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EXPERIMENTAL ARTICLES

On the Problem of Anaerobic Methane Oxidation

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Abstract—To clarify the biological mechanism of anaerobic methane oxidation, experiments were performed with samples of the Black Sea anaerobic sediments and with the aerobic methane-oxidizing bacterium *Methylomonas methanica* strain 12. The inhibition–stimulation analysis did not allow an unambiguous conclusion to be made about a direct and independent role of either methanogenic or sulfate-reducing microorganisms in the biogeochemical process of anaerobic methane oxidation. Enrichment cultures obtained from samples of water and reduced sediments oxidized methane under anaerobic conditions, primarily in the presence of acetate or formate or of a mixture of acetate, formate, and lactate. However, this ability was retained by the cultures for no more than two transfers on corresponding media. Experiments showed that the aerobic methanotroph *Mm. methanica* strain 12 is incapable of anaerobic methane oxidation at the expense of the reduction of amorphous FeOOH.

Key words: methane, anaerobic oxidation, inhibitory analysis, methanotrophs, methanogens, sulfate reducers.

The distribution pattern of the methane concentration in marine sediments suggests that methane does not accumulate in the sediments but is consumed by microorganisms. Numerous experiments with radioactive [1–3] and stable isotopes unambiguously demonstrated the occurrence of not only aerobic but also anaerobic methane oxidation, at least in marine ecosystems. The biological nature of this process has been actively discussed in the microbiological and geochemical literature over the last three decades. The main role in anaerobic methane oxidation was initially ascribed to sulfate-reducing bacteria. Methane oxidation at the expense of sulfate is theoretically feasible; however, thermodynamic calculations show that the direct reaction of sulfate and methane cannot provide for the synthesis of an amount of ATP sufficient for bacterial development.

Earlier [4], enzymatic oxidation of small amounts of methane (0.3%) was demonstrated in cultures of methanogenic archaea. It was hypothesized that anaerobic methane oxidation under natural conditions may occur due to joint activity of microorganisms of different physiological groups: methanogens could oxidize methane to methanol or acetate, which could be metabolized by other anaerobes, e.g., sulfate-reducing bacteria [5]. Valentine and Reeburgh [6] substantiated, both theoretically and experimentally, the possibility of anaerobic methane oxidation by methanogenic archaea followed by the oxidation of the acetate formed by sulfate reducers. The possibility of such a mechanism of anaerobic methane oxidation was confirmed by Lein and coworkers [7] based on data of analyses of the stable-isotopic composition ($\delta^{18}O$) of the oxygen of water, sulfates, and autigenic carbonates and bicarbonates in the Black Sea.

In bottom sediments of Lake Mendota, oxidation of $^{14}CH_4$ to $^{14}CO_2$ was revealed, which was inhibited by oxygen [8]. Enrichment cultures obtained from the sediments could oxidize methane only in the presence of sulfate and acetate or lactate. During this oxidation, the labeled carbon of methane passed to carbon dioxide, whereas the carbon of acetate appeared in microbial cells. However, lack of progress in the obtaining of stable pure or enrichment cultures of anaerobic methanotrophs imparts a mysteriousness to the microbiological, biogeochemical, and evolutionary aspects of the problem of anaerobic methane oxidation.

For bottom sediments, the reduction of Fe(III) to Fe(II) during bacterial mineralization of butyrate, ethanol, methanol, or trimethylamine but not methane was demonstrated [9]. Potekhina and coworkers [10] reported "anaerobic" growth at the expense of FeOOH reduction of our isolate (strain 12) [11] of the aerobic methane-oxidizing bacterium *Methylomonas methanica.* However, the authors did not provide direct evidence of methane oxidation by this culture under anaerobic conditions.

Thus, the analysis of published data leads to the conclusion that we do not yet know either the chemical compound that oxidizes methane under anaerobic conditions or the physiological group of microorganisms playing the major role in this process, although over the last two to three years many experimental works were performed and

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Table 1. Effect of different compounds on the rates of methane oxidation, methane production, and sulfate reduction in anaerobic sediments of st. 806-3 (% of the control without additives)

Compound	Concentration	Methane oxidation			Methane production	Sulfate reduction
		to CO_2	to cells and exometabolites	total		
Imidazole	2 mM	100	134	111	ND	ND
CCl_4	2 mM	85	46	78	62	53
BES	50 mM	103	89	96	17	105
Na_2MoO_4	20 mM	100	21	85	153	38
S^0	40 mg/g	122	20	103	33	130
$FeSO_4$	10 μg/g	112	14	94	483	127
Methanol	40 μg/g	116	329	155	112	57
Methylamine	50 μg/g	123	39	107	25	58
Acetate	50 μg/g	128	14	107	831	56
Lactate	50 μg/g	123	23	105	59	44
Formate	50 μg/g	69	160	86	69	130
Glutaraldehyde	10 mg/g	0	0	0	0	0
Autoclaving		0	0	0	0	0

Note: ND stands for "not determined."

significant advances have been made in solving the riddle of anaerobic methane oxidation [12–16].

The present paper presents the results of experiments that aimed to reveal microorganisms responsible for anaerobic methane oxidation and unravel the reactions they perform.

MATERIALS AND METHODS

Geography of investigations and sample collection. The materials for investigations were obtained in October–December 1984 during the eighth cruise of the R/V *Vityaz'* at polygons located in different regions of the Black Sea and in December 1990 during an expedition on board the *Bentos-300* submersible laboratory (in tandem with the R/V *Divnyi*) [3]. Experiments were conducted aboard the ship or the submersible and in the laboratories of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino), or the Institute of Microbiology, Russian Academy of Sciences (Moscow). Samples of bottom sediments were taken with an Okean dredger and straight-flow geological tubes with an inner diameter of 12 cm; water samples were taken with General Oceanics bathometers (United States) and through the Kingston valves of the submarine; carbonate construction samples were taken with a hydraulically manipulated dipper of the submarine.

Isolation and enumeration of aerobic methanotrophs was performed by the serial dilution method in Hungate tubes (Bellco Glass, United States) with liquid mineral NH_4NO_3-containing medium under a methane–air (30 : 70) atmosphere [3].

Anaerobic enrichment procedures. Liquid media were prepared from sterile solutions composed of (g/l distiled water) (**I**) NaCl, 24; $MgCl_2 \cdot 6H_2O$, 11; Na_2SO_4, 4; KCl, 0.8; and NH_4Cl, 0.5; (**II**) KH_2PO_4, 15, and $Na_2HPO_4 \cdot 5H_2O$, 30; (**III**) $CaCl_2$, 100; (**IV**) NaBr, 8, and $SrCl_2 \cdot 6H_2O$, 2; (**V**) $KF \cdot 2H_2O$, 1; (**VI**) H_3BO_3, 0.3; $CoCl_2$, 1; $CuSO_4 \cdot 5H_2O$, 0.1; $MnCl_2$, 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.3; $ZnSO_4 \cdot 7H_2O$, 2; $NiCl_2$, 0.05; and H_2SO_4, 2 ml; (**VII**) $FeSO_4$, 10 (in 1% solution of HCl); (**VIII**) $NaHCO_3$, 20; (**IX**) Na_2S, 100 (in 5% solution of $NaHCO_3$); (**X**) FeS (amorphous), 10; (**XI**) S^0, 50 (10% solution in acetone); (**XII**) KNO_3, 50; (**XIII**) acetate, 50; (**XIV**) lactate, 50; (**XV**) formate, 50; (**XVI**) methylamine, 50; and (**XVII**) methanol, 50. The solutions were autoclaved at 1 atm for 1 h.

To prepare the media (eight variants; see Table 1), 900 ml of solution **I** was aseptically supplemented with solutions **II**, 20 ml; **III–V**, 10 ml; **VI**, 1 ml; **VII**, 10 ml (except the Ser variant); **VIII**, 10 ml; **IX**, in drops until the medium acquired gray color; **X** and **XI**, 10 ml (only the Ser and ALF variants); **XII** (only the Nit variant); **XIII–XVII**, 10 ml (only the corresponding medium variants). Anaerobiosis was controlled with the help of resazurin (0.002%, pH 9.0). A total of eight variants of the medium were used (Table 1): media with acetate (Ace), lactate (Lac), formate (For), methanol (Met), methylamine (MA), sulfur (Sul), and nitrate (Nit) and ALF medium with acetate, lactate, and formate (solutions **X–XII** and **XIII–XV**, 2 ml each). Medium without organic substrates and additives **X–XII** was used as the control.

Samples of reduced bottom sediments or hydrogen sulfide–containing water (1 cm^3) were introduced into

10 ml of an appropriate medium in 17-ml Hungate tubes closed with gastight butyl rubber stoppers (Bellco Glass, United States). The tubes were sparged with nitrogen passed through a copper catalyst at 800°C to remove oxygen admixture and then with high-purity methane (99.99%). The cultures were incubated at room temperature for one to six months.

Methane-oxidizing activity of anaerobic enrichment cultures was determined from the rate of oxidation of $^{14}CH_4$ to $^{14}CO_2$ [3]. A 1-ml aliquot of the culture liquid was taken from the experimental tubes and transferred to 4 ml of an appropriate medium in a 6-ml Hungate tube sparged in advance with nitrogen from which oxygen admixture had been removed. Then, 100 μl of a solution of $^{14}CH_4$ in sterile degassed water (total activity, 0.19 μCi) was introduced and the tubes were incubated for 16 h at room temperature, after which the samples were fixed with 0.5 ml of 20% KOH, transferred to a system for driving off gases from aqueous solutions, and supplemented with 1 ml of strong orthophosphoric acid. The labeled CO_2 formed was driven off with a flow of nitrogen for 1 h and trapped in a reaction with 2-phenylethylamine in the scintillation cocktail; the radioactivity of the $^{14}CO_2$ trapped was determined in a RackBeta scintillation counter (Sweden).

Determination of the rates of biogeochemical processes in samples of water and bottom sediments was performed as described earlier [3]. The rate (cpm) of methane oxidation was evaluated from the formation of $^{14}CO_2$ and ^{14}C-biomass + ^{14}C-exometabolites from $^{14}CH_4$. The rate of methanogenesis was judged from $^{14}CH_4$ production from $NaH^{14}CO_3$. The rate of sulfate reduction was evaluated from the production of ^{34}S-hydrogen sulfide, ^{34}S-pyrite, ^{34}S-elemental sulfur, and ^{34}S-organic sulfur from $Na_2{}^{34}SO_4$.

Experiments on inhibition and stimulation of bacterial processes [3]. Bottom sediments were sampled at deep-sea station 806-3 (western gyristase, 2142 m) with an Okean dredger. With the help of a plastic cylinder 20 cm in diameter, a sediment block was extracted and transferred to a double-walled polyethylene bag sparged with oxygen-free nitrogen, and the contents of the bag were thoroughly mixed. Samples of the mixed ("averaged") sediment were taken with glass tubes analogously to native samples. The tubes were hermetically closed with Balch butyl rubber stoppers (Bellco Glass, United States). Solutions (200 μl) of inhibitory or stimulatory compounds were introduced with a syringe. After 12 h of incubation at 8°C, the samples were supplemented with 100 μl of a solution of $^{14}CH_4$, $NaH^{14}CO_3$, or $Na_2{}^{34}SO_4$ with a total activity of 3, 20, and 50 μCi, respectively, and the incubation was continued for 72 h at 8°C, after which the samples were fixed with 1 ml of a 2 N KOH solution.

Tests of the ability of *Methylomonas methanica* to oxidize methane with FeOOH under anaerobic conditions were performed in three experimental variants [3]:

Experimental variant I. A 3-day culture (20 ml) of *Methylomonas methanica* strain 12 was introduced into a flask with 200 ml of liquid mineral P medium [11]. Then, 3 ml of a suspension of amorphous FeOOH was added to a final concentration of 30 mg/l. The mixture was sparged for 1 h with argon (without elimination of the traces of oxygen present in argon) and dispensed into Hungate tubes with gastight butyl rubber stoppers. After that, 100 μl of a solution of $^{14}CH_4$ in sterile degassed distilled water (total activity, 0.38 μCi) was added. Incubation was performed at room temperature. The $^{14}CO_2$ formed upon methane oxidation was trapped with 2-phenylethylamine in the scintillation cocktail, and radioactivity was determined in a liquid scintillation counter.

Experimental variant II. All manipulations were analogous to experimental variant I with the following exceptions: (1) after the introduction of FeOOH, the reducing agent Na_2S was introduced to a final concentration of 50 mg/ml; (2) the mixture was sparged with argon passed through a copper catalyst at 800°C to remove oxygen admixture. The rate of methane oxidation was determined as in experimental variant I. The radioactivity of the $^{14}CH_4$ solution introduced was 0.19 μCi.

Experimental variant III. All manipulations were performed in an anaerobic glove box. A culture of *Methylomonas methanica*, fresh medium, and a suspension of FeOOH were kept in separate flasks with cotton plugs for a day in the anaerobic box in an atmosphere of CO_2 + H_2 with a platinum catalyst for removal of traces of oxygen. After that, 20 ml of the methanotroph culture and 20 ml of the FeOOH suspension were introduced into 200 ml of medium. After mixing, the inoculated medium was dispensed into Hungate tubes. Sodium sulfide was not added. The rate of methane oxidation was determined as in experimental variant I. The radioactivity of the $^{14}CH_4$ solution introduced was 0.38 μCi.

Ultrathin sections of bacterial mats from carbonate constructions in the regions of the Black Sea methane seeps were obtained as follows. Samples were processed aboard the R/V *Professor Vodyanitskii* immediately after withdrawing a carbonate construction from the sample collector of the *Bentos* submarine in December 1989. Mat samples measuring 3 × 3 mm were fixed with 3.2% glutaraldehyde solution in cacodylate buffer for 4 h; postfixed with 1% solution of OsO_4; dehydrated in ethanol, acetone, and finally propylene oxide; and embedded in an Epon–araldite mixture [3]. The sections were obtained on an LKB-8802A ultramicrotome and examined under a JEM-100C transmission electron microscope.

RESULTS AND DISCUSSION

Aerobic methanotrophs have been detected in and isolated from shelf bottom sediments contacting with

oxygen-containing waters, as well as from these waters themselves, but not from bottom sediments or the water column of the anaerobic zone. Nevertheless, occurrence of methane oxidation has been shown by the radioisotopic method in all samples of bottom sediments and water in both aerobic and anaerobic zones, the integral rate of anaerobic methane oxidation being higher than that of the aerobic process. It is beyond doubt that the biogeochemical/microbiological process of methane oxidation in the Black Sea as a whole is a sum of aerobic and anaerobic processes [1, 3].

In the experiments that involved inhibition and stimulation by various compounds of methane oxidation, methane production, and sulfate reduction in reduced sediments, attempts were undertaken to outline the range of microorganisms responsible for anaerobic methane oxidation (Table 1). Imidazole, an inhibitor of aerobic methane oxidation, not only failed to inhibit the process but, on the contrary, caused certain stimulation of methane oxidation and, especially, of methane carbon incorporation into bacterial cells and exometabolites. Carbon tetrachloride, an inhibitor of methanogenesis, decreased the rates of all of the three processes investigated. Bromoethanesulfonic acid (BES), another methanogenesis inhibitor, suppressed methane production but not methane oxidation or sulfate reduction. Sodium molybdate, an inhibitor of sulfate reduction, decreased the rate of methane oxidation but not as significantly (by 15%) as the rate of sulfate reduction, which was decreased by 62%. Molybdate, sulfate, and acetate stimulated methanogenesis; the stimulation caused by sulfate and acetate was considerable, 5- and 8-fold, respectively. The process of bacterial sulfate reduction was slightly stimulated by elemental sulfur, sulfate, and formate. Other compounds tested did not significantly influence the integral rate of methane oxidation under anaerobic conditions but notably inhibited methane carbon incorporation into microbial cells and organic exometabolites. Only methanol and formate stimulated methane carbon incorporation into microbial cells and exometabolites. Glutaraldehyde and autoclaving completely inhibited all three processes—methane production, sulfate reduction, and methane oxidation.

From bottom sediments and water of different regions of the Black Sea, primary enrichment cultures were obtained under methane atmosphere on media with additional carbon sources. Some enrichment cultures of presumed anaerobic methanotrophs exhibited methane-oxidizing activity that exceeded the control values by 3- to 380-fold. The most significant activities were recorded on media with acetate and formate, as well as on media where acetate, formate, and lactate were present simultaneously (Table 2). However, after the third transfer, these enrichment cultures of anaerobic methanotrophs completely lost their ability to oxidize methane. It is possible that these cultures contained syntrophic consortia of microorganisms for which conditions promoting methane oxidation existed only in the primary cultures due to the presence of a sufficient amount of a natural substrate significant for these organisms.

Thus, the integrity of the data obtained does not allow unambiguous conclusions to be made about the nature of the microorganisms responsible for anaerobic methane oxidation. Inhibitory analyses carried out by other researchers [17] also failed to unravel the mechanism of this microbiological process. All these data create an impression that, taken alone, neither methanogens nor sulfate reducers can be direct and sole catalysts of anaerobic methane oxidation.

We investigated two polygons in the region of methane seeps with carbonate constructions (expedition on board the *Bentos-300* submersible, anaerobic part of the Black Sea, 44°41′70 N, 31°40′70 E, depth of 205–230 m). The fields with carbonate constructions (methane seeps) are located either immediately under oxygen-containing waters (depth, 60–80 m) or at considerable depths in anaerobic conditions (presently, many more fields of the latter type are known). The rates of anaerobic methane oxidation in the water column of the region that we investigated were considerably higher than the rates of the aerobic process and peaked in near-bottom layers (368 and 558 ng C/(l day), respectively) [18]. From a depth of 220 m, we lifted a carbonate (aragonite) construction above which there was a 60-m layer of anaerobic (hydrogen sulfide–containing) water column (Eh from –153 to –276 mV; H_2S, 300–970 μl/l; CH_4, 44–145 μl/l; no O_2).

The gas sampled from a neighboring carbonate construction mainly consisted of methane (80%) with a carbon isotopic composition characterized by $\delta^{13}C = -58.2‰$. The $\delta^{13}C$ value of the aragonite carbonate carbon varied from –32.5 to –40.4‰ (Table 3), whereas the $\delta^{13}C$ of the seawater carbonate ranged from –7 to –12‰. The organic matter of the slimy bacterial mats on the carbonate construction had an extremely light isotopic composition of carbon ($\delta^{13}C$ from –75.6 to –83.8‰; Table 3); this fact, together with data on the isotopic composition of aragonite carbonate, allows us to infer that the carbonate constructions are formed as a result of the activity of methanotrophic bacteria, which oxidize methane to CO_2 under anaerobic conditions of the environment.

During the submarine expedition, we visually observed bacterial mats about 5 cm thick that completely covered the bottom sediments between the plates with carbonate constructions. Due to the intense oxidation of the methane seeping through the bacterial mat and the consequent formation of carbon dioxide, the carbonate equilibrium is shifted toward carbonate precipitation, which results in the production of aragonite [18]. The occurrence of the initial stages of this process in the bacterial mat can be judged from the presence of small (0.5–2 mm) carbonate inclusions (*bacterial pearls*). In certain sites of the mat, the amount of the carbonate inclusions gradually increases

Table 2. Oxidation of $^{14}CH_4$ to $^{14}CO_2$ by primary enrichment cultures of anaerobic microorganisms, obtained from samples of bottom sediments and water

Station	Depth, m	Horizon, cm	Methane oxidation rate (% of the rate in medium without additives)							
			Nit	Sul	Met	Ace	For	MA	Lac	AFL
823	101	20–30	47	67	78	**29553***	80	84	140	221*
833	108	3–6	106	64	53	**1304**	132	74	119	118*
848	118	20–25	53	55	109	**682**	124	628	174	130
795	150	2–6	107	75	90	103	200	55	206	**649***
		6–11	76	56	146	72	134*	**11187***	182	**411***
		11–25	34	82	101	80	79	88	151	**438**
817	1420	100–110	48	46	81	308*	64	48	79	98*
805	1583	0–3	157	176	70**	215**	**401**	223**	307	**469****
		15–25	60	135	122	**925**	305	180**	128	187
		35–45	77	150	222	117	273	275	168	**376236***
		54–64	95	98	78	109	316	105	156	112
806	2141	1–5	108	151	253	142	376	261	301	231**
		20–25	195	204	245	74	**497**	211	200	173*
		50–60	267	253	286**	90	**382**	210	240	130
839	2154	145–147	37	32	74	27	83	**482**	137	56
839 (water)	2154	1500 m	94	100	81	68	57	67	61	**3143**

Notes: The additives were Nit, nitrate; Sul, elemental sulfur; Met, methanol; Ace, acetate; For, formate; MA, methylamine; Lac, lactate; AFL, mixture of acetate, formate, and lactate.

* Enrichment cultures of anaerobic methanotrophs in which significant consumption of methane was revealed chromatographically (a 4- to 80-fold decrease of the CH_4 content in the gas phase).

** Sediment samples from which methanogenic enrichments were obtained that produced significant amounts of methane (0.15–0.6 ml CH_4/ml gas phase).

and they come into physical contact. Then, these inclusions merge, with the formation of a carbonate crust; it becomes thicker and thicker and a massive plate is formed, which usually has one outlet (channel) through which methane is released. The conditions most favorable for methanotrophs and, hence, for the bacterial mat occur in the immediate vicinity of the channel. In this zone, vertical growth of the bacterial mat occurs, accompanied by further aragonite precipitation. Finally, a construction of an intricate shape (towerlike, coral-like, funguslike) is formed on the plate. These constructions invariably have a channel in the middle; at its outlet, a "bag" is formed by a bacterial film, which has a perforation that periodically releases methane bubbles (N.V. Pimenov, personal communication supported by a video recording). The outer surface of the construction is covered by a bacterial mat 0.5–2 mm thick; no mat occurs in the channel. During the development of the carbonate construction, the plate, whose diameter may reach 1–2 m, plays the role of a trap for methane that seeps from the sediments and is released through the channel of the construction. This mechanism of the formation of carbonate constructions is supported by the presence on the surface of the constructions of bacterial pearl inclusions, which are cemented but easily

Table 3. Stable-isotopic composition of the carbon of methane, organic matter of bacterial mats, carbonates in the mats, and carbonate in the construction aragonite

Sample	$\delta^{13}C$, ‰
Methane	–58.2
Bacterial mats (C_{org}):	
no. 1, grayish yellow	–78.7
no. 2, grayish pink	–83.8
no. 3, light brown	–79.3
no. 4, pink	–80.2
Carbonate inclusions from the mats (bacterial pearl, C_{min}):	
from mat no. 1	–46.4
from mat no. 2	–43.5
from mat no. 3	–46.1
from mat no. 4	–40.3
Aragonite carbonates:	
saw cut no. 1	–32.5…–40.0*

Note: Determinations were performed by Yu.M. Miller.

* Data of A.Yu. Lein.

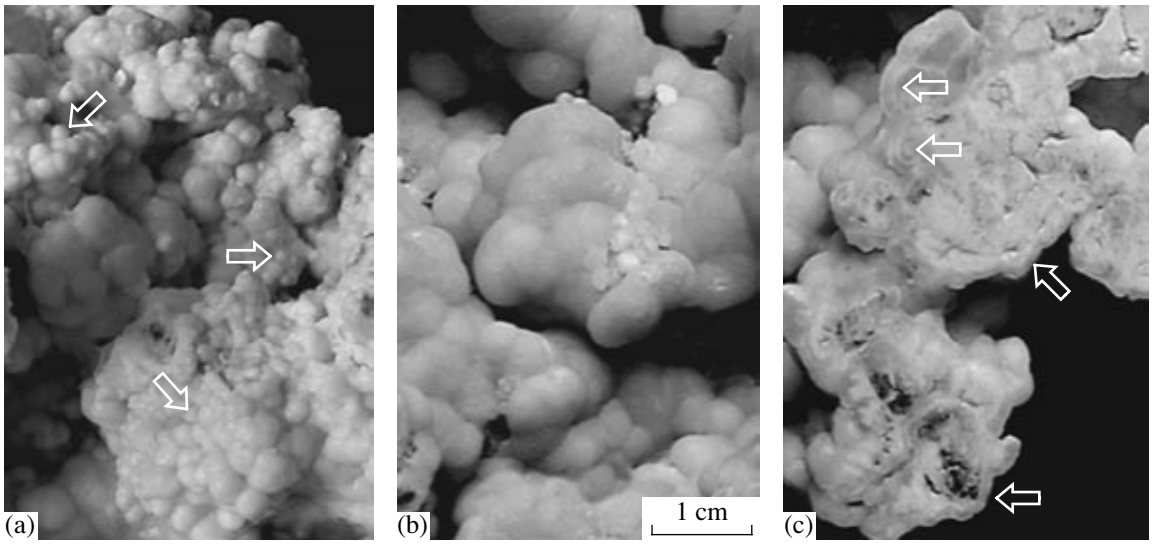

Fig. 1. Carbonate (aragonite) construction from the methane seep region, Black Sea: (a) outer side of the construction (small carbonate spheres under bacterial mat); (b) inner side of a sawed construction (large carbonate spheres inside the gas channel); and (c) saw cut showing layered structure of large spheres.

recognizable; they vary in shape, reaching 1–2 cm in diameter, and have a lamellar structure (Fig. 1).

In ultrathin sections of bacterial mats, among morphologically diverse bacteria (Figs. 2e, 2f), cysts (Fig. 2d), and residues of lysed bacteria (Figs. 2a–2c), chains of long cells with tapered ends predominated, often surrounded by a considerable amount of slime (Figs. 2e, 2f). Depending on the orientation of the section plane, these cells of microorganisms appeared either as cocci (transverse section, Fig. 2e) or as spindles (oblique section, Figs. 2a–2c). In lysed cells, numerous strips were observed, which either connected opposite cell walls or hung down from the walls as short segments. These cells often displayed shrunk cytoplasm containing the nucleoid (Figs. 2a, 2b) [3].

Analogous cells were revealed by Pimenov and coworkers [19] in a mat on a carbonate construction sampled during trawling of a methane seep at a depth of 180–190 m. In the opinion of these researchers, these organisms are morphologically and ultrastructurally similar to the methanogens of the genus *Methanothrix (Methanosaeta)*, which are heterotrophic archaea that produce methane at the expense of acetate decomposition. It is so far hardly possible to definitely establish whether the microorganisms revealed are indeed *Methanothrix* representatives. It should be noted that both of the *Methanothrix* species recognized today, *Methanothrix soehngenii* and *Methanothrix thermophila*, have optimum growth temperatures (35–40 and 55–65°C) that are considerably higher that those recorded at the Black Sea methane seeps. Another type of cells revealed in electron microscopic specimens is cells of irregular shape (Fig. 2), very similar to gram-negative bacteria (sulfate reducers?).

American researchers [20] reported the results of experiments that they performed with bottom sediments of a marine lagoon with the aim to prove the involvement of a syntrophic consortium of methanogens and sulfate-reducing bacteria in methane oxidation under anaerobic conditions. The authors put forward a "biological energy" model of CO_2 reduction in the sediments investigated. According to their calculations, the processes of methanogenesis at the expense of carbon dioxide reduction can be observed only at hydrogen concentrations above 2.4 nM. As for the anaerobic methane oxidation by methanogenic archaea (methane oxidation and hydrogen production via a reversed reaction of CO_2 reduction), it can proceed only at H_2 concentrations below 0.29 nM. The hydrogen produced is efficiently consumed and maintained at a low concentration level by sulfate-reducing microorganisms, which thus provide for methane oxidation by methanogens. The role of the hydrogen sink might be played not only by sulfate reducers but also by bacteria that reduce NO_3^-, Fe^{3+}, or Mn^{4+}.

Valentine and Reeburgh [6] also propose a mechanism of methane oxidation to CO_2 and H_2 via reversed methanogenesis with subsequent utilization of hydrogen by sulfate reducers for the reduction of sulfate, i.e., a mechanism based on interspecies hydrogen transfer in a microbial consortium.

Variant I:

$$CH_4 + 2H_2O \longrightarrow CO_2 + 4H_2 \text{ (MO–MG)},^1 \quad (1)$$

$$SO_4^{2-} + 4H_2 + H^+ \longrightarrow HS^- + 4H_2O \text{ (SRB)}, \quad (2)$$

[1] MO–MG, methane-oxidizing methanogens, SRB, sulfate-reducing bacteria.

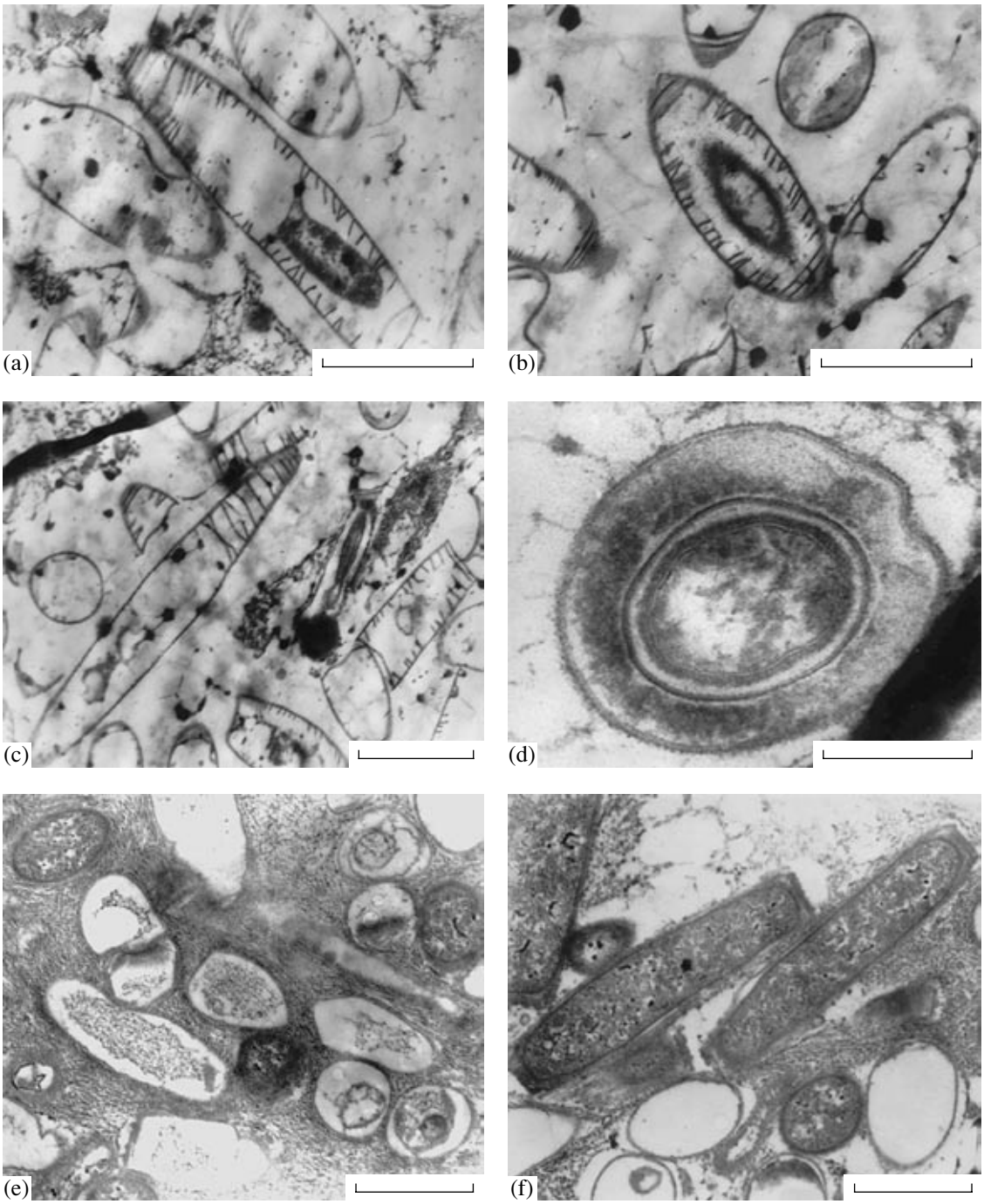

Fig. 2. Ultrastructure of microorganisms in the mats on the aragonite construction. The bar in (e) corresponds to 0.5 μm; all other bars correspond to 1 μm. See text for other details.

$$SO_4^{2-} + CH_4 \longrightarrow HCO_3^- + HS^- + H_2O \text{ (MO–MG + SRB)}. \quad (3)$$

The authors also suggested several alternative mechanisms of anaerobic methane oxidation. One of them involves the formation of, instead of carbon dioxide, acetate and hydrogen (reaction (4)), which are further used by sulfate-reducing bacteria (reactions (5)–(7)).

Variant II:

$$2CH_4 + 2H_2O \longrightarrow CH_3COOH + 4H_2 \text{ (MO–MG)}, \quad (4)$$

$$4H_2 + SO_4^{2-} + H^+ \longrightarrow HS^- + 4H_2O \text{ (SRB)}, \quad (5)$$

$$CH_3COOH + SO_4^{2-} \longrightarrow 2HCO_3^- + HS^- + H^+ \text{ (SRB)}, \quad (6)$$

$$2CH_4 + 2SO_4^{2-} \longrightarrow 2HCO_3^- + 2HS^- + 2H_2O \text{ (MO–MG + SRB)}. \quad (7)$$

A third variant, proposed by Zender and Brock [5] and Hoehler *et al.* [20], also involves the formation of acetate in the reaction of methane with bicarbonate; however, hydrogen is not formed (reactions (8)–(10)).

Variant III:

$$CH_4 + HCO_3^- \longrightarrow CH_3COOH + H_2O \text{ (MO–MG)}, \quad (8)$$

$$CH_3COOH + SO_4^{2-} \longrightarrow 2HCO_3^- + HS^- \text{ (SRB)}, \quad (9)$$

$$CH_4 + SO_4^{2-} \longrightarrow HCO_3^- + HS^- + H_2O \text{ (MO–MG + SRB)}. \quad (10)$$

Thermodynamically, variant II is more beneficial than III; however, at a methane pressure of 50 atm, the production of acetate from methane and bicarbonate also provides for a biologically sufficient amount of energy (for ATP formation, ~20 kJ/mol is required).

Based on the data on the stable-isotopic composition of the oxygen ($\delta^{18}O$) of water, sulfates, carbonates of constructions, and bottom sediment bicarbonates in the regions of methane seeps in the Black Sea, Lein and coworkers [7] confirmed the feasibility of the variant III mechanism of anaerobic methane oxidation. Judging from its $\delta^{13}C$ values (up to 83.8‰), the carbon of carbonates and organic matter of the constructions originates from the carbon of methane [19, 3], which in these regions is mainly oxidized anaerobically [19]. It turned out [7] that the $\delta^{18}O$ values of these autigenic carbonates are close to the $\delta^{18}O$ value of the bottom sediment bicarbonate but not to those of the Black Sea water or sulfates (the $\delta^{18}O$ values are 0.4–1.2‰ for carbonates, –0.5 to –3.2‰ for bicarbonates, –31.5 to –33.1‰ for water, and –15.5 to –20.0‰ for sulfates). It was with good reason inferred [7] that it is the bicarbonate oxygen that is used by the microbial community for methane oxidation under anaerobic conditions.

The distribution and composition of lipid components in the bacterial mats at methane seeps and in bottom sediments [13] of the Black Sea (the C_{20} isoprenoid crocetane, the C_{25} isoprenoid pentamethylicosan, and C_{40} isoprenoids) suggest the predomination of methanogenic archaea in these ecological niches. Thiel *et al.* [12] believe that the high content of *iso*- and *anteiso*-branched fatty acids indicates the presence of sulfate-reducing bacteria in these econiches. In both of the above-cited papers, data are presented on the stable-isotopic composition of the carbon of the lipids; these data are indirect evidence of the origination of these lipids from methane that underwent biological oxidation under anaerobic conditions.

Phylogenetic analysis of archaeal 16S rDNA sequences retrieved from samples of bottom sediments of Eel River Basin (California), where active anaerobic methane oxidation was demonstrated, revealed the presence of methanogens of the orders *Methanosarcinales* and *Methanomicrobiales*, which, however, were remote from the known species of these orders [14, 15]. On the other hand, the sediments rich in methane hydrates and exhibiting considerable rates of sulfate reduction were shown by German researchers [16] to contain bacterial conglomerates consisting of cells of archaea surrounded by sulfate-reducing bacteria. The authors assume that the sulfate reducers probably belong to *Desulfosarcina/Desulfococcus.* The presence of the archaeal and sulfate-reducing components in these conglomerates was confirmed by fluorescent in situ hybridization with 16S rRNA–targeted nucleotide probes. However, specially designed experiments failed to confirm the affiliation of the archaea present in the conglomerates with *Methanosarcinales* or *Methanomicrobiales* [16].

Lovley and Phillips [9] studied anaerobic oxidation of various organic compounds at the expense of different forms of iron oxyhydroxide (hematite, goethite, akaganeite, and amorphous oxyhydroxide) in reduced freshwater and saline sediments of the Potomac River estuary. The reduction of Fe(III) to Fe(II) was shown to occur during mineralization of butyrate, ethanol, methanol, and trimethylamine but not methane. Potekhina and coworkers [10] reported "anaerobic" growth at the expense of FeOOH of the obligately aerobic methanotrophic bacterium *Methylomonas methanica* strain 12, which had been isolated and characterized by us [3, 11]. However, Potekhina and coauthors did not present direct evidence (chromatographic, radioisotopic, or stable-isotopic) of the oxidation of CH_4 to CO_2 or of CH_4 carbon incorporation by the culture under anaerobic conditions.

I performed a series of experiments with radiolabeled methane that aimed to reveal the ability of *Methylomonas methanica* strain 12 to oxidize methane under anaerobic conditions in the presence of FeOOH. Data of Table 4 show that in the experiments where the anaerobic conditions were rigorously maintained, no methane oxidation by *Methylomonas methanica* strain 12 at the expense of FeOOH occurred. In the controls, the background radioactivity strongly correlated with the radioactivity of the solution of $^{14}CH_4$ introduced (in the second experiment, a twofold less radioactive solution of labeled methane was introduced).

The high amounts of labeled carbon dioxide revealed in experimental variant I (two orders of magnitude higher than in other experimental variants) can be explained by the presence of traces of oxygen. To maintain anaerobiosis, Potekhina and coworkers used in their experiments argon that did not undergo purification from oxygen over a copper catalyst at 800°C (personal communication). Commercial argon is

Table 4. Oxidation of $^{14}CH_4$ to $^{14}CO_2$ (10^3 cpm) by cells of *Methylomonas methanica* 12 under anaerobic conditions in the presence of amorphous FeOOH

Exposure, h	Experimental variant I		Experimental variant II		Experimental variant III	
	Control	Experiment	Control	Experiment	Control	Experiment
0.5	11.6	2029.9	4.7	10.3	12.7	11.5
1	–	–	4.6	17.2	6.8	7.1
3	12.6	1816.6	5.1	10.8	–	–
6	18.7	2113.8	6.2	15.9	7.9	14.2
18	17.2	2193.3	7.2	7.2	12.1	10.4

Note: "–" stands for "not determined."

known to contain significant oxygen contamination, sometimes reaching 1–2%. This oxygen could have provided for the short-term process of methane oxidation to CO_2 by the aerobic bacterium *Methylomonas methanica* strain 12 observed in the first minutes in the "anaerobic" conditions of Potekhina's experiments; this assumption agrees with the lack in those experiments of a further increase in the radioactivity of labeled CO_2. Unfortunately, the discussed work of Potekhina *et al.* [10] does not contain data proving the microbiological purity of the experiments. Surely, the monitoring of culture growth in experiments yielding such disputable results should have been conducted via determination of protein or nucleic acid dynamics rather than via cell counting under a microscope. Moreover, the authors did not specify the method that they used to count bacterial cells sorbed on particles of amorphous iron hydroxide. Our experiments on direct radioisotopic determination of the occurrence of the process did not confirm the capacity of *Methylomonas methanica* strain 12 for "anaerobic methane oxidation coupled with Fe(III) reduction."

Thus, it follows from our data presented in this paper and from data available in the literature that, currently, no direct proof exists of the involvement of these or other physiological groups of microorganisms in anaerobic methane oxidation. Nevertheless, there are grounds to state that the involvement of a consortium of methanogenic archaea and sulfate-reducing bacteria in this microbiological (biogeochemical) process seems most substantiated and probable. The problem is, however, complicated by the fact that, most probably, different microbial communities with different mechanisms of anaerobic methane oxidation may be involved in this process under different physicochemical (ecological) conditions [6].

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