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EXPERIMENTAL ARTICLES

Anaerobic Methane Oxidation and Sulfate Reduction in Bacterial Mats on Coral-Like Carbonate Structures in the Black Sea

N. V. Pimenov and A. E. Ivanova[1]

Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia

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Abstract—A detailed study of the processes of anaerobic methane oxidation and sulfate reduction in the bacterial mats occurring on coral-like carbonate structures in the region of methane seeps in the Black Sea, as well as of the phenotypic diversity of sulfate-reducing bacteria developing in this zone, has been performed. The use of the radioisotopic method shows the microbial mat structure to be heterogeneous. The peak activity of the two processes was revealed when a mixture of the upper (dark) and underlying (intensely pink) layers was introduced into an incubation flask, which confirms the suggestion that methanotrophic archaea and sulfate-reducing bacteria closely interact in the process of anaerobic methane oxidation. Direct correlation between the rate of anaerobic methane oxidation and the methane and electron acceptor concentrations in the medium has been experimentally demonstrated. Several enrichment and two pure cultures of sulfate-reducing bacteria have been obtained from the near-bottom water and bacterial mats. Both strains were found to completely oxidize the substrates to CO_2 and H_2S. The bacteria grow at temperatures ranging from –1 to 18 (24)°C, with an optimum in the 10–18°C range, and require the presence of 1.5–2.5% NaCl and 0.07–0.2% $MgCl_2 \cdot 6H_2O$. Regarding the aggregate of their phenotypic characteristics (cell morphology, spectrum of growth substrates, the capacity for complete oxidation), the microorganisms isolated have no analogues among the psychrophilic sulfate-reducing bacteria already described. The results obtained demonstrate the wide distribution of psychrophilic sulfate-reducing bacteria in the near-bottom water and bacterial mats covering the coral-like carbonate structures occurring in the region of methane seeps in the Black Sea, as well as the considerable catabolic potential of this physiological group of psychrophilic anaerobes in deep-sea habitats

Key words: methane seeps in the Black Sea, microbial mats, anaerobic methane oxidation, sulfate reduction, psychrophilic bacteria.

The phenomenon of methane seepage in the Black Sea was discovered in the late 1980s [1]. Since then, the researchers of the Institute of the Biology of Southern Seas, National Academy of Sciences of Ukraine, have pinpointed many hundreds of methane seeps, located predominantly at the continental slope in the northwestern part of the Black Sea [2]. It was shown in the 1990 expedition on board the *Bentos-300* submarine laboratory that coral-like carbonate structures, which may be several meters high, are formed in the anaerobic zone (at depths more than 160–170 m) of the regions of methane seepage [3]. The structures are covered with dense bacterial mats of up to several centimeters thick. The extremely light isotopic composition of the carbon found in these bacterial mats (the $\delta^{13}C$ value varies between –75.6 and –83.3‰ [3]) unambiguously indicates the involvement of methane carbon in the formation of the microbial biomass. Later, radioisotopic, isotopic–geochemical, and molecular–biological data substantiating anaerobic methane oxidation by a community of archaea and sulfate-reducing bacteria were presented in a series of works [4–7]. Moreover, it was shown that the formation of the carbonate structures in the region of methane seeps is a consequence of a large-scale microbial process of anaerobic methane oxidation resulting in the formation and sedimentation of light-isotope carbonates [5].

The study of methane seeps was continued in July 2001 using the German habitable apparatus *Jago*, capable of sinking to a depth of 400 m. The aim of our work was to carry out an in detail study of the process of microbial methane oxidation and sulfate reduction in the bacterial mats, as well as to obtain enrichment and pure cultures of the sulfate-reducing bacteria forming part of the microbial community developing on the coral-like carbonate structures. Considering the environmental conditions in the region of the Black Sea methane seeps, the investigation was focused on the search for psychrophilic and psychrotolerant sulfate-reducing bacteria active at low temperatures.

[1] Corresponding author; e-mail: aivan@newmail.ru

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MATERIALS AND METHODS

Sampling. Samples of the carbonate structures with bacterial mats were collected in the zone of methane seeps at the northwestern shelf of the Black Sea from a depth of 182–326 m in July 2001, during the international expedition on board the research vessel *Professor Logachev*, as part of the GOSTDABS project. The sampling was carried out using a scoop furnished with a television camera (part of the standard equipment of the research vessel *Professor Logachev*) or a manipulator on the German habitable apparatus *Jago*. Immediately after being lifted on board the vessel, 2-cm^3 portions of bacterial mat samples were placed into 10-ml glass flasks completely filled with hydrogen sulfide–containing near-bottom water and closed with gas-tight stoppers made of butyl rubber.

Radioisotopic studies. The rates of the microbial processes occurring in the bacterial mats were determined on board the vessel with the radioisotopic method, using ^{14}C-methane, ^{14}C-acetate, ^{14}C-bicarbonate, and ^{35}S-sulfate. In order to perform the determination, 0.2 ml of labeled ^{14}C-methane (4 μCi), ^{14}C-bicarbonate (10 μCi), ^{14}C-acetate (20μCi, methyl group–labeled), or ^{35}S-sulfate (10 μCi), as well as the necessary amount of unlabeled methane, was injected into the bacterial mat samples through the rubber stoppers with a syringe. The samples were incubated in a refrigerator at 7–8°C for 4–6 h. When the incubation was completed, the samples were fixed with 0.5 ml of a saturated KOH solution or 1 ml of 25% glutaraldehyde. The samples into which the fixing solution was introduced before the addition of the labeled substrate served as controls. The samples were further treated at a stationary laboratory according to the technique described in detail previously [8, 9].

Obtaining enrichment and pure cultures of sulfate-reducing bacteria. The primary detection of cultivated sulfate-reducing bacteria in the bacterial mat and near-bottom water samples was carried out on board the vessel on the day of sampling by means of the SRB-BART™ (biodetector for sulfate-reducing bacteria) system (Droycon Bioconcepts Inc., Regina, SK, Canada, S4S 5H4). For this purpose, the sample to be studied (1 cm^3 of the mat or near-bottom water) was introduced into a plastic test tube containing a weighed portion of an anhydrous nutrient medium, and the cultures were incubated in the refrigerator at 7–8°C for 10–15 days. Darkening of the originally colorless commercial medium occurred in case of the development of sulfate reduction.

The work was continued at the stationary laboratory. The method of end-point dilutions in a liquid medium was used to determine the cell numbers and obtain enrichments of the sulfate-reducing bacteria. An inoculate aliquot (0.5 ml of the contents of the positive test tubes) was introduced with a sterile syringe into a Hungate tube with an anaerobic mineral medium for marine bacteria [10] supplemented with lactate (10 mM), acetate (10 mM), or butyrate (10 mM), and a series of sequential dilutions was prepared in the same medium. For some samples, inoculations into media with propionate (10 mM) or sodium benzoate (5 mM) and methanol (10 mM) were made. The tubes were incubated at 4, 10, 16, 21, and 28°C. The development of the sulfate-reducing bacteria was judged from the hydrogen sulfide increment in tenfold dilutions during the subsequent 24 months.

Pure cultures from single colonies were isolated from a solid medium according to the modified method of dilutions in a deep agar layer [11] to avoid the long-term effect of increased temperatures on the bacteria. The culture purity was controlled microscopically and by inoculating the medium that contained 0.1% yeast extract, 0.1% tryptone, and 0.1% glucose.

The study of cell morphology. The morphology of the microbial cells was studied in living and fixed preparations under a Jenaval light microscope equipped with a phase-contrast device at a 1500× magnification. The cell size was determined in the middle of the stationary phase of culture growth with an ocular screw micrometer.

Analytical methods. The growth of significantly enriched nutrient cultures and pure cultures was recorded by a direct cell count. The hydrogen and carbon dioxide present in the gas phase, as well as the C_2–C_4 fatty acids, were assayed by gas chromatography [12]. Soluble sulfides and the free hydrogen sulfide content were determined by the colorimetric method, using *N*,*N*-dimethyl-1,4-phenylenediamine chloride [13].

RESULTS

The rates of biogenic processes. Anaerobic methane oxidation was revealed in all the bacterial mat samples studied (Table 1). The experiments used near-bottom water sampled by the *Jago* apparatus in immediate proximity to the methane seeps at a depth of 226 m. The methane content in this water was about 80 μl/l. The addition of 1.5 μl/l of [^{14}C]methane to the experimental flask barely altered the methane content in the sample.

The addition of different amounts of unlabeled methane to the incubation flasks led to a substantial change in the methane oxidation rate. As follows from the data listed in Table 1, methane oxidation by the bacterial mat community was significantly activated at a methane concentration of 160 μl/l and higher. Therefore, further experiments exploring the stimulation of the process of methane oxidation were performed at a methane concentration of 320 μl/l.

The distribution of [^{14}C]carbon in the methane oxidation products (Table 1) showed that the bulk of the methane was oxidized to carbon dioxide. The biomass and soluble organic matter incorporated no more than 25% of the methane carbon consumed.

Figure 1 shows the dynamics of methane consumption. It can be seen that the rate of methane oxi-

Table 1. Rate of anaerobic methane oxidation in the bacterial mats*

Station no./depth, m; coordinates	Amount of unlabeled methane introduced, μl/l	Methane oxidation, nl CH_4/(cm^3 h)
14/182 44°46.647′ N 31°58.877′ E	Without addition	0.12 (0)**
	320	3.23 (2.5)
55/226; 44°46.479′ N 31°59.530′ E	Without addition	0.08 (0)
	80	0.33 (6)
	160	0.91(15)
	320	5.08 (7)
	640	15.57 (25)
21/230 44°46.455′ N 31°59.674′ E	Without addition	0.08 (0)
	320	4.91 (8)
10/235 44°46.515′ N 31°59.559′ E	320	1.35 (7)
68/326 44°44.112′ N 31°47.309′ E	Without addition	0.10 (0)
	320	3.40 (6)

* Determinations were carried out at the stationary laboratory at the Institute of Microbiology, Russian Academy of Sciences, by I.I. Rusanov.

** The percentage of methane carbon incorporation into organic matter is shown in brackets.

dation was virtually constant over the first 12 h of incubation and then decreased.

In a number of earlier publications [2, 4, 5] it was noted that the bacterial mats are pink in color. However, viewing the video records made with the *Jago* apparatus, as well as the observers' reports, showed that the bacterial mats on the carbonate structures are characterized by a layered structure. The external layer of the bacterial mats directly in contact with the seawater appears to be quite a dense black formation. The thickness of this external layer did not normally exceed 0.5 cm. A looser layer, of an intense pink color, occurs at a deeper level. The thickness of this layer changed depending on the size of the structure and could reach several centimeters. In the lowermost mat layer, the coloration became paler due to the appearance of a large amount of carbonate granules, of which the structure is formed.

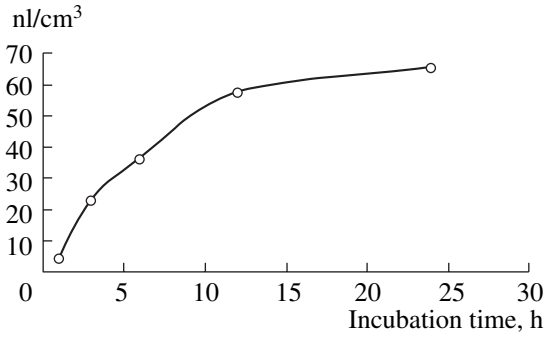

Fig. 1. Dynamics of methane oxidation in the microbial mats.

We staged a series of experiments to investigate the rate of anaerobic methane oxidation in the different layers of the microbial mats (Fig. 2). The highest rate of methane oxidation was recorded in samples composed of a mixture of the black and pink mat layers. Taken separately, the black and pink layers were characterized by a lower intensity of methane oxidation.

Along with the investigation of the rate of methane oxidation, the rate of sulfate reduction was determined using [^{35}S]sulfate (Fig. 3). The lowest activity was recorded in the lower pink layer of the bacterial mats. The maximum rate of sulfate reduction was revealed in the samples in which the dark surface layer and the intensely pink layer immediately underlying it were present simultaneously. The addition of 1 ml of unlabeled methane to the incubation flasks led to a sharp increase in the rate of sulfate reduction (Table 2).

We attempted to measure the rates of autotrophic and aceticlastic methanogenesis in the microbial mats. However, in short-term experiments (10–20 h of incubation), weak aceticlastic methanogenesis with a rate not exceeding 0.01 nl/(cm^3 h) was recorded only in single samples.

The occurrence of sulfate-reducing bacteria in the samples of near-bottom water and bacterial mats on the carbonate structures. Along with the determination of the rates of microbial processes, sulfate-reducing bacteria were revealed in the bacterial mats by inoculating the nutrient media. As early as the third day of incubation of the tubes containing a commercial medium, bacterial growth was noted in some of them. The most active development of sulfate-reducing bacteria occurred in the tubes into which the upper, dark-colored, part of the bacterial mat was introduced. After 15 days of incubation, bacterial sulfate reduction was revealed in all of the samples studied. Five tubes with considerable sulfide formation (Table 3) were selected for a detailed study of the microbial population.

Obtaining enrichment and pure cultures of sulfate-reducing bacteria. Inoculation of the media with certain organic substrates with aliquots of serial dilutions of the primary cultures (Table 3, 1–5), followed by cultivation at different temperatures, revealed some specific features of the population of marine sulfate-reducing bacteria. On all of the utilizable substrates, bacterial growth, accompanied by the formation of a considerable amount of sulfide, was noted at 4 and 10°C. As a rule, the maximum cell number was recorded at a cultivation temperature of 16°C. With an increase in temperature to the next test point (21°C), the bacterial numbers decreased, while growth at 28°C was observed only in single tubes. Exceptions to this pattern

were the sample 1 dilutions in a medium with propionate, in which the bacteria did not develop at a temperature of 16°C and incubation at 10°C yielded the maximum number of sulfate-reducing bacteria, and cultures in a medium with acetate, where the bacterial number was 10^2–10^4 cells/ml at 21°C and was lower at other cultivation temperatures.

The organisms depending on lactate and butyrate for growth were present in all of the samples at the amount of 10^4–10^6 cells/ml. The sulfate-reducing bacteria using propionate were revealed episodically, and their number did not exceed 10^2 cells/ml. Growth with acetate as the single source of carbon and energy was very poor and was insignificantly stimulated by the addition of yeast extract or Casamino acids (100 mg/l). The first signs of activity of the sulfate-reducing bacteria (a hydrogen sulfide increment of 15–20 mg/l) in the acetate medium were observed after five months of incubation at 21°C and after nine or more months of incubation at 28, 10, or 4°C. Despite its extremely low rate, sulfide formation continued during the subsequent months. When media with methanol and benzoate were used, no significant H_2S increment was revealed in any of the samples studied despite long-term cultivation (24 months).

Microscopic studies showed the development of sulfate-reducing bacteria whose cell shape was vibrioid or spiral-like (lactate, ethanol, and formate media), rod-like (butanol, formate, butyrate, crotonate media), spherical to lemonlike (propionate medium), or oval (media with valerate, caproate, and some other substrates). Irrespective of the substrate used, the primary enrichment cultures revealed a small amount of spirochete-like cells and slightly curved filaments, which were most probably satellites and appeared to be unstable during subsequent culture transfers. Spore-forming bacteria were not prevalent. No cell aggregates or multicellular filaments typical of the genera *Desulfosarcina* or *Desulfonema* occurred on media containing any substrate. A particularly large morphological diversity was observed in the formate medium when inoculated with any of the samples and in the valerate medium when inoculated with sample 3. Many of the enrichment cultures of the sulfate-reducing bacteria were close in their cell phenotypes, and, at this investigation stage, we regarded them as identical. At the same time, others differed in at least one of the characteristics considered. Based on preliminary phenotypic identification, 11 enrichment cultures were selected for further study.

Pure cultures of psychrophilic sulfate-reducing bacteria, designated as strain 3BSr24 and strain 2VSr10, were isolated on media containing *n*-butanol (12 mM) and sodium valerate (5 mM) from enrichment cultures that originated from the primary inoculation of the lactate medium with sample 3 and the butyrate medium with sample 2, with cultivation at 10 and 6°C, respectively.

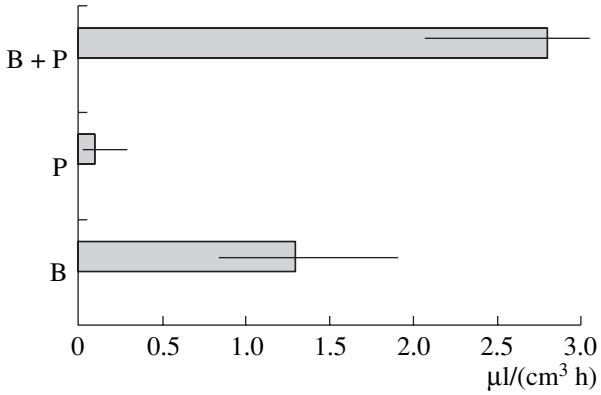

Fig. 2. Methane oxidation in different microbial mat layers. B + P indicates a mixture of the black and pink layers in equal amounts; P, the pink mat layer; and B, the black mat layer. The line shows the dispersion in the methane oxidation rate values in independent measurements.

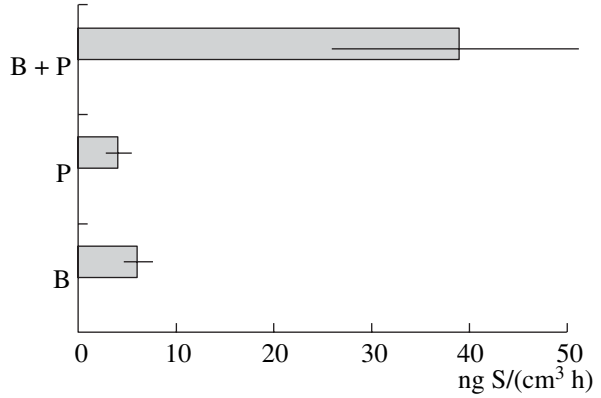

Fig. 3. Rate of sulfate reduction in different microbial mat layers. B + P indicates a mixture of the black and pink layers in equal amounts; P, the pink mat layer; and B, the black mat layer. The line shows the dispersion in the sulfate reduction rate values in independent measurements.

Physiology of culture growth. As was shown by the studies of significantly enriched and pure cultures, psychrophilic microorganisms formed the basis of the sulfate-reducing community studied. Most of the cultures were obtained at 4–10°C, and only three could grow at 28°C (Table 4). Even those enrichment cultures that did

Table 2. Rate of sulfate reduction in the microbial mats

Station no./depth, m	Sulfate reduction rate, ng S/(cm^3 h)	
	without methane addition	with the addition of 1 ml of CH_4
14/182	9.6	–
55/226	4.6	42
21/230	25	137
85/295	6.9	–
68/326	102	157

Note: "–" means "no data are available".

Table 3. Characteristics of the sampling points and bacterial mat samples used to obtain enrichment and pure cultures of marine sulfate-reducing bacteria

Designation*	Sampling site at the northwestern shelf of the Black Sea	Sample characteristics
1	Station 38; depth, 235 m	Bacterial mat from the carbonate structures occurring in the hydrogen-sulfide–containing zone (H_2S, 2–3 mg/l)
2	Station 68; depth, 326 m	Near-bottom water from the bathometer, sampled by *Jago* at a distance of several meters from the methane seeps
3	Station 68; depth, 326 m	Bacterial mat with near-bottom water
4	Station 55; depth, 226 m	The same as variant 3
5	Station 85; depth, 295 m	The same as variant 4

* Tubes with primary cultures obtained from the samples on a commercial medium and selected for further studies of sulfate-reducing bacteria.

Table 4. The effect of temperature on the growth of the enrichment and pure cultures of marine psychroactive sulfate-reducing bacteria (SRB) from the samples of bacterial mats

Sample	The SRB cultures obtained	Cell shape*	Substrate (temperature, °C)**	SRB growth at a temperature (°C) of					
				4	10	16	21	28	37
1	1HSr5	R	$H_2 + CO_2$*** (10)	+	+	+	+	–	–
	1BtSr5	O	Butyrate (10)	+	+	+	+	–	–
	1CapSr8	R	Caproate (8)	+	+	+	–	–	–
	1CrSr5	S	Crotonate (10)	+	+	+	–	–	–
	1FSr6	R	Formate*** (4)	+	+	+	+	–	–
	1PSr4	S	Propionate (4)	+	+	–	–	–	–
2	Strain 2VSr10	O	Valerate (6)	+	+	+	–		–
3	3HSr6	S	$H_2 + CO_2$*** (4)	+	+	+	–	–	–
	Strain 3BSr24	R	Butanol (10)	+	+	+	+	–	–
	3VSr6	R	Valerate (18)	–	+	+	+	+	–
4	4FSr6	R	Formate*** (16)	–	+	+	+	+	–
5	5FSr5	R	Formate*** (8)	+	+	+	–	–	–
	5EtSr6	V	Lactate (10)	+	+	+	+	–	–

Note: "+" denotes occurrence of growth at a particular temperature; "–" denotes absence of growth.
* The shape of the bacterial cells: R stands for rodlike; O, for oval; V, for vibrioid; and S, for spherical or lemonlike.
** The carbon substrate and the cultivation temperature used for the enrichment (or isolation) of sulfate-reducing bacteria.
*** 2 mM of acetate was added as the source of carbon.

not grow at 4°C (cultures of 3VSr6 and 4FSr6) were capable of sulfate reduction at this and even lower temperatures (the minimal temperature tested was –1°C), and, with an insignificant increase in the cultivation temperature (to 6–8°C), weak cell growth did occur. Nevertheless, despite the capacity of some of the cultures for growth at 28–30°C, their growth optimum was considerably lower (19 and 22°C for enriched cultures 3VSr6 and 4FSr6, respectively). At 37°C, neither growth nor sulfate reduction were observed.

Chromatographic analysis of the metabolic products showed that most of the cultures were characterized by the accumulation of acetate in the medium; no active utilization of acetate occurred subsequently. These results should be regarded as preliminary, and are to be verified in further work with pure cultures of the bacteria studied.

Some specific phenotypic features of the pure cultures of psychrophilic sulfate-reducing bacteria. Strain 3BSr24 cells were motile straight or slightly curved rods with rounded ends, and they stained gram-positive. While they had an almost constant cell width (2.2–2.4 μm), their length varied considerably, from 4.6 to 8.1 μm. In some of the cells, gas vacuoles were present. In the process of sulfate reduction, the bacterium formed an appreciable amount of extracellular mucous material, and the medium became more viscous.

The cells of strain 2VSr10 were oval-shaped, measured 2.6–2.8 × 3.0–4.2 μm in size, and were characterized by an absence of motility. The growth of this bac-

terium was accompanied by the formation of short chains, irregular aggregates, and cell aggregates remotely resembling the rosettes of the methanotrophic bacterium *Methylosinus trichosporum.* The bacteria stained gram-negative.

We performed a chromatographic analysis of the oxidation products formed upon the oxidation of valerate by strain 2VSr6 and the oxidation of butanol, butyrate, caproate, and crotonate by strain 3BSr24. In all cases, the only metabolic products were H_2S and CO_2; acetate was not accumulated in the medium. Despite the ability of the bacteria to completely oxidize organic substrates, acetate failed to support growth. Some of the phenotypic characteristics of the new isolates (the required mineral composition of the cultivation medium, the growth pH and temperature values, the electron acceptors, and the spectrum of the substrates used) are listed in Table 5.

DISCUSSION

At the present time, there are a series of studies available presenting evidence that archaea (ANMI, a cluster of the subdivision *Euryarchaeota*) and sulfate-reducing bacteria (the *Desulfosarcina–Desulfococcus* group) are the dominant members of the microbial community that drives large-scale methane oxidation in the anaerobic zone of the Black Sea in the regions of methane seeps [6, 7, 14, 15]. In our experiments, it was shown by radioisotopic analysis, that the structure of the microbial mats on the carbonate structures is heterogeneous. The slow rate of anaerobic methane oxidation in the dark upper layer and the results of the direct experiments with $^{35}SO_4^{2-}$ lead us to suggest that sulfate-reducing bacteria dominate in this layer. The intensely pink layer, where the sulfate reduction rate is considerably lower, lies underneath. The pink layer seems to consist of the biomass of anaerobic methanotrophic archaea. According to our observations, only the pink mat portion adjacent to the dark layer should be considered as active. The underlying mat portion, whose thickness can sometimes reach several centimeters, is inactive, and no $^{14}CO_2$ or $H^{35}S^-$ formation was noted in this layer upon incubation with labeled methane and sulfate.

The layer-by-layer measurement of methane oxidation and sulfate reduction activities showed that the highest rates of these two processes are recorded when a mixture of the dark and pink layers is introduced into the incubation flasks. Thus, direct radioisotopic experiments confirm the suggestion that methanotrophic archaea and sulfate-reducing bacteria interact closely in the process of anaerobic methane oxidation. It is possible that the denser dark layer prevents rapid gas exchange with the surrounding seawater and contributes to the maintenance of high methane concentrations in the zone of its oxidation.

Table 5. Some phenotypic characteristics of the new strains of marine psychrophilic sulfate-reducing bacteria

Characteristic	New strains	
	3BSR24	2VSR10
Optimal concentration of salts (%):		
NaCl	1.5–2.5	2.0–2.5
$MgCl_2 \cdot 6H_2O$	0.07–0.2	0.1–0.3
Optimum pH	7.2–7.4	7.4–7.6
Temperature range (optimum) for growth, °C	from –1 to 24 (16–18)	from –1 to 18 (10–12)
Temperature range (optimum) for sulfate reduction interval, °C	from –1 to 30	from –1 to 24
Electron donors:		
$(H_2 + CO_2)$ + acetate	++	++
formate	++	++
acetate	–	–
propionate	+–	+++
butyrate	+++	++
valerate	–	+++
caproate	+++	–
palmitate	ND	++
stearate	–	ND
crotonate	++	+++
lactate	++	++
pyruvate	++	++
malate	+–	ND
succinate	++	–
fumarate	++	–
ethanol	+–	++
propanol	–	++
butanol	+++	–
alanine	+–	–
asparagine	+–	–
glutamate	+–	–
Electron acceptors:		
sulfate*	+	+
thiosulfate	+	+
sulfite	+	+
Fe(III) citrate	+	–
anthraquinone-2,6-disulfonate	+	–
Capacity for autotrophic growth with $H_2 + CO_2$	+	–

Note: "+" denotes the presence of a characteristic, "–" denotes absence of a characteristic, and "ND" means "no data available".

* With sulfate as an electron acceptor, none of the strains oxidized methanol, phenol, benzoate, nicotinate, cyclohexane carboxylate, glucose, fructose, peptone, or yeast extract. "+++" denotes rapid culture growth, "++" denotes slow growth, and "+–" denotes very poor and slow growth with insignificant accumulation of H_2S.

It should also be noted that the high rate of anaerobic methane oxidation was observed only when there were high methane and electron acceptor (sulfate) concentrations in the medium (Table 1). Hence, in natural habitats, the large-scale processes of anaerobic methane oxidation should be sought for in methane-enriched anaerobic zones containing the sulfate ions required for the development of sulfate-reducing bacteria. The fact that all the known sites where high rates of anaerobic methane oxidation are found are situated in methane-enriched anaerobic water columns or marine bottom sediments supports this suggestion [16, 17].

In deep-sea habitats, characterized by permanently low temperatures (2–8°C), sulfate is one the most readily available electron acceptors used in the process of the anaerobic degradation of organic matter. The high rates of the process of sulfate reduction in situ allow us to suggest the existence of microorganisms optimally adapted to these specific conditions. At the same time, most of the known sulfate-reducing bacteria are incapable of actively functioning at temperatures characteristic of anaerobic sea depths (<8°C) [10]. The psychrophilic representatives of this physiological group of microorganisms have been described only in recent years [11, 18], and their presence in river estuaries and marine arctic sediments has been shown [19, 20]. The isolated bacteria are represented by four genera that include six species. They grow in a broad temperature range (–1.8 to 27°C), with the optimum being at 7–19°C, and metabolize a wide spectrum of substrates. Except for the bacterium *Desulfofrigus oceanense*, all of the isolates are incomplete oxidizers whose metabolic end product is acetate.

We obtained microbiological evidence of the existence of a community of sulfate-reducing microorganisms with a psychrophilic growth potential (below 20°C) and extensive metabolic abilities in the region of methane seeps found in the Black Sea at a depth of over 180 m. The psychrophilic and psychrotolerant sulfate-reducing bacteria adapted to the environmental conditions dominate in the community. This fact is especially interesting because the mild conditions of the experiments on the primary detection of marine sulfate-reducing microorganisms (7–8°C) did not exclude mesophiles, which could be retained in a viable state with subsequent development at more favorable temperatures.

This work demonstrates the prevalence of psychrophilic sulfate-reducing bacteria in the near-bottom water and bacterial mats covering the coral-like carbonate structures occurring in the Black Sea, as well as the diversity of their phenotypic properties. The pure cultures we isolated, along with the characteristics described for other psychrophilic isolates, have a number of novel distinctive features: the capacity for autotrophic growth with hydrogen; a specific spectrum of oxidized substrates; the ability to use anthraquinone-2,6-disulfonate, an analogue of humic acid, as an electron acceptor alternative to sulfate; and other properties. These microorganisms are capable of complete oxidation of various low-molecular-weight substrates representing a wide spectrum of bacterial fermentation products, with the formation of hydrogen sulfide and carbon dioxide as the only end products; thus, they can provide for the effective mineralization of organic matter. It should be noted that hydrogen, as well as many organic compounds such as lactate, propionate, valerate, and some amino acids used by the new isolates for sulfate reduction, may act as the substrates for sulfate reduction within marine sediments in situ [21].

However, as far as the real carbon substrates for sulfate-reducing bacteria in low-temperature marine habitats are concerned, many problems remain unsolved. Among other things, the role of acetate under these conditions has not yet been determined. In marine ecosystems, acetate is traditionally considered to be the main substrate, accounting for no less than 70% of all the sulfate reduced [22]. On the other hand, some authors [21] emphasize the relationship between the rate of sulfate reduction and the substrates used for this process. The inhibitory analysis and radioisotopic studies we performed allowed us to establish that the importance of individual compounds varied in different habitats. In estuaries and within marine sediments, where the sulfate reduction rate is quite high, acetate is not the dominant substrate, and other sources of carbon and energy (hydrogen, lactate, propionate, *n*- and *iso*-butyrate, *iso*-valerate, and some amino acids) play a significant role in sulfate reduction [21, 22].

Our results on the phenotypic diversity of the sulfate-reducing bacteria found in the region of the Black Sea methane seeps indirectly confirm the point of view of Parkes *et al.* [21] (see above) and agree with the data of other authors who have studied the population of sulfate-reducing bacteria in sediments taken from arctic marine habitats [18–20]. The psychrophilic sulfate-reducing bacteria (the maximum cell number is recorded at temperatures below 16°C) that mediate incomplete oxidation of organic substrates predominate in the composition of the microbial community developing on coral-like structures and in near-bottom water.

The cell number of acetate-utilizing microorganisms is, on average, two orders of magnitude lower than that of microorganisms using lactate as the substrate. The maximum cell number and high growth rates are attained at higher temperatures (21°C). Inoculation of the nutrient media with the bacterial mat samples failed to reveal sulfate-reducing bacteria specialized in acetate oxidation and/or capable of its rapid utilization (similar to the mesophilic representatives of the genus *Desulfobacter* and the spore-forming bacterium *Desulfotomaculum acetoxidans*).

Interestingly, in contrast to the psychrophilic isolates from arctic habitats, most of which were isolated on a lactate medium [18, 19], lactate was not the preferred substrate for the sulfate-reducing bacteria from

the region of the Black Sea methane seeps. At the same time, as is illustrated by this and several other studies, another substrate, formate, is actively metabolized by most psychrophilic cultures [11, 17]. Moreover, as is shown by our observations, it maintains the maximum morphological diversity in the microbial community studied. Taking into account these circumstances, as well as the fact that, from the point of view of the bioenergetics and kinetics of the process, it is formate that is the most suitable interspecies electron carrier in anaerobic methane oxidation [23], this substrate seems to merit much attention.

The contributions of psychrophilic sulfate-reducing bacteria to the decomposition of aromatic compounds and methanol, an important one-carbon substrate, also remain an unsolved problem. None of the earlier described [11, 18] or newly isolated (this investigation) psychrophilic isolates are able to oxidize these substrates for the purpose of sulfate reduction. We suggest that a correlation of the data on the qualitative and quantitative composition of the biologically available sources of carbon with the rate of the process of sulfate reduction may be useful for revealing the relationships between substrates and the presence of specific groups of sulfate-reducing bacteria in permanently cold marine habitats.

The data presented in this study show that the diversity of psychrophilic sulfate-reducing bacteria is not limited to the recently described taxa, and a further search for such bacteria, including those involved in the process of anaerobic methane oxidation, is undoubtedly a promising direction of investigation.

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REFERENCES

1. Polikarpov, G.G., Egorov, V.N., Nezhdanov, A.I., Gulin, S.B., Kulev, Yu.D., and Gulin, M.B., A Phenomenon of Active Gas Release from Elevations at the continental slope in the Western part of the Black Sea, *Dokl. Akad. Nauk Ukr. SSR, Ser. Biol.*, 1989, vol. 12, pp. 13–16.
2. Egorov, V.N., Polikarpov, G.G., Gulin, S.B., Artemov, Yu.G., Stokozov, N.A., and Kostova, S.K., Modern Concepts of Ecological Role of Methane Seeps at the Bottom of the Black Sea, *Morskoi Ekol. Zh.*, 2003, vol. 2, no. 3, pp. 5–26.
3. Ivanov, M.V., Polikarpov, G.G., Lein, A.Yu., Gal'chenko, V.F., Egorov, V.N., Gulin, S.B., Gulin, M.B., Rusanov, I.I., Miller, Yu.M., and Kuptsov, V.M., Biogeochemistry of the Carbon Cycle in the Zone of Methane Seeps in the Black Sea, *Dokl. Akad. Nauk SSSR*, 1991, vol. 320, no. 5, pp. 1235–1245.
4. Pimenov, N.V., Rusanov, I.I., Poglazova, M.N., Mityushina, L.L., Sorokin, D.Yu., Khmelenina, V.N., and Trotsenko, Yu.A., Bacterial Mats on Coral-like Structures at Methane Seeps in the Black Sea, *Mikrobiologiya*, 1997, vol. 66, no. 3, pp. 421–428.
5. Lein, A.Yu., Ivanov, M.V., Pimenov, N.V., and Gulin, M.B., Geochemical Peculiarities of the Carbonate Constructions Formed during Microbial Oxidation of Methane under Anaerobic Conditions, *Mikrobiologiya*, 2002, vol. 71, no. 1, pp. 89–102.
6. Michaelis, W., Seifert, R., Nauhaus, K., Treude, T., Thiel, V., Blumenberg, M., Knittel, K., Gieseke, A., Peterknecht, K., Pape, T., Boetius, A., Amann, R., Jorgensen, B.B., Widdel, F., Peckmann, J., Pimenov, N.V., and Gulin, M.B., Microbial Reefs in the Black Sea Fueled by Anaerobic Oxidation of Methane, *Science*, 2002, vol. 297, pp. 1013–1015.
7. Tourova, T.P., Kolganova, T.V., Kuznetsov, B.B., and Pimenov, N.V., Phylogenetic Diversity of the Archaeal Component in Microbial Mats on Coral-like Structures Associated with Methane Seeps in the Black Sea, *Mikrobiologiya*, 2002, vol. 71, no. 2, pp. 230–236.
8. Ivanov, M.V., Pimenov, N.V., Rusanov, I.I., and Lein, A.Yu., Microbial Processes of the Methane Cycle at the North-Western Shelf of the Black Sea, *Estuarine, Coastal Shelf Sci.*, 2002, vol. 54, pp. 589–599.
9. Lein, A., Pimenov, N., Guillou, C., Martin, J.-M., Lancelot, C., Rusanov, I., Yusupov, S., Miller, Yu., and Ivanov, M., Seasonal Dynamics of the Sulphate Reduction Rate on the North-Western Black Sea Shelf, *Estuarine, Coastal Shelf Sci.*, 2002, vol. 54, pp. 385–401.
10. Widdel, F. and Bak, F., Gram-Negative Mesophilic Sulfate-Reducing Bacteria, *The Prokaryotes, 2nd ed.*, Balows, H. *et al.*, Eds., New York: Springer, 1992, vol. 3, pp. 3352–3378.
11. Isaksen, M.F. and Teske, A., *Desulforhopalus vacuolatus* gen. nov., sp. nov., a New Moderately Psychrophilic Sulfate-Reducing Bacterium with Gas Vacuoles Isolated from a Temperate Estuary, *Arch. Microbiol.*, 1966, vol. 166, pp. 160–168.
12. Nazina, T.N., Rozanova, E.P., Belyaev, S.S., and Ivanov, M.V., *Khimicheskie i mikrobiologicheskie metody issledovaniya plastovykh zhidkostei i kernov neftyanykh mestorozhdenii* (Chemical and Microbiological Methods for the Analysis of Formation Waters and Cores from Oil Fields), Pushchino, 1988.
13. Trüper, H.G. and Schlegel, H.G., Sulphur Metabolism in *Thiorhodaceae.* I. Quantitative Measurements on Growing Cells of *Chromatium okenii, Antonie van Leeuwenhoek*, 1964, vol. 30, pp. 225–238.
14. Thiel, V., Peckmann, J., Richnow, H.H., Luth, U., Reitner, J., and Michaelis, W., Molecular Signals for Anaerobic Methane Oxidation in Black Sea Seeps Carbonates and a Microbial Mat, *Mar. Chem.*, 2001, vol. 73, pp. 97–112.
15. Schouten, S., Wakeham, S.G., and Damste, J.S.S., Evidence for Anaerobic Methane Oxidation by Archaea in

Euxinic Waters of the Black Sea, *Org. Geochem.*, 2001, vol. 32, pp. 1277–1281.

16. Boetius, A., Ravenschlag, K., Schubert, G.J., Rickert, D., Widdel, F., Gieseke, A., Amann, R., Jorgensen, B.B., Witte, U., and Pfannkuche, O., A Marine Microbial Consortium Apparently Mediating Anaerobic Oxidation of Methane, *Nature*, 2000, vol. 407, pp. 623–626.
17. Pimenov, N.V., Savvichev, A.S., Rusanov, I.I., Lein, A.Yu., and Ivanov, M.V., Microbiological Processes of Carbon and Sulfur Cycles at Cold Methane Seeps of the North Atlantic, *Mikrobiologiya*, 2000, vol. 69, no. 6, pp. 831–843.
18. Knoblauch, C., Sahm, K., and Jorgensen, B.B., Psychrophilic Sulfate-Reducing Bacteria Isolated from Permanently Cold Arctic Marine Sediments: Description of *Desulfofrigus oceanense* gen. nov., sp. nov., *Desulfofaba gelida* gen. nov., sp. nov., *Desulfotalea psychrophila* gen. nov., sp. nov. and *Desulfotalea arctica* sp. nov., *Int. J. Syst. Evol. Bacteriol.*, 1999, vol. 49, pp. 1631–1643.
19. Knoblauch, C., Jorgensen, B.B., and Harder, J., Community Size and Metabolic Rates of Psychrophilic Sulfate-Reducing Bacteria in Arctic Marine Sediments, *Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 4230–4233.
20. Sahm, K., Knoblauch, C., and Amann, R., Phylogenetic Affiliation and Quantification of Psychrophilic Sulfate-Reducing Isolates in Marine Arctic Sediments, *Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 3976–3981.
21. Parkes, R.J., Gibson, G.R., Mueller-Harvey, J., Buckingham, W.J., and Herbert, R.A., Determination of the Substrates for Sulfate-Reducing Bacteria within Marine and Estuarine Sediments with Different Rates of Sulfate Reduction, *J. Gen. Microbiol.*, 1989, vol. 135, pp. 175–187.
22. Sorensen, J., Christensen, D., and Jorgensen, B.B., Volatile Fatty Acids and Hydrogen as Substrates for Sulfate Reducing Bacteria in Anaerobic Marine Sediment, *Appl. Environ. Microbiol.*, 1981, vol. 42, pp. 5–11.
23. Sorensen, K.B., Finster, K., and Ramsing, N.B., Thermodynamic and Kinetic Requirements in Anaerobic Methane Oxidizing Consortia Exclude Hydrogen, Acetate, and Methanol as Possible Electron Shuttles, *Microb. Ecol.*, 2001, vol. 42, pp. 1–10.