied by the synthesis of a minor amount of hydrogen [3], which may be captured by its other potential consumers.

The dominant anaerobic organisms isolated from the UGSF samples in the course of previous studies [4] proved to be related to the species *Eubacterium limosum, Sporomusa sphaeroides, Methanobacterium formicicum, Desulfovibrio desulfuricans*, and *Methanosarcina barkeri* and were mostly methylotrophic. The special feature of the isolated strains is their ability to develop under enhanced methanol concentrations typical of the North Stavropol UGSF. The combination of high concentrations of methanol, acetate, and iron with very low (for stratum waters) concentrations of other salts makes it a unique object.

The goal of this work was to study the trophic relations between microbial strains isolated from the UGSF during their joint cultivation in the media with high methanol concentrations.

[1] Corresponding author; e-mail: tarasov@yandex.ru

Table 1. Consumption of the substrates and formation of the products by the culture of acetogenic *E. limosum* AG12 and the binary culture of the acetogen with methanogenic *Mb. formicicum* MG134.

Cultures	Methanol, μmol-C	Acetate, μmol-C	Butyrate, μmol-C	Hydrogen (maximum), μmol	CH_4, μmol-C
E. limosum AG12	−585.9 ± 29.3	−126.7 ± 6.34	711.1 ± 35.55	5.18 ± 0.26	0.0
E. limosum AG12 + *Mb. formicicum* MG134	−456.0 ± 22.8	−60.00 ± 3.00	488.9 ± 24.45	0.51 ± 0.03	66.8 ± 3.34

The vial contained 20 ml of the medium (7500 μmol methanol, 100 μmol acetate, and 100 μmol bicarbonate). Negative values correspond to substrate consumption

MATERIALS AND METHODS

Cultivation methods. The inoculum and binary cultures were cultivated on the previously described medium [4] supplemented after sterilization with: $NaHCO_3$, up to a final concentration of 20 mM; cysteine and sulfide, up to 0.5 mM; acetate, up to 2–3 mM; and final pH 7.0.

Monocultures of acetogenic strains *Eubacterium limosum* AG12 and *Sporomusa sphaeroides* AG8-2 and the methanogenic strain *Methanosarcina barkeri* MGZ3 were maintained in the medium with methanol (3 g/l). For cultivation of the methanogenic strain *Methanobacterium formicicum* MG134, which utilizes only hydrogen, the nitrogen gas phase was replaced by the $CO_2 + H_2$ mixture (1 : 4); the strain of sulfate-reducing bacteria (SRBs) *Desulfovibrio desulfuricans* SR12 able to grow on hydrogen was also cultivated in the $CO_2 + H_2$ atmosphere in the presence of Na_2SO_4 (7.4 mM) and $FeSO_4$ (2 mM).

The experiments with binary cultures were carried out under the atmosphere of nitrogen in the base medium supplemented with methanol, acetate, sodium, and iron sulfates in the concentrations mentioned above.

The concentrations of CH_4, CO_2, and H_2 were determined by gas chromatography in a Crystal 5000.1 chromatograph (Chromatec, Russian Federation) with CaA 0.2–0.4 and Hayexp N 80–100 packed columns (2 m × 3 mm), using argon as a carrier gas and a katharometer or flame ionization detector. Methanol and volatile fatty acids were detected in the Crystal 5000.2 chromatograph with a Sovpol B metal packed column (1 m ± 2 mm), using argon as a carrier gas and flame ionization detector. The concentrations of sulfides, sulfates, and iron ions were determined with Merck test kits (Germany) in the photometric variant.

RESULTS AND DISCUSSION

Eubacterium limosum, similarly to other acetogens, is known to produce hydrogen when grown on certain substrates, including methanol [3, 5]. Hydrogen concentrations in the culture liquid and in the gas phase depend on the ratio of kinetic parameters of hydrogen production and uptake. The presence of a hydrogen pool during the growth of acetogens creates a possibility for hydrogen uptake by sulfate reducers or methanogens if they possess systems with higher affinity to hydrogen, which is characterized by a threshold hydrogen concentration for a given culture [6].

The strain *E. limosum* AG12 isolated from the UGSF samples was cultivated jointly with the pure culture of the methanogen *Methanobacterium formicicum* MG134 isolated from the same object. The methanogen grew on the $H_2 + CO_2$ mixture in the presence of acetate as a carbon source. The strain could not grow on acetate or methanol, but the presence of methanol (up to 1.25 M) had no significant inhibitory effect during its growth on hydrogen. Initial substrate concentrations were chosen as consistent with those actually observed in the gas storage; methanol, acetate, and bicarbonate were introduced in the concentrations of 312, 5, and 5 mM, respectively. In the course of binary culture development, methane in the concentration of 37 430 ppm (3.34 mmol/l of the medium) was present in the gas phase after 14 days of incubation. In case of the binary culture, hydrogen concentration in the gas phase did not exceed 300 ppm (0.247 mmol/l of the medium), i.e., it was ten times lower than in case of the acetogen monoculture (2760 ppm). The absolute values of consumption of the substrates and synthesis of the products are given in Table 1. In the presence of the methanogen, butyrate production and acetate consumption were somewhat lower than in the acetogen monoculture. A decrease in the share of butyrate and increase in the share of acetate show the shift of acetogenesis toward more oxidized products in the case of hydrogen efflux to methanogenesis. The dynamics of acetate concentration in *E. limosum* is known to vary depending on its initial level [3, 5]. In this experiment, acetate consumption or production was determined by the balance of all reactions involving butyrate, acetate, and hydrogen, as well as by consumption of acetate as a carbon source by the methanogen.

The hydrogen pool decreased by an order of magnitude as a result of methane formation, with about 1 mol methane carbon produced per 7 mol butyrate carbon. Thus, in the ecotope under study, acetogenesis may be accompanied by methanogenesis carried out by hydrogen-consuming methanogens under conditions of exhaustion of hydrogen reserves.

The next experiment was aimed at studying the interaction between the acetogen *E. limosum* AG12

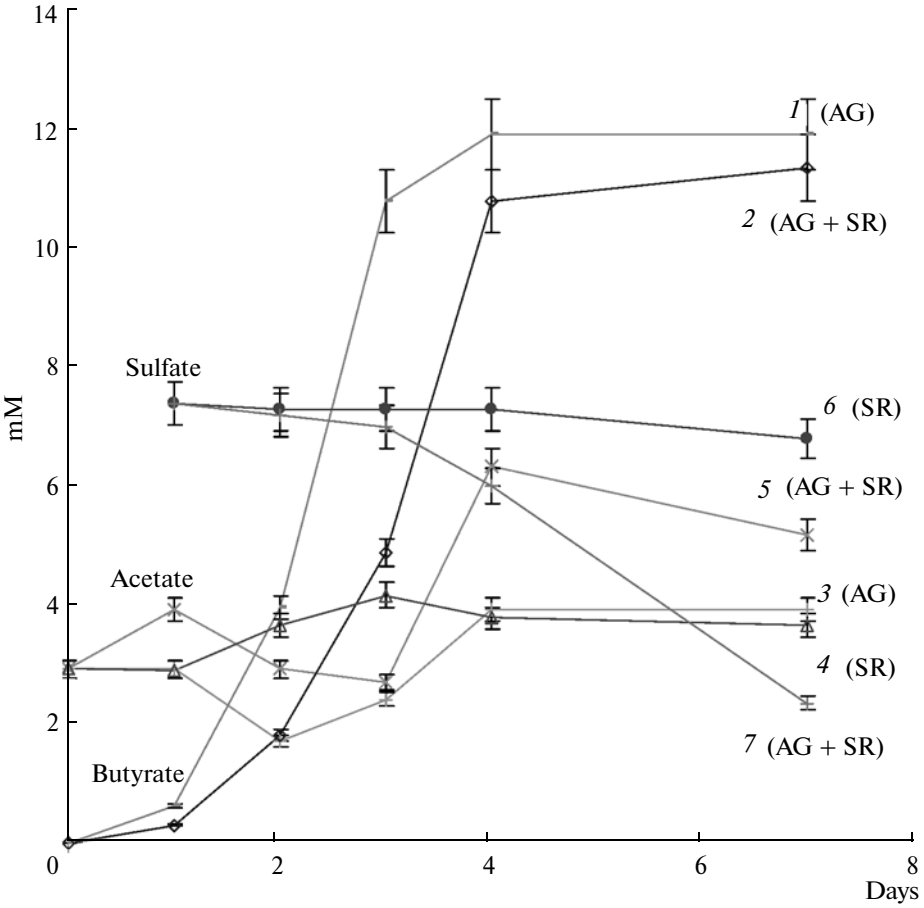

Fig. 1. Dynamics of the variations in butyrate, acetate, and sulfate concentrations during the development of monocultures of acetogenic *E. limosum* AG12 (AG) and sulfate-reducing *Dv. desulfuricans* AS12 (SR) and their binary culture (AG + SR). Butyrate (*1, 2*), acetate (*3–5*), sulfate (*6, 7*).

and the SRB strain *Desulfovibrio desulfuricans* SR12 isolated at the same UGSF object. Methanol in the concentration of 7 g/l usual for the UGSF did not inhibit the development of the SRB culture on hydrogen. The SRB strain SR12 cannot grow on acetate but grows well on hydrogen at its high partial pressure in the presence of sulfate and bicarbonate, which has been used for cultivation of the inoculum.

Figure 1 shows the dynamics of changes in acetate, butyrate, and sulfate concentrations for the mono- and binary cultures of the acetogen and the sulfate reducer under conditions of growth on the medium with methanol (224 mM), acetate (2.92 mM), and bicarbonate (5 mM). Fe^{2+} concentration in the UGSF samples was 50–500 mg/l (at the stage of gas extraction); therefore, for reducing the toxicity of produced hydrogen sulfide, the medium was also supplemented with $FeSO_4$ (1.8 mM). SRB growth was controlled by depletion of sulfate in the medium.

Development of the SRB monoculture on methanol was rather slow. Sulfate consumption and, accordingly, development of sulfate-reducing bacteria was substantially stimulated in the presence of acetogens. In this case, hydrogen concentration in the gas phase was 2150 ppm on day 7, while the pure acetogen culture accumulated 8770 ppm of hydrogen in the gas phase, i.e., SRBs decreased the hydrogen pool (Table 2). In the presence of SRBs, the synthesis of butyrate slightly decreased, while the synthesis of acetate increased, demonstrating the shift of acetogenesis products toward more oxidized compounds resulting from hydrogen efflux.

In contrast to the previous experiment with the methanogen *Methanobacterium formicicum* MG134, the sulfate-reducing bacterium had much lesser effect both on the hydrogen pool and on butyrate production. The methanogenic strain MG134 probably possesses a system with higher affinity to hydrogen than the SRB strain SR12 and captures hydrogen more effectively. However, the literature data suggest that sulfate reducers will substantially inhibit the development of acetogenic bacteria, which are characterized by the higher threshold values and lower K_m values for hydrogen [7, 8]. The main substrate for the isolated SRB strain SR12 in the UGSF system is probably

Table 2. Quantities of the substrates and products during the development of the culture of acetogenic *E. limosum* AG12, sulfate-reducing *Dv. desulfuricans* SR12, and their joint cultivation on methanol.

Cultures	Acetate, μmol-C	Butyrate, μmol-C	Hydrogen (maximum), μmol	Sulfate, μmol	Methanol, μmol-C
E. limosum AG12	78.67 ± 3.93	954.55 ± 47.72	15.66 ± 0.72	0.00	−1012.50 ± 50.62
Dv. desulfuricans SR12	58.67 ± 2.93	0.00	0.27 ± 0.03	−11.88 ± 0.63	−181.25 ± 9.06
E. limosum AG12 + *Dv. desulfuricans* SR12	90.00 ± 4.93	909.09 ± 47.72	3.85 ± 0.19	−100.97 ± 5.05	−1187.50 ± 59.62

The vial contained 25 ml of the medium (methanol, 4690 mmol; acetate, 117 mmol; HCO_3^-, 100 mmol; sulfates, 148 mmol, including 36 mmol $FeSO_4$.

hydrogen in high concentrations, which is typical of the stage of gas flooding into the UGSF.

Apart from the acetogenic bacteria utilizing hydrogen or methanol as substrates, enrichment cultures were shown to contain methanogenic archaea of the genus *Methanosarcina*.

Trophic interrelationships between *Ms. barkeri* and *Desulfovibrio vulgaris* when grown on methanol were described in 1973 by Zhilina and Zavarzin [9]. It was subsequently shown that a decrease in methane production by one-third was accompanied by a decrease of the hydrogen pool in the gas phase from 10 to 2 μmol/l [10], i.e., approximately from 200 to 40 ppm.

Development of the strain *Ms. barkeri* MGZ3 on the medium containing methanol (234 mM), acetate (5 mM), bicarbonate (5 mM), $NaSO_4$ (7.4 mM), and $FeSO_4$ (1.8 mM) occurred usually after a long lag phase. However, methanogenesis was noticeably stimulated in the presence of the SRB strain SR12 (Fig. 2). In the binary culture, the lag phase decreased by more than 2 days in the course of methanosarcina development, although the rate of methane production was practically the same. After 2 weeks of incubation, methane concentrations were rather close (18 and 20%). A similar observation was made in work of T.N. Zhilina [11]; however, the cause of this effect needs further research.

In contrast to acetogens, the hydrogen pool formed during the growth of the methanosarcina was much lower. The hydrogen concentration in the gas phase was did not exceed 200 ppm, and no reliable difference was shown between hydrogen concentrations in the methanosarcina monoculture and the binary culture with SRB. No noticeable growth acceleration was observed for the SRB strain SR12 in the presence of the methanosarcina, and sulfate consumption was practically the same in the SRB binary culture and monoculture (about 36.4 μmol per 20 ml of the medium). Obviously, the SRB strain SR12 cannot compete with the methanogen MGZ3 for hydrogen at low concentrations of the latter.

In our experiment, the hydrogen pool size (200 ppm, i.e., about 10 μmol/l) for the methanosarcina culture coincided with the data obtained by other authors [10]. The inability to consume hydrogen at a concentration corresponding to 200 ppm in the gas phase indicates that upon exhaustion of high hydrogen concentrations in the gas the strain *Desulfovibrio desulfuricans* SR12 should utilize the hydrogen efflux from acetogens or switch over to methanol.

Methanol does not belong to the substrates "convenient" for SRBs, since few species can utilize it, and the rate of SRB growth on methanol is much lower than that on hydrogen [12]. It is also known that methanogenesis on methanol in marine sediments is either not suppressed by sulfate reducers (in contrast to methanogenesis on hydrogen or acetate), or this effect is minor [6, 13].

The culture *Sporomusa sphaeroides* AG8-2 grows quicker than the methanosarcina strain MGZ3 and produces high hydrogen concentrations during the growth on methanol, which is in full agreement with the literature data [7]. Joint cultivation with other cultures was carried out in the medium containing methanol (123 mM), acetate (2.8 mM), and bicarbonate (5 mM) in the presence of Na_2SO_4 (7.4 mM) and $FeSO_4$ (2 mM). We found that under joint cultivation of the strain *Sm. sphaeroides* AG8-2 with the hydrogen-consuming methanogen *Mb. formicicum* MG134, a considerable decrease occurred in the maximum concentration of the hydrogen pool in the gas phase, from 19 600 to 4170 ppm (Fig. 3b). It was accompanied by growth of the archaeon and methane accumulation and a considerable increase in methanol uptake compared to the acetogen monoculture (Fig. 3c, Table 3). The quantities of produced acetate in the mono- and binary cultures were practically the same; i.e., growth of the acetogen culture was not inhibited. Total CO_2 consumption in the binary culture with the methanogen was lower compared to the acetogen monoculture. The hydrogen efflux to methanogenesis curtails the CO_2 uptake due to partial production of methane, which is a more reduced product than acetate. *Mb. formicicum* MG134 did not suppress the development of the acetogen in spite of a considerable decrease of the hydrogen pool in the monoculture, and the same amount of acetate was formed in the binary culture (Fig. 3a, Table 3). This suggests syntrophic interrelationships between the cultures of

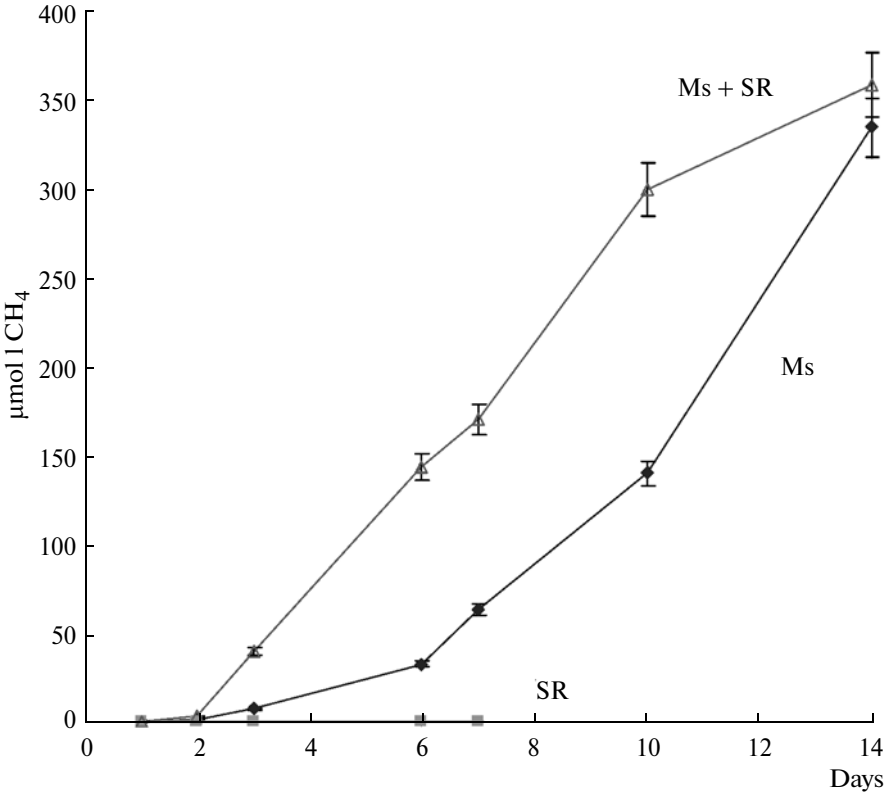

Fig. 2. Methane production by the *Ms. barkeri* MGZ3 culture in the presence of sulfate-reducing bacteria *Dv. desulfuricans* SR12 (Ms + SR), in the *Ms. barkeri* MGZ3 monoculture (Ms), and in the *Dv. desulfuricans* SR12 monoculture (SR).

Sporomusa sphaeroides AG8-2 and *Mb. formicicum* MG134. Characteristically, the binary culture of *Sm. sphaeroides* AG8-2, which is liable to form microcolonies as small flakes, includes the filamentous cells of the methanogen into these microcolonies. Theoretically, acetogens in a syntrophic association with hydrogenotrophic methanogens can perform the same process as methylotrophic methanogens, when methane synthesis is accompanied by CO_2 production and acetate is not synthesized under conditions of hydrogen efflux.

Under joint cultivation of the *Sm. sphaeroides* AG8-2 culture with the sulfate reducer *Dv. desulfuricans* SR12, the hydrogen concentration decreased less (to 6590 ppm) compared to the cultivation with *Mb. formicicum* MG134. In the binary culture, methanol

Table 3. Substrates and products during development of the *Sm. sphaeroides* AG802 culture in the presence of *Dv. desulfuricans* SR12 or *Mb. formicicum* MG134. The vial contained 25 ml of the medium (methanol, 3080 μmol; acetate, 100 μmol; HCO_3^-, 100 μmol; sulfates, 230 μmol, including 54 μmol $FeSO_4$)

Cultures	Methanol, μmol-C	Acetate, μmol-C	Methane, μmol-C	H_2S, μmol-C	CO_2 and HCO_3^-, μmol-C	pH
Sm. sphaeroides AG8-2	−663 ± 33	970 ± 48	0	0	−227 ± 11	5.46 ± 0.07
Sm. sphaeroides AG8-2 + *Mb. formicicum* G134	−1403 ± 70	976 ± 48	423 ± 21	0	−84 ± 4	5.56 ± 0.07
Dv. desulfuricans SR12	−639 ± 25	190 ± 8	0	34 ± 2	512 ± 26	7.01 ± 0.07
Sm. sphaeroides AG8-2 + *Dv. desulfuricans* SR12	−515 ± 25	275 ± 12	0	116 ± 6	458 ± 23	6.98 ± 0.07

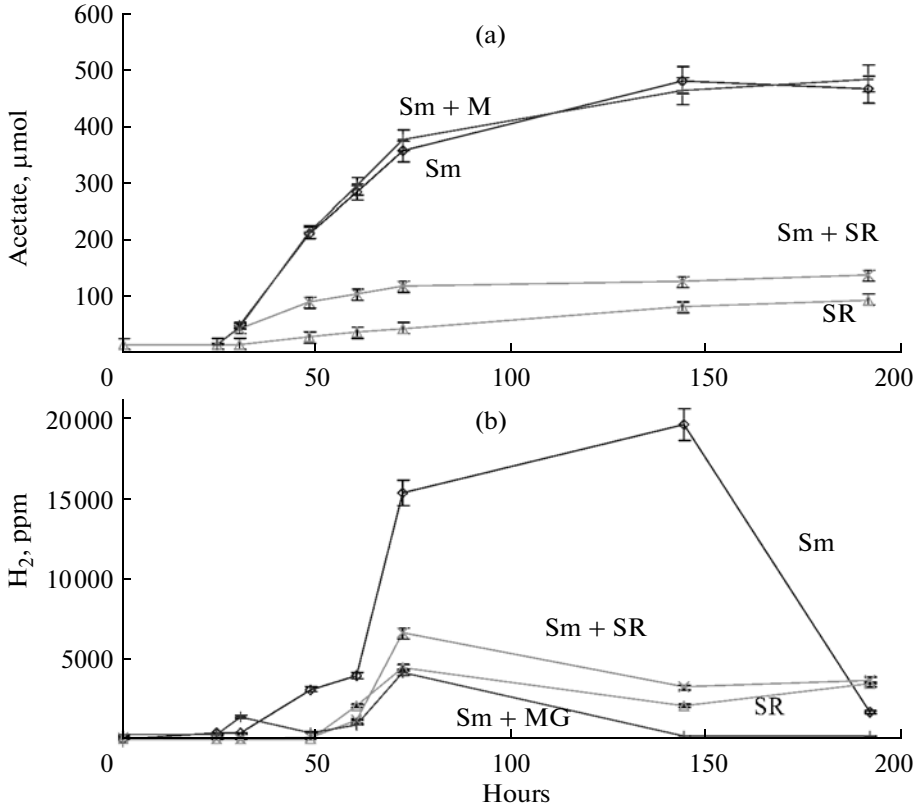

Fig. 3. Dynamics of the changes in acetate (a), hydrogen (b), methanol (c), sulfide (d), and methane (e) concentrations during the development of monocultures of the acetogen *Sm. sphaeroides* AG8-2 (Sm) and the sulfate reducer *Dv. desulfuricans* SR12 (Sr), their binary culture (Sm + SR), and methane formation in the binary culture of *Mb. formicicum* MG134 and the acetogen (Sm + MG) during the growth on methanol.

was utilized much more rapidly than in the SRB monoculture, while rapid accumulation of sulfide in the medium was observed (Figs. 3a, 3d; Table 3). However, methanol consumption in the case of the binary SRB-acetogenic culture does not reach such values as in the binary culture with the methanogen MG134 or in the *Sm. sphaeroides* AG8-2 monoculture. It may result from a very high sulfide concentration, which only decreased to 100 mM due to the presence of iron ions, exceeding by many times the threshold of 2 mM, above which the development of the *Sm. sphaeroides* AG8-2 culture is inhibited. Nevertheless, iron ion concentrations in some samples from the UGSF objects are as high as 10 g/l, and joint cultivation of sulfate reducers and acetogens in storage objects is quite possible.

It is obvious that the isolated SRB culture has a much higher threshold value for hydrogen than the methanogen and is able to take away hydrogen only under joint cultivation with acetogens. Moreover, compared to the cases of joint methanol consumption by cultures of the genera *Sporomusa* and *Desulfovibrio* described in the literature, our SRB isolate has low competitiveness for hydrogen [7, 14]. This confirms the assumption of its adaptation to high hydrogen concentrations.

At the stage of gas flooding, the main substrates (hydrogen, CO_2, sulfate) continuously enter the storage facility. Their reserves are renewed. These conditions are favorable for development of acetogens such as rapidly growing *Sporomusa* and *Eubacterium* and specialized hydrogen-utilizing methanogens (*Mb. formicicum*) and sulfate reducers (*Dv. desulfuricans*). Adaptation of the strain *Dv. desulfuricans* SR12 to high hydrogen concentrations shows that isolation of these SRBs from the samples at the stage of gas flooding is not accidental. During gas extraction, the inflow of substrates (except for methanol) is stopped and their concentrations decrease. First of all, this concerns hydrogen, which is a universal substrate for many groups of microorganisms.

Under hydrogen deficiency, acetogenic bacteria utilize methanol, creating a hydrogen pool, which is captured by hydrogen-utilizing microorganisms such as *Mb. formicicum* or *Dv. desulfuricans*. It is quite probable that a variant of syntrophic nutrition can be formed between homoacetogenic bacteria capable of methanol utilization and methanogens growing on hydrogen and CO_2. Sulfate reducers in the presence of

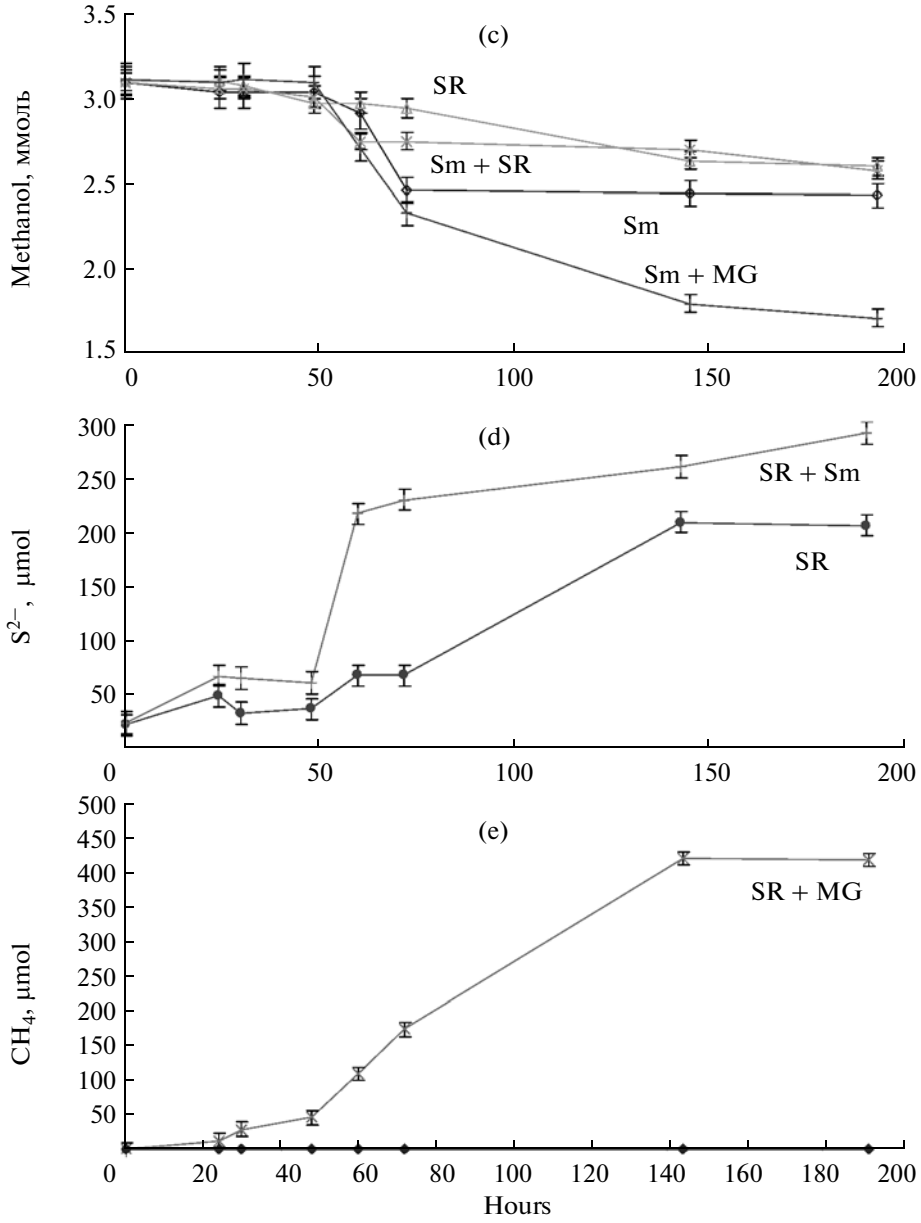

Fig. 3. Contd.

sulfate are forced to utilize more "difficult" methanol instead of hydrogen or enter into trophic relations with methylotrophs possessing a sufficient intracellular hydrogen pool.

As CO_2 reserves are exhausted and, accordingly, bicarbonate concentration in the water decreases, methanosarcinas with relatively slow growth rates may gain an advantage over acetogens, because CO_2 is not required for methanogenesis on methanol. Moreover, methanogenesis from methanol is accompanied by CO_2 synthesis, partially replenishing this electron acceptor for acetogens.

Thus, the role of interspecies hydrogen transfer increases in the UGSF under the conditions of microorganisms switching over to methanol as the main substrate in the period of gas extraction. The ecosystem as a whole continues to function, performing a number of physicochemical processes influencing the corrosion of equipment and the permeability of stratum rocks.

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