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EXPERIMENTAL ARTICLES

Phylogenetic Diversity of the Sulfur Cycle Bacteria in the Bottom Sediments of the Chersonesus Bay

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Abstract—The Black Sea is the largest meromictic basin, in the bottom sediments of which a powerful biogenic process of sulfide production occurs. The goal of the present work was to obtain data on phylogenetic diversity of the sulfur cycle microorganisms (sulfate-reducing and sulfur-oxidizing bacteria) in the Black Sea coastal gas-saturated bottom sediments. The samples were collected in the Chersonesus (Blue) Bay near Sevastopol from whitish bacterial mats of sulfurettes, and from the upper layer of the nearby seabed. Using DNA isolated from the native samples and obtained enrichment cultures, PCR analysis was performed with oligonucleotide primers specific to the fragments of the 16S rRNA genes of the main subgroups of sulfate-reducing bacteria (SRB) and to the fragments of the *dsrB* gene (both reductive and oxidative types), encoding the β-subunit of dissimilatory (bi)sulfite reductase, the key enzyme in the sulfur cycle, inherent in both sulfate-reducing and sulfur-oxidizing microorganisms. The presence of 16S rRNA gene fragments specific to the genera *Desulfobacterium*, *Desulfobacter*, *Desulfococcus–Desulfonema–Desulfosarcina*, and *Desulfovibrio–Desulfomicrobium* was detected in the DNA samples isolated from coastal bottom bacterial mats. Usage of denaturing gradient gel electrophoresis (DGGE) with subsequent sequencing of reamplified *dsrB* gene fragments revealed that according to deduced amino acid sequences encoded by the *dsrB* gene (reductive type), SRB from the coastal gas-saturated bottom sediments of the Black Sea had the highest homology (92–99%) with the *dsrB* gene of cultured SRB belonging to the genera *Desulfovibrio*, *Desulfatitalea*, *Desulfobacter*, and *Desulfobacterium*, as well as with uncultured SRB strains from various marine habitats, such as bottom sediments of the Northern and Japanese seas. Deduced amino acid sequences encoded by the oxidative *dsrB* gene had the highest homology (90–99%) with the relevant sequences of the genera *Thiocapsa*, *Thiobaca*, *Thioflavicoccus*, and *Thiorhodococcus*.

Keywords: sulfate-reducing bacteria, sulfur-oxidizing (thionic) bacteria, the Black Sea, bottom sediments, microbial mats, microbial communities, dissimilatory (bi)sulfite reductase, *dsrB* gene

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The Black Sea is the world largest meromictic basin, in the bottom sediments of which active biogenic sulfidogenesis occurs. The sulfur cycle microorganisms are known to play a crucial role in the biogeochemical processes occurring in the bottom sediments of the World Ocean. Undoubtedly, the study of sulfate reduction, as well as sulfur oxidation coupled with it and of the microorganisms involved in these processes, is one of the key vectors of investigating the biogeochemistry and the taxonomic structure of marine ecosystems in general and those of the Black Sea in particular.

Radioisotope, microscopic, and molecular biological methods used in several works showed a high metabolic activity and the presence of sulfate-reducing bacteria (SRB) in the water column at different depths (Vetriani et al., 2003; Neretin et al., 2007; Bryukhanov et al., 2011), as well as in deep-sea sediments (Leloup et al., 2009; Blazejak and Schippers, 2011; Schippers et al., 2012) and microbial mats of carbonate structures in the regions of cold methane seeps of the Black Sea continental slope (Pimenov and Ivanova, 2005; Wrede et al., 2008; Kleindienst et al., 2012). Determination of the rates of microbiological processes of the methane cycle (methane oxidation and methanogenesis), as well as of the process of sulfate reduction, which is coupled with anaerobic methane oxidation in reduced areas of bottom sediments and microbial mats, makes it possible to quantify the scales of involvement of the Black Sea methane seepage in the global biogeochemical cycle. As for the Black Sea sulfur-oxidizing (thionic) bacteria, 16S rRNA gene sequencing demonstrated the presence of *Beggiatoa*-related filamentous sulfur bacteria in

microbial mats in the region of the shelf margin (at the depth of 150–170 m) in the north-western part of the Black Sea near the Crimean Peninsula. Importantly, organic matter accumulation and intense sulfate reduction were observed under these mats (Jessen et al., 2016).

However, no molecular ecological studies of the phylogenetic diversity and identification of sulfate-reducing and sulfur-oxidizing bacteria have been conducted up to date at the sites of shallow shelf gas seepage and in gas-saturated sediments of the Black Sea coastal zone, which actually remain unexplored.

In the upper horizons of anaerobic marine deposits, the terminal phase of mineralization of organic substances in the presence of sulfates is known to occur with the involvement of strictly anaerobic SRB, which predominantly utilize available low-molecular weight organic substances or molecular hydrogen, reducing sulfate to sulfide. During the period of the highest sulfate reduction rates, H_2S may penetrate the water column and cause mass death of the near-bottom fauna. However, in anaerobic sediments of coastal highly productive sea regions, sulfate concentration in pore waters decreases significantly due to the high sulfate reduction rate, and in the terminal phase, over 50% of organic matter deposits are decomposed by methanogenic archaea (Jørgensen et al., 1990). Some of the methane formed is oxidized by aerobic methanotrophs inhabiting the oxidized surface layer of the bottom sediments and the water column (Ivanov et al., 2002), and in the anoxic zone of marine sediments, near the transition zone from sulfate to methane silts (SMTZ), the process of anaerobic methane oxidation is carried out by consortia of sulfate-reducing bacteria and methanogenic archaea (Hoehler et al., 1994; Boetius et al., 2000). Nevertheless, a considerable portion of the methane formed in shallow marine bottom sediments escapes into the atmosphere as gas seepages (Hovland et al., 1993; Judd, 2004). Such methane gas jets (predominantly of biogenic origin) along the Black Sea northern and western coasts (Crimean, Caucasian, and Bulgarian) are widespread and exert a noticeable influence on the biogeochemical processes in the Black Sea region (Tkeshelashvili et al., 1997; Amouroux et al., 2002; Dimitrov et al., 2002; Michaelis et al., 2002; Egorov et al., 2012). However, discharge of thermogenic methane can not be ruled out in shallow seepages near the Southern Coast of Crimea (Lysenko and Shik, 2013).

Investigation of shallow seeps in the Sevastopol bays provided the first quantification of the rates of microbial processes of methane formation and oxidation, as well as of sulfate reduction, in the bottom sediments with high organic matter content in the upper layers (Pimenov et al., 2013). In the revealed areas of coastal gas seepages in the Chersonesus Bay, Herakles Peninsula (Sevastopol), methane of biogenic origin (the isotopic composition $\delta^{13}C\text{-}CH_4$ was –60.4‰; the methane content was up to 70%) was shown to predominate in the composition of the bubbling gas being discharged from the underlying geological structures. The maximal sulfate reduction rate in these bottom sediments was detected in the subsurface horizon (2–4 cm), and deeper, at the SMTZ, the process of anaerobic methane oxidation coupled with sulfate reduction was observed. Whitish bacterial films of the *Thiodendron*-type microbial communities, in which spirochete-like filamentous cells were seen under the microscope, were detected in the areas of gas seepages there (Malakhova et al., 2015).

Due to the fact that sulfate-reducing bacteria are a phylogenetically heterogeneous group of microorganisms, the *dsrB* gene encoding the β-subunit of dissimilatory (bi)sulfite reductase (EC 1.8.99.1), the key enzyme in reduction of sulfite to sulfide inherent in all sulfate- and sulfite-reducing microorganisms, is more often used as the main phylogenetic marker for their detection in ecosystems (Geets et al., 2006; Bagwell et al., 2009). Many sulfur-oxidizing prokaryotes (especially those accumulating sulfur in the cells), being the central component in the global biogeochemical sulfur cycle, possess dissimilatory (bi)sulfite reductase, which catalyzes a reverse reaction—in this case the *dsrB* gene of the oxidative type is used for phylogenetic analysis virtually coinciding with the 16S rRNA-based dendrograms (Loy et al., 2009; Müller et al., 2015).

The goal of the present work was to obtain data on the phylogenetic diversity of microbial communities of the biogeochemical sulfur cycle (sulfate-reducing and sulfur-oxidizing bacteria) in the Black Sea coastal gas-saturated bottom sediments in the region of the Crimean Peninsula (Sevastopol) using PCR analysis and denaturing gradient gel electrophoresis with the subsequent sequencing of the fragments of the key functional gene *dsrB*.

MATERIALS AND METHODS

Sampling of bottom sediments. The samples were collected in August 2015 on board the boat equipped with a SD204 hydrological CTD system (SAIV A/S, Norway) in the Chersonesus (Blue) Bay of the Black Sea near Sevastopol (the station with the coordinates 44.5647° N, 33.3993° E) at a depth of 5 m from the surface whitish bacterial mats of sulfurettes in the area of methane gas emissions, as well as from the upper layer (0–5 cm) of the seabed adjacent to gas seepages and microbial mats, as a background sample. The seabed granulometric composition was determined by the percentage-expressed mass content of particles of different sizes in relation to the mass of the dry sediment sample (analysis was performed using the sieve method of dry dispersion with a set of sieves with 5.0-, 2.0-, 1.2-, 0.9-, 0.2-, and 0.09-mm mesh). The bottom sediment samples collected into screw-capped sterile plastic containers were filled to capacity

at a 2 : 1 ratio with a buffer solution (50 mM Tris-HCl, pH 8.0; 150 mM NaCl and 100 mM Na_2-EDTA) and stored at 4°C. These samples were used for further molecular phylogenetic analysis of the native microbial community as well as for preparation of enrichment cultures of sulfate-reducing and sulfur-oxidizing (thionic) bacteria.

Enrichment cultures of sulfate-reducing and sulfur-oxidizing bacteria. To obtain enrichment cultures, strongly reduced sediments with a layer of microbial mat rich in free sulfide, as well as nearby bottom sediments, were used as inocula. Enrichment cultures of SRB on Postgate C liquid nutrient medium for marine SRB (DSMZ 163 according to the catalog of nutrient media of the German collection of microorganisms and cell cultures https://www.dsmz.de) were obtained using the method of end-point dilutions up to 10^{-9} (cultivation was carried out under anaerobic conditions at 4 and 22°C); columns of agar medium were then inoculated with the subsequent transfer of the resulting separate colonies into Postgate C liquid nutrient medium with different electron donors. Culture development was assessed by biomass growth and sulfide formation determined by colorimetry using *N*,*N*-dimethyl-*p*-phenylenediamine (Trüper and Schlegel, 1964). Enrichment cultures of sulfur-oxidizing (thionic) bacteria were obtained in a similar way on liquid Pfennig I nutrient medium for purple sulfur bacteria (DSMZ 28) and Pfennig II nutrient medium for green sulfur bacteria (DSMZ 29). Microscopy was carried out at 1000× using an Axio Imager D1 microscope (Carl Zeiss, Germany) with an Axio Cam HRc digital camera.

Amplification of the 16S rRNA and *dsrB* gene fragments. The total DNA was isolated from native bacterial mats, bottom sediments, and enrichment cultures using the Genomic DNA Purification Kit (Thermo Fisher Scientific, United States) according to the manufacturer's protocol. The isolated DNA was additionally reprecipitated for 20 min at −20°C with 3 M sodium acetate (0.1 volume) and 96% ethanol (3 volumes) to remove low-molecular weight compounds.

PCR analysis with total DNA was performed using the oligonucleotide primers specific to the fragments of the *dsrB* gene (both reductive and oxidative types) encoding the β-subunit of dissimilatory (bi)sulfite reductase. In order to detect the presence of the 16S rRNA gene fragments characteristic of the main SRB phylogenetic subgroups in the samples, a more sensitive nested PCR method was employed when the products of amplification of the *Bacteria* 16S rRNA gene fragments were used as a template instead of the native DNA extracted from the samples. The list of the PCR primers (synthesized by Syntol, Russia) used in the study is shown in Table 1.

The reaction mixtures for PCR contained ~1–3 ng/μL of template DNA; 2.0 mM $MgCl_2$; 400 μM dNTPs (Thermo Fisher Scientific, United States); 500 nM of each primer, and 0.1 U/μL of *Taq* DNA-polymerase (Thermo Fisher Scientific, United States). The template DNA isolated from *Desulfovibrio vulgaris* Hildenborough and *Allochromatium vinosum* was used as a positive control for sulfate-reducing and sulfur-oxidizing bacteria, respectively. The PCR was performed using a GeneAmp PCR System 9700 amplifier (Thermo Fisher Scientific, United States) in the following mode: 10 min at 95°C; 35 cycles—30 s at 95°C, 40 s at the annealing temperature (T_a) of the relevant pair of primers, 3 min at 72°C; and 10 min at 72°C. All PCR were performed in three replicates to obtain reliable results.

Amplification products were detected using electrophoresis in 1% agarose gel in 1× TAE buffer solution (pH 8.0) with subsequent staining of the gel with ethidium bromide (0.5 μg/mL) and visualization of the bands in the passing UV light (λ 254 nm) on an ECX-15.C transilluminator (Vilber Lourmat, France). MassRuler DNA-markers (Thermo Fisher Scientific, United States) were used as markers for determining the size and concentration of the amplified DNA fragments.

The amplification products were purified using the DNA Gel Extraction Kit (Thermo Fisher Scientific, United States) and the innuPREP DOUBLEpure Kit/innuPREP Gel Extraction Kit (Analytic Jena, Germany) according to the manufacturer's protocol.

Denaturing gradient gel electrophoresis (DGGE). Amplification products of the *dsrB* gene fragments were separated by the degree of strength of the secondary structure using denaturing gradient gel electrophoresis (DGGE), which is a common method for amplicon analysis to reveal the structure of microbial communities. To improve the separation of the DNA fragments in the course of DGGE performance, a 40-bp GC-rich fragment was incorporated into the sequences of direct PCR primers DSRp2060F and dsrB F1a (Table 1) specific to the *dsrB* gene (reductive and oxidative type, respectively) from the 5'-terminus (Muyzer et al., 1993); in this case, PCR was performed with the denaturing and annealing time increased to 1 min. The PCR amplification products were applied directly into 8% (vol/vol) polyacrylamide gel with an acrylamide concentration gradient of 35–65% in 0.5× TAE buffer solution (20 mM Tris-acetate, pH 7.4; 10 mM sodium acetate; 0.5 mM Na_2-EDTA). In order to prepare 8% polyacrylamide gel, commercial acrylamide/*N*,*N*-methylene-bis-acrylamide solution (37.5 : 1) (Bio-Rad Laboratories, United States) was used; the 35–65% concentration gradients were formed by 8% (vol/vol) acrylamide/*N*,*N*-methylene-bis-acrylamide solutions, which contained 0 and 100% of the denaturing agent, respectively. Urea (7 M, Bio-Rad Laboratories, United States) and 40% deionized formamide (Merck, Germany) were used simultaneously as denaturing agents for the preparation of 100% solution. Ammonium persulfate was added as an

Table 1. Primers used in the study and PCR analysis of the phylogenetic composition of the sulfur cycle bacterial community in the bottom sediments of the Chersonesus Bay (Sevastopol)

Primer pairs	Target gene (fragment position)	Nucleotide sequence, 5'–3' (reference)	PCR results	
			bacterial mat	seabed
		For 16S rRNA gene		
Eub63F Eub1387R	16S rRNA *Bacteria* (63–1387)*	CAGGCCTAACACATGCAAGTC GGGCGGWGTGTACAAGGC (Marchesi et al., 1998)	+	+
DFM140 DFM842	16S rRNA subgroup 1 SRB *Desulfotomaculum* (140–842)*	TAGMCYGGGATAACRSYKG ATACCCSCWWCWCCTAGCAC (Daly et al., 2000)	–	–
DBB121 DBB1237	16S rRNA subgroup 2 SRB *Desulfobulbus* (121–1237)*	CGCGTAGATAACCTGTCYTCATG GTAGKACGTGTGTAGCCCTGGTC (Daly et al., 2000)	–	–
DBM169 DBM1006	16S rRNA subgroup 3 SRB *Desulfobacterium* (169–1006)*	CTAATRCCGGATRAAGTCAG ATTCTCARGATGTCAAGTCTG (Daly et al., 2000)	+	–
DSB127 DSB1273	16S rRNA subgroup 4 SRB *Desulfobacter* (127–1273)*	GATAATCTGCCTTCAAGCCTGG CYYYYYGCRRAGTCGSTGCCCT (Daly et al., 2000)	+	+
DCC305 DCC1165	16S rRNA subgroup 5 SRB *Desulfococcus-Desulfonema-Desulfosarcina* (305–1165)*	GATCAGCCACACTGGRACTGACA GGGGCAGTATCTTYAGAGTYC (Daly et al., 2000)	+	–
DSV230 DSV838	16S rRNA subgroup 6 SRB *Desulfovibrio-Desulfomicrobium* (230–838)*	GRGYCYGCGTYYCATTAGC SYCCGRCAYCTAGYRTYCATC (Daly et al., 2000)	+	+
		For *dsrB* gene		
DSR1Fmix DSR4Rmix	*dsrAB* reductive type (187–2129)**	RKYYAYTGGAARCAYG GTRWAGCARTTGCCGCA (Pester et al., 2010)	+	+
DSRp2060F DSR4R	*dsrB* reductive type (1752–2129)**	§ CAACATCGTTCATACCCAGGG GTGTAGCAGTTACCGCA (Geets et al., 2006)	+	+
rDSRA240F rDSRB808R	*dsrB* oxidative type (172–2027)***	GGNTAYTGGAARGGNGG CCDCCNACCCADATNGC (Lenk et al., 2011)	+	+
dsrB F1a 4RSI2a	*dsrB* oxidative type (1684–2027)***	§ CACACCCAGGGCTGG CAGGCGCCGCAGCAGAT (Lever et al., 2013)	+	+

* Position for *Escherichia coli* K12 16S rRNA gene.
** Position for *Desulfovibrio vulgaris* Hildenborough *dsrAB* gene.
*** Position for *Allochromatium vinosum dsrAB* gene.
§GC-rich 5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3' fragment was attached to the primer 5'-terminus for DGGE performance (according to Muyzer et al., 1993).

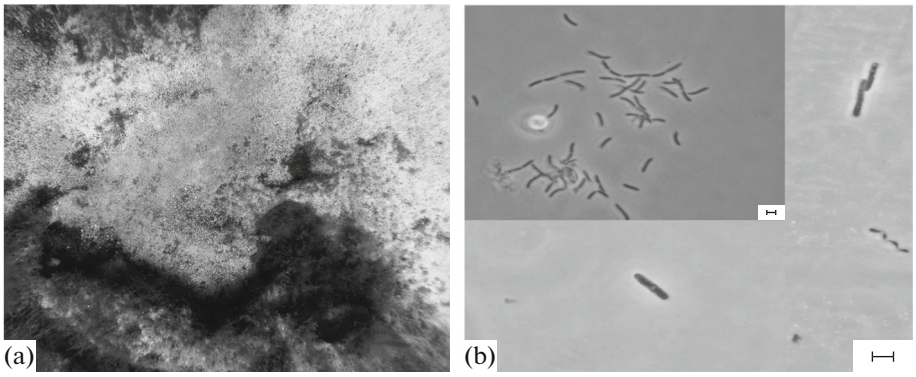

Fig. 1. Photograph of the sea bottom in the Chersonesus (Blue) Bay (Sevastopol) at a depth of 5 m with bacterial mats of sulfurettes above the gas-saturated sediments (a) and micrograph of the cells of the predominant morphology from the line of enrichment cultures of sulfate-reducing bacteria isolated from these mats; phase contrast light microscopy, the scale bar is 1 μm (b).

initiator of the polymerization process, and TEMED as a catalyst of this process. A Scie-Plas device for the performance of denaturing gradient gel electrophoresis (Great Britain) was used in this work.

Electrophoresis was performed at a constant voltage of 70 V and at 60°C for 16–17 h. After electrophoresis, the gel was washed with distilled water and then stained with SYBR® Gold (Thermo Fisher Scientific, United States) for 40 min in the dark. After staining, the gel was visualized on a transilluminator; separate bands were excised, placed in the test tubes with 20 μL of sterile distilled water, and stored at 4°C for 16 h for the DNA fragments to be eluted from gel.

Sequencing of the *dsrB* gene fragments, phylogenetic analysis. Using the DNA fragments eluted from the DGGE-bands as a template, preparative PCR (using a pair of primers DSRp2060F/DSR4R for the reductive *dsrB* gene and dsrB F1a/4RSI2a for the oxidative *dsrB* gene) was performed for reamplification of the corresponding DNA fragments, and the fragments obtained were purified from 1% agarose gel using the Cleanup Mini Kit for DNA isolation from agarose gel and reaction mixtures (Evrogen, Russia). The nucleotide sequences of the *dsrB* gene fragments (the DNA concentration in the preparations obtained was 52–93 ng/μL) were identified according to Sanger's method using the BigDye Terminator v. 3.1 Cycle Sequencing Kit and the primer DSRp2060F or dsrB F1a on an automatic ABI PRISM 3730 gene analyzer (Thermo Fisher Scientific, United States) at the Institute of Bioengineering, Research Center of Biotechnology, Russian Academy of Sciences.

Analysis of the similarity between the corresponding amino acid sequences encoded by the *dsrB* gene of the reductive and oxidative types with known sequences from the GenBank database was performed after alignment in the ClustalW software using the BioEdit and the BLAST packages. Phylogenetic trees were constructed with the Maximum Likelihood method implemented in the MEGA 7.0.21 software (Kumar et al., 2016) using homologically close reference sequences from the GenBank. The nucleotide sequences of the *dsrB* gene fragments obtained in the study were deposited in the GenBank under the numbers KY983391–KY983397 (*dsrB* of the reductive type) and MF041801–MF041804 (*dsrB* of the oxidative type).

RESULTS AND DISCUSSION

General characterization of the benthic samples and the enrichment cultures of the sulfur cycle bacteria isolated from them. According to the CTD system data, in August 2015, the salinity of bottom water at the sampling point was 17.664‰; its temperature was 26.18°C. The bottom sediments with bacterial overgrowth appeared to be silt sandstone (the predominant particle size 0.2–1.2 and 0.09–0.2 mm); the background bottom sediments represented sand with shell rock residues and a small amount of aleuropelites (the predominant particle size 0.2–1.2 mm). The bacterial mats of sulfurettes were clearly localized in space (Fig. 1a). The gas-saturated organogenic silt containing a large amount of detritus and macrophyte tissue fragments (mainly *Cystoseira* sp.) at a depth of 10–15 cm underlaid the upper sand layer covered by a whitish bacterial film. Emission of large gas bubbles from the silt was observed in the process of sampling.

The studied bottom sediments from the area of gas seeps of the Chersonesus Bay were characterized by a virtually complete absence of the oxidized layer. Strongly reduced conditions (E_h = from −245 to −330 mV) contributing to active processes of sulfate reduction and methanogenesis (upon sulfate depletion) and the formation of gas-saturated silts with a stable flow of H_2S and CH_4 into the water column developed at the center of gas seepage sites. The water content and fineness of such silts contributed to a certain degree of gas retention in the sediment layers;

however, sulfide discharge was probably responsible for the extinction of macrophytes often occurring in this region. Earlier, the physicochemical and radioisotope analysis showed a high rate of sulfate reduction in this area of the Chersonesus Bay (Pimenov et al., 2013).

Microscopy revealed high morphological diversity of the microbial community in the collected native samples: numerous filamentous forms, vibrios, rod-shaped forms of various lengths; cocci, spirilla, and bacteria with the plectridial type of spore formation also occurred. Earlier, it was suggested that the main part of the whitish-gray bacterial mats of sulfurettes in the surface layer of microbial mats of the Chersonesus Bay may be formed by the filamentous sulfur bacteria morphologically similar to members of the genus *Beggiatoa*, and such mats can also be assigned to the *Thiodendron* type (Malakhova et al., 2015). However, no high homology with the representatives of *Beggiatoa* sulfur bacteria was detected in the enrichment cultures of sulfur-oxidizing bacteria obtained by us from similar microbial mats (collected in the same seabed area next year) by DGGE with the subsequent sequencing of the oxidative *dsrB* gene fragments.

The SRB enrichment cultures from the coastal bottom sediments of the Chersonesus Bay grew well and produced sulfide at room temperature; at 4°C, the growth was slower. Microscopy of enrichment cultures from bacterial mats revealed thick and long thin rods (including numerous motile ones), spirilla, and vibrios (Fig. 1b). During the first reinoculations of the seabed enrichment cultures (used as a background sample), short thick motionless rods, long thin rods, as well as cells with the plectridial type of spore formation, spirilla, and vibrios were predominantly detected. Stable lines of SRB enrichment cultures after several reinoculations on Postgate C nutrient medium with acetate or ethanol (10 mM each) as electron donors contained the cells with an obviously predominant similar morphological type (mainly motile and, rarer, motionless straight rods of an average size; the number of vibrios decreased sharply in the course of reinoculations).

The enrichment cultures of sulfur-oxidizing bacteria from bacterial mats appeared purple and had coccoid/rod-shaped cells; those from the seabed background point were almost colorless with the predominance of bacteria in the form of short thick rods.

Phylogenetic analysis of sulfate-reducing bacteria in the coastal bottom sediments of the Chersonesus Bay. Nested PCR using the oligonucleotide primers for the 16S rRNA gene fragments of the main SRB phylogenetic subgroups showed the presence of genetic material of the representatives of the genera *Desulfobacterium* (subgroup 3 SRB), *Desulfobacter* (subgroup 4 SRB), *Desulfococcus–Desulfonema–Desulfosarcina* (subgroup 5 SRB), and *Desulfovibrio–Desulfomicrobium* (subgroup 6 SRB) in the DNA samples isolated from microbial mats. In the DNA samples from the seabed near these microbial mats, SRB from subgroups 3 and 5 were not detected in contrast to those of subgroups 4 and 6 (Table 1). The phylogenetic composition of SRB community similar enough to the above-mentioned microbial mats, except for the absence of subgroup 3 members, was detected by us earlier using the same method in gas-saturated anaerobic bottom sediments of the Streletskaya Bay that is strongly contaminated by anthropogenic organic matter (horizons 0–2, 2–4, and 4–6 cm) and in the bottom sediments from the area of methane gas jets of the Sevastopol Bay (horizons 2–4 and 8–10 cm) of the Black Sea (unpublished data). Importantly, in the latter case, the representatives of subgroup 6 SRB were detected only in the subsurface 2–4-cm horizon where the maximal rate of sulfate reduction was observed.

Positive PCR-signals for the presence of the *dsrB* gene of the reductive type were revealed in the DNA samples isolated from both bacterial mats of sulfurettes of coastal gas-saturated bottom sediments and from the seabed of the Chersonesus Bay (Sevastopol) near the microbial mats. After DGGE, it was found that the profile of amplicons of the reductive *dsrB* gene fragments from the bacterial mats of sulfurettes contained six individual bands; the profile from the seabed near the microbial mats contained only two separate bands.

Analysis of eight translated amino acid sequences encoded by the *dsrB* gene of the reductive type showed that the sulfate-reducing bacteria revealed by us in the coastal bottom microbial mats of the Chersonesus (Blue) Bay of the Black Sea had the highest homology (92–99%) with the corresponding *dsrB* gene sequences of cultured SRB of the genera *Desulfovibrio* (*D. piezophilus*, *D. desulfuricans*, *D. oxyclinae*, *D. aespoeensis*, *D. longus,* etc.), *Desulfatitalea* (*D. tepidiphila*), *Desulfobacter* (*D. latus*, *D. curvatus*, *D. vibrioformis*), and *Desulfobacterium* (*D. autotrophicum*), as well as with uncultured SRB strains from various habitats, predominantly marine ones, such as the coastal bottom sediments of the North Sea and the Sea of Japan, corrosive metalwork on the Atlantic coast of France, and anaerobic sludge of sulfate-containing wastewater from bioreactors in Denmark. The translated amino acid sequence encoded by the reductive *dsrB* gene from the seabed near the microbial mats had the highest homology (99%) with the corresponding sequences from the bottom sediments of the Baltic Sea (near the Swedish coast) and the Sea of Japan. In the phylogenetic tree (Fig. 2), the corresponding sequences are designated as $Sev1_{red}$ (appeared to be identical two sequences from the seabed) and $Sev2_{red}$–$Sev7_{red}$ (the sequences from the bacterial mat).

Phylogenetic analysis of sulfur-oxidizing (thionic) bacteria in the coastal bottom sediments of the Chersonesus Bay. Positive PCR signals for the presence of the *dsrB* gene of the oxidative type were obtained in

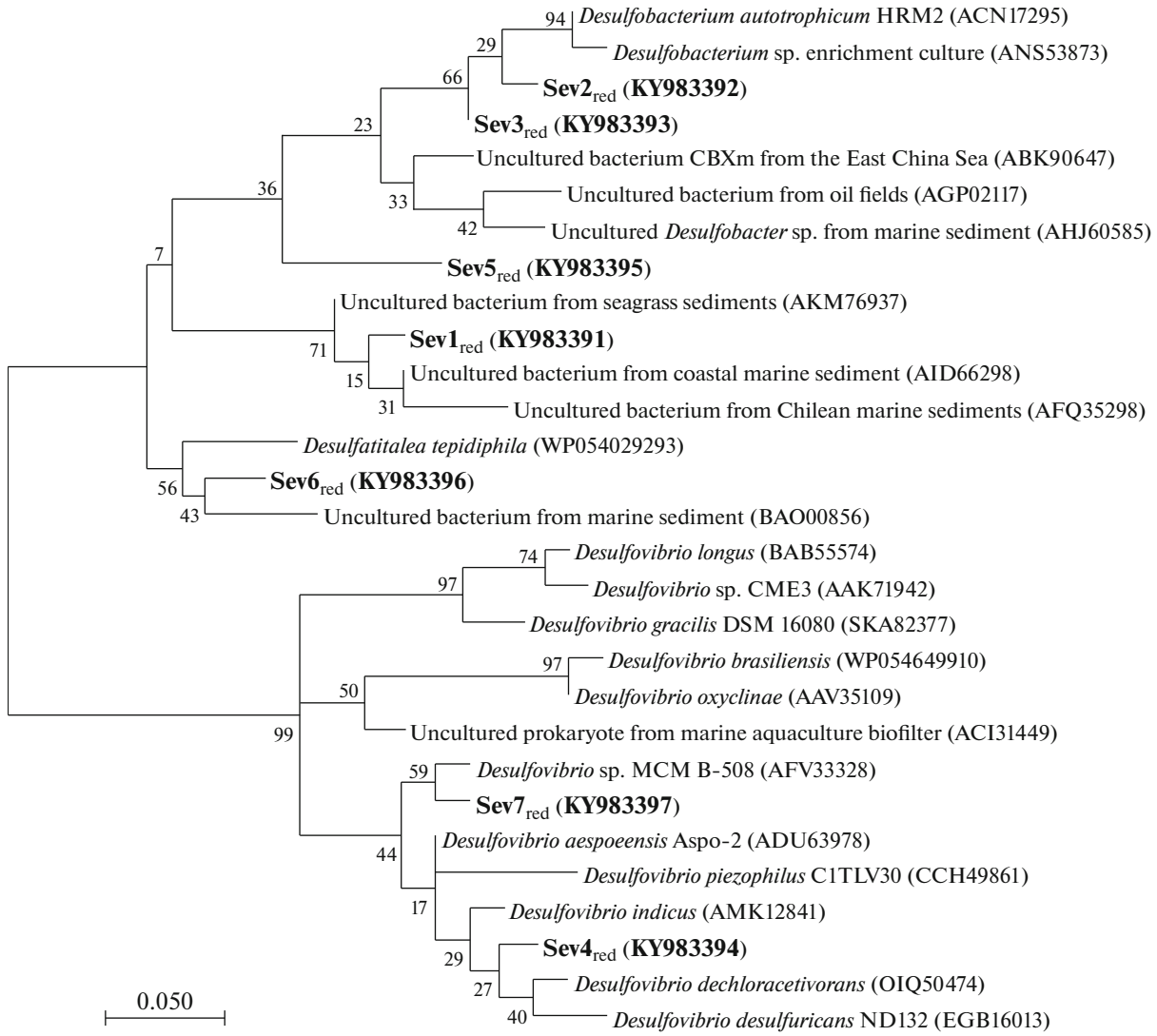

Fig. 2. Phylogenetic tree of sulfate-reducing bacteria obtained by comparative analysis of amino acid sequences encoded by the *dsrB* gene of the reductive type. The sequences obtained in the course of sequencing the *dsrB* gene fragments after DGGE of the amplification products of the reductive *dsrB* gene from the coastal gas-saturated bottom sediments of the Chersonesus Bay are designated in boldface. The scale bar corresponds to 5% of computational sequence divergence.

the DNA samples isolated from both bacterial mats of sulfurettes of reduced bottom sediments and from the nearby seabed of the Chersonesus Bay (Sevastopol). It is necessary to note that the use of the dsrB F1a/4RSI2a pair of primers yielded better results in PCR detection of the presence of the oxidative *dsrB* gene in the investigated samples compared to the usage of a mixture of the dsrB F1a-h/4RSI2a-h primers (Lever et al., 2013).

Since, in contrast to the reductive *dsrB* gene, it was difficult to obtain the amplicons of the oxidative *dsrB* gene fragments at concentrations sufficient for DGGE from native samples, it was decided at first to obtain enrichment cultures of sulfur-oxidizing bacteria (using both microbial mats and seabed as inocula) and to isolate DNA from these cultures. This mode proved to be successful.

After DGGE separation of the amplification products of the oxidative *dsrB* gene using the DNA template from the enrichment cultures of sulfur-oxidizing bacteria (bacterial mats were used as inoculum), a total of eight separate bands were detected. Only one band was seen in the DGGE profile of the oxidative *dsrB* gene of the enrichment cultures of sulfur-oxidizing bacteria from the seabed. This gives evidence of a wider phylogenetic diversity of the sulfur cycle bacteria in bacterial overgrowth compared with the seabed outside the microbial mats and gas seepage.

The analyzed eight translated amino acid sequences encoded by the *dsrB* gene of the oxidative type of sulfur-oxidizing bacteria from bacterial mats in

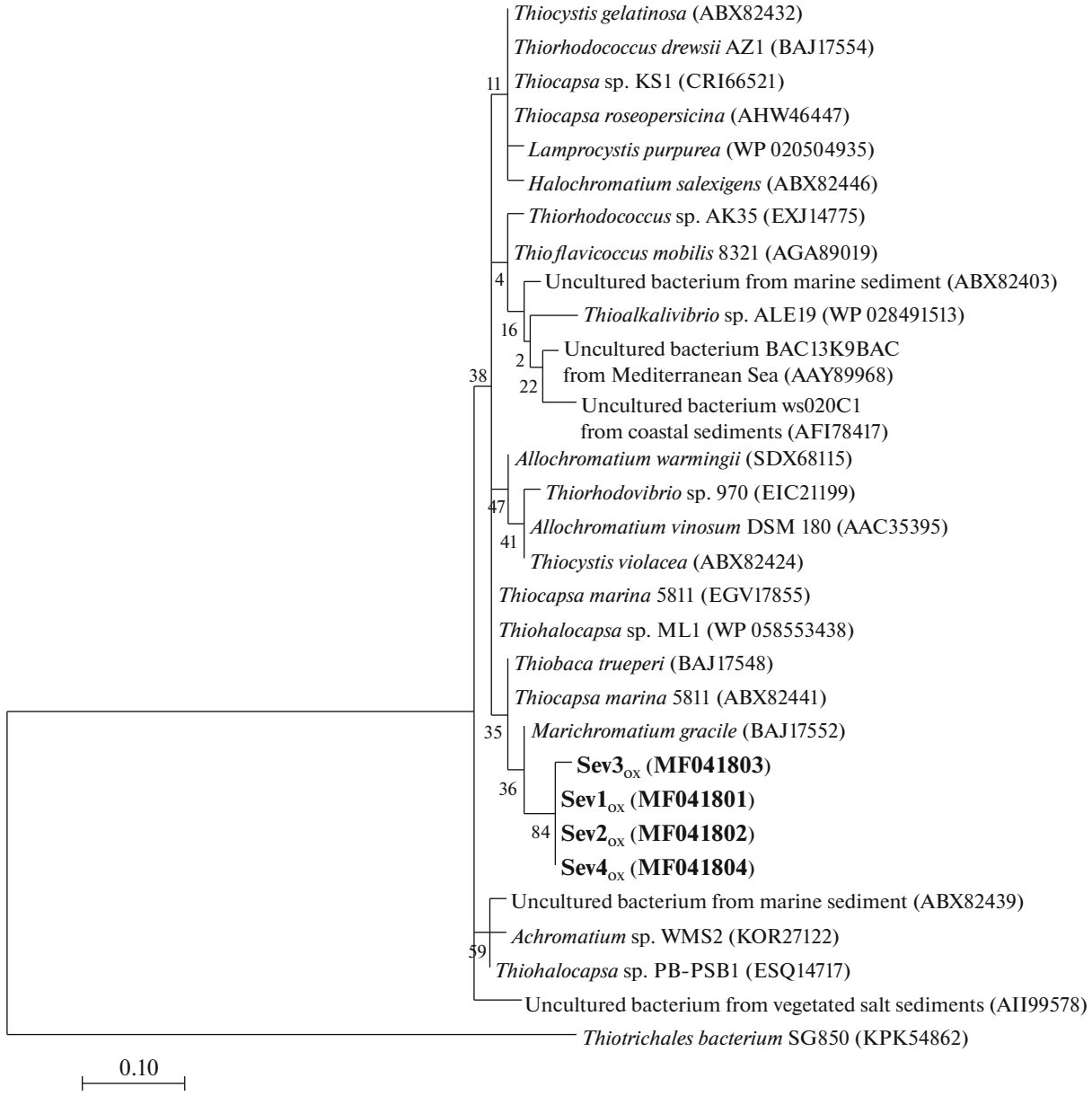

Fig. 3. Phylogenetic tree of sulfur-oxidizing bacteria obtained by comparative analysis of amino acid sequences encoded by the *dsrB* gene of the oxidative type. The sequences obtained in the course of sequencing the *dsrB* gene fragments after DGGE of the amplification products of the oxidative *dsrB* gene from the coastal gas-saturated bottom sediments of the Chersonesus Bay are designated in boldface. The scale bar corresponds to 10% of computational sequence divergence.

the Chersonesus Bay had the highest homology (90–99%) with the β-subunit of dissimilatory (bi)sulfite reductase of the representatives of sulfur-oxidizing bacteria of the genera *Thiocapsa* (*T. marina*, *T. roseopersicina*), *Thiobaca* (*T. trueperi*), *Thioflavicoccus* (*T. mobilis*), and *Thiorhodococcus* (*T. drewsii*), as well as to *Marichromatium gracile*. The translated amino acid sequence encoded by the oxidative *dsrB* gene from the seabed near the sulfurettes also had a relatively low homology (85–89%) with the corresponding sequences of *Achromatium* spp., *Thiohalocapsa* spp., and uncultured sulfur-oxidizing bacteria from the salt marsh bottom sediments on the Plum Island (Atlantic coast, Massachusetts, United States). In the phylogenetic tree (Fig. 3), the corresponding sequences are designated as Sev1$_{ox}$ (appeared to be identical one sequence from the seabed and two sequences from the microbial mat), Sev2$_{ox}$ (appeared to be identical four sequences from the microbial mat), and Sev3$_{ox}$–Sev4$_{ox}$ (two sequences from the microbial mat).

Interestingly, the representatives of three out of at least 13 reductive *dsrAB*-based phylogenetic lineages at

the family level, which presently have no exactly identified and cultured strains, were detected exactly in marine ecosystems (Müller et al., 2015). On the border of sulfate and methane silts of the Black Sea deep bottom sediments, where the SRB number was at its maximum constituting 30% of all microorganisms, *Desulfobacteraceae* was the predominant SRB subgroup based on the library of the *dsrAB* gene clones (Leloup et al., 2007). Investigations using CARD–FISH showed that SRB from the *Desulfosarcina–Desulfococcus* subgroup predominated in hydrocarbonate structures in the regions of marine methane seeps, including the Black Sea ones (Kleindienst et al., 2012), which may well be consistent with our analysis data on the 16S rRNA-based SRB communities in the coastal bottom sediments of the Sevastopol bays. Undoubtedly, the phylogenetic composition of the sulfur-oxidizing component of the mat microbial communities of various areas of the coastal zone of the Crimean Peninsula requires further close study.

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