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MICROBIOLOGY

Detection of Anaerobic Sulfate-Reducing Bacteria in Oxygen-Containing Upper Water Layers of the Black and Baltic Seas

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Abstract—Fluorescent in situ hybridization (FISH) and PCR were used for analysis of phylogenetic structure of anaerobic sulfate-reducing bacterial communities in oxygen-containing upper water layers of meromictic basins: the Black Sea and the Gdansk Deep of the Baltic Sea. In the Black Sea (continental slope at depths 30–70 m), cells of sulfate-reducing bacteria (SRB) hybridizing with 16S rRNA-specific FISH-probes for *Desulfotomaculum, Desulfobacter*, and *Desulfovibrio* genera were revealed, whereas *Desulfomicrobium*-related bacteria were prevalent in the chemocline zone at a 150-m depth. Besides *Desulfotomaculum* (SRB subgroup 1), *Desulfobacter* (SRB subgroup 4), and *Desulfovibrio-Desulfomicrobium* (SRB subgroup 6), nested PCR with the use of 16S rRNA gene-specific primers detected the presence of *Desulfococcus–Desulfonema–Desulfosarcina* (SRB subgroup 5) in the oxygen-containing water column of the Black and Baltic seas. Active enrichment SRB culture that contained bacterium *Desulfosporosinus* sp. as a major component was obtained from the Black Sea water sample collected at a 70-m depth.

Keywords: sulfate-reducing bacteria, *Desulfosporosinus*, fluorescent in situ hybridization, nested PCR, meromictic basin, Black Sea, Gdansk Deep of the Baltic Sea.

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INTRODUCTION

The Black Sea is the largest meromictic basin and reservoir of dissolved hydrogen sulfide and methane in the world. On the continental slope, oxygen-containing waters (~300 μM O_2 in subsurface layer) extend to the depths of 140–175 m where concentration of oxygen drops to 2–10 μM. Chemocline zone is normally located between 95 m (start of rapid decrease of concentration of oxygen) and 157–167 m (presence of traces of hydrogen sulfide) [1, 2]. Anoxic water column containing H_2S (up to 370 μM at depths over 1500 m) is located below the chemocline zone in the Black Sea. In the Gdansk Deep of the Baltic Sea, the chemocline zone starts at the depth of 80 m (oxygen amount: 69 μM) and H_2S appears at the bottom layer at 102 m [3]. Thus, upper water layers of the Black Sea and deeps of the Baltic Sea appear to be perfect models to study microbial distribution on one vertical section with various hydrochemical conditions.

Microbial communities of the oxic–anoxic interface are especially interesting. Thus, noticeable microbial processes (dark CO_2 fixation, transformation of sulfur compounds, production and oxidation of methane) were discovered in the chemocline zone of the Black Sea [4].

Sulfate-reducing bacteria (SRB) are a phylogenetically diverse group utilizing hydrogen and, presumably, low-molecular-weight organic compounds as electron donors for sulfate reduction to form hydrogen sulfide. SRB are regarded as strict anaerobes, though many of the species have systems of antioxidant defense and can exist in habitats exposed to the oxygen [5]. SRB play the most important role in H_2S accumulation in the deep waters of the Black Sea. However, with the help of labeled $^{35}S–SO_4^{2-}$, sulfate reduction was found to occur not only in anoxic water column but in oxygen-containing waters of the Black Sea as well [2].

SRB cells were first identified by fluorescent in situ hybridization (FISH) in oxygen-containing waters of the Black Sea recently [2]. Also, the presence of endospores of *Desulfotomaculum* spp. was determined in the waters of the Baltic Sea at depths of 1 m and 14 m by 16S rRNA gene analysis [6]. To date, PCR primers specific to 16S rRNA genes of the main phylogenetic subgroups of SRB were designed [7], though they have not been used before to detect SRB in sea waters. The main aim of this study was to investigate phylogenetic affiliation of SRB that were previously

detected in oxygen-containing water layers of the Black and the Baltic seas.

MATERIALS AND METHODS

In the Black Sea, water samples from the depths up to 200 m were collected using a Sea-Bird 19 CTD-probe (United States) equipped with 10-L Niskin bathometers and submerged pump, from onboard RV *Ashamba*. Station of sample collecting (N44.458°, E37.882°, depth 1300 m) was located at the continental slope in 16 km from the Blue Bay (Gelendzhik). In the Gdansk Bay of the Baltic Sea, water samples from the depths up to 107 m were collected at the station 22 (N54.860°, E19.333°) from onboard trawler MRTK-1073.

Water samples for FISH analysis were fixed in 4% formaldehyde in PBS (pH 7.0) and stored at 4°C. Cells were concentrated on GTBP 2500 membrane filters (Millipore, United States). Hybridization was carried out according to the previously described protocol [2]. Cyanine-3 labeled 16S rRNA-specific probes for Bacteria and Archaea domains were used [8] as well as for the main phylogenetic subgroups of SRB [9–11]. Total abundance of the microorganisms was determined using DNA-dye 4',6-diamidino-2-phenylindole (0.5 ng/μL). Visualization was carried out in 30 fields of view at 1000× magnification with an Axio Imager.D1 epifluorescence microscope (Carl Zeiss, Germany) with digital camera Axio Cam HRc and Zeiss 20/49 color filters.

For total DNA isolation, 5 L of water samples were sequentially filtered through fiberglass filters GF/C (Whatman, United States) and membrane filters with pore diameter 0.22 μm (Millipore, United States). Filters then were disrupted in liquid nitrogen before Genomic DNA Purification Kit (Fermentas, Lithuania) was used. PCR mixture contained ~25 ng of DNA matrix; 2.0 mM $MgCl_2$, 400 μM dNTP, 500 nM of each primer, and 2.5 units of *Taq* DNA template. PCR was carried out according to the following scheme: 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at the respective temperature of annealing and 1 min at 72°C (6 min for primers pA and pH'), 10 min at 72°C. Primers for 16S rRNA genes of Bacteria (pA and pH') [12] and six phylogenetic subgroups of SRB [7] as well as for gene *dsrB* [13] were used.

Enrichment cultures were obtained anaerobically at Widdel medium for marine SRB [14]. Capron filters with concentrated from 100 mL of water cells were used as inoculum. SRB culture growth at 22°C was determined by hydrogen sulfide production, which was detected colorimetrically (λ 670 nm) after staining of the samples with paraphenylenediamine [15]. Identification of SRB was carried out according to the following scheme: fragments of Bacterial 16S rRNA gene were amplified using isolated DNA and primers 341F/907R [16], and obtained amplicons were cloned in pGEM-T vector (Promega, United States) and analyzed using restriction endonuclease *Hae*III with subsequent sequencing.

RESULTS AND DISCUSSION

The abundance of microorganisms in the chemocline zone of the Black Sea was 20% higher than in subsurface waters (1.06×10^6 cells/mL); the ratio of bacteria in the chemocline zone reduced from 75 to 40%, while the ratio of archaea increased by four times. In the Gdansk Deep of the Baltic Sea, the amount of bacteria also decreased from 65% in the oxygenated layers (30–50 m) to 28% in the chemocline zone, while the ratio of archaea increased from 4 to 12% of total microbial number.

PCR with total DNA isolated from the water samples and primers for gene *dsrB* (encoding for β-subunit of dissimilatory sulfite reductase, the key enzyme of sulfate reduction) has shown the presence of genetic material of SRB in oxygen-containing layers of both the Black (from 30 to 200 m) and the Baltic (from 10 to 107 m) seas.

The amount of bacterial cells hybridizing with FISH probes specific for genera *Desulfotomaculum* (Dtm229), *Desulfovibrio* (DSV1292), and *Desulfobacter* (DSB129) in subsurface waters of the Black Sea at depths 30–70 m reached 2×10^5, 1.2×10^5, and 4.4×10^4 cells/mL, respectively (Fig. 1a). Bacteria of the genus *Desulfomicrobium* (DSV214) represented the dominant phylogenetic subgroup of sulfate reducers near the lower boundary of chemocline of the Black Sea at depths 157–167 m (1×10^5 cells/mL), while the abundance of *Desulfovibrio* spp. at those depths decreased remarkably (Fig. 1a). Cells of *Desulfotomaculum* spp. were not detected in the chemocline zone of the Black Sea by FISH. However, sequences of 16S rRNA gene of this genus were detected at depths of 100 and 145 m by nested PCR indicating the possible presence of nonactive minor members of the genus *Desulfotomaculum* in the chemocline. In the previous studies of the Black Sea, the peak of abundance of sulfate reducers in the chemocline zone was discovered using FISH-probe SRB385 targeting SRB that belong to δ-Proteobacteria [17] and using real-time PCR to evaluate the number of copies of gene *dsrA* [18].

It is important to note that the real amount of SRB in oxygen-containing waters of the Black Sea can be lower due to non-absolute specificity of the known FISH-probes. But there are also other explanations of possible high abundance of SRB in upper water layers. Thus, many of the SRB species (especially members of the genera *Desulfovibrio* and *Desulfotomaculum*) possess effective enzymatic systems of protection from oxygen stresses and are able to form cell aggregates and symbiotic consortia with aerobic microorganisms [5].

Nested PCR with preliminarily amplified fragments of Bacterial 16S rRNA gene and primers specific to six main phylogenetic subgroups of SRB is a

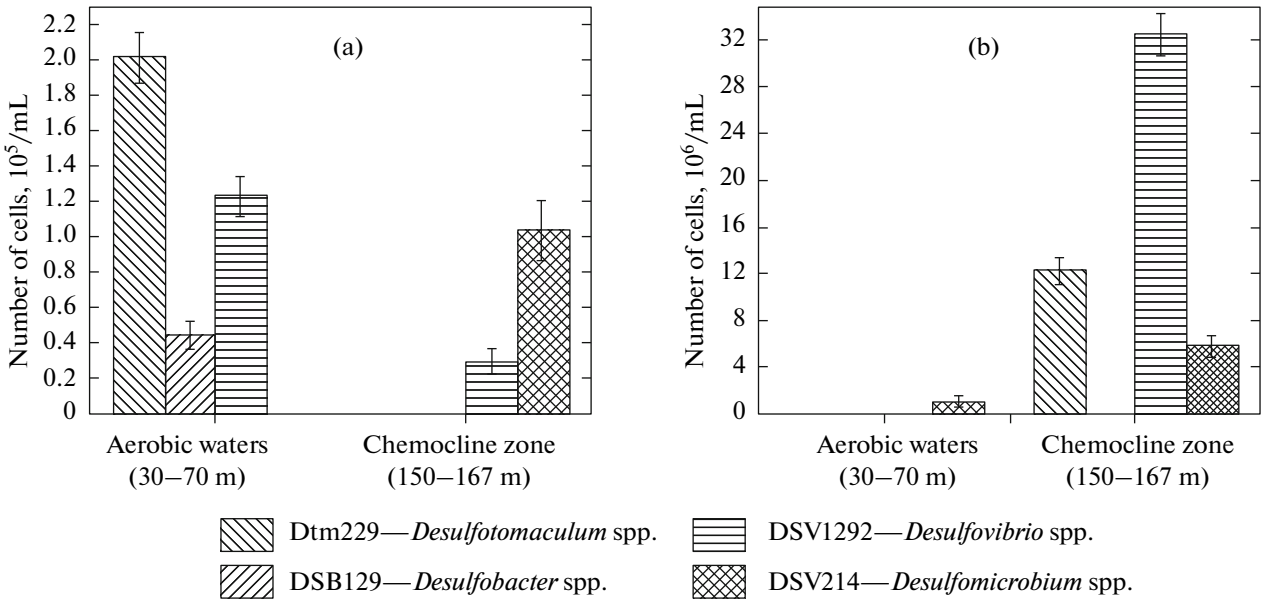

Fig. 1. FISH detection of abundance and phylogenetic composition of communities of sulfate-reducing bacteria (a) in oxygen-containing water column of continental slope of the Black Sea and (b) in enrichment cultures isolated from the water samples from the respective depths.

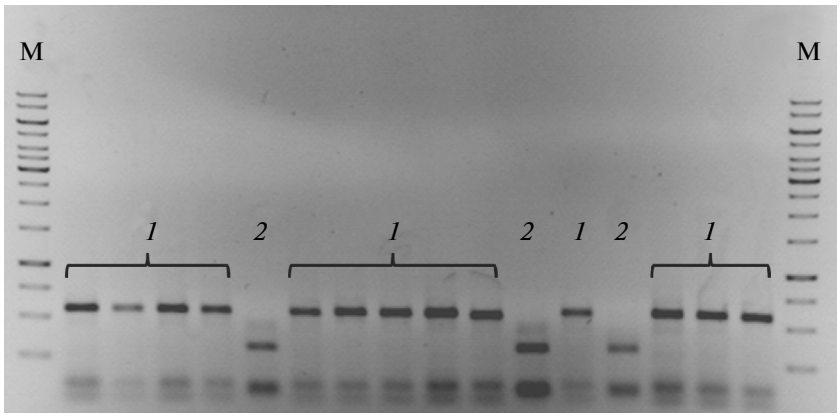

Fig. 2. Restriction analysis of 16S rRNA gene fragments of the Black Sea SRB enrichment culture obtained from the water sample from the 70 m depth. Lines: (*1*) fragments without restriction site for *Hae*III (*Desulfosporosinus* sp.), (*2*) fragments with a single restriction site for *Hae*III (*Vibrio* sp.); (M) DNA-marker GeneRuler 1 kb DNA Ladder (Fermentas, Lithuania).

more reliable method than FISH. Nested PCR helped to confirm the results of FISH analysis demonstrating the presence in the Black Sea and in the Gdansk Deep of the Baltic Sea at a 30-m depth of not only *Desulfotomaculum* (first subgroup of SRB), *Desulfobacter* (fourth subgroup of SRB), and *Desulfovibrio-Desulfomicrobium* (sixth subgroup of SRB) but also of DNA of the fifth subgroup of SRB (*Desulfococcus-Desulfonema-Desulfosarcina*) as well. Nested PCR helped to detect the members of the first, fifth, and sixth subgroups of SRB in the chemocline zone also, while 16S rRNA gene fragments specific for SRB of the second (*Desulfobulbus*) and third (*Desulfobacterium*) subgroups were not discovered in oxygen-containing water layers of both meromictic basins. According to the obtained results, the members of *Desulfococcus-Desulfonema-Desulfosarcina* and *Desulfovibrio-Desulfomicrobium* are supposed to be dominant SRB in the upper horizons of the Black and the Baltic seas up to the lower border of chemocline according to the following presumption. In natural samples, microorganisms whose 16S rRNA gene can be detected only by nested PCR are represented in a less amount than those detected also by the less sensitive direct PCR as well [7].

In subsurface waters and in the chemocline zone, PCR with total DNA isolated from water samples that were filtered only through GF/C filters (to assess the

presence of not only free-living SRB but of associated with organic suspended matter as well), detected the presence of 16S rRNA gene fragments specific for the first, fourth, and sixth subgroups of SRB in the Black Sea and for the first, fifth, and sixth subgroups in the Baltic Sea. Interestingly, 16S rRNA gene fragments of *Desulfobacter* genus were not registered in the biomass samples from the lower zones of chemocline of the Black Sea concentrated on membrane filters. This might be an evidence of the presence of *Desulfobacter* spp. cells in the chemocline zone only as a part of suspended particles.

Active enrichment cultures of SRB were obtained from oxygen-containing waters of the Black Sea (30, 70, and 157 m depths) and the Gdansk Deep of the Baltic Sea (30 m depth). Structure of SRB community in enrichment cultures differed significantly from that in native water samples. Thus, enrichments from aerobic zone of the Black Sea contained mostly *Desulfomicrobium* spp. cells, while the members of the genera *Desulfovibrio* and *Desulfotomaculum* dominated the enrichment cultures from the chemocline zone (Fig. 1b).

Enrichment culture from a 70-m depth of the Black Sea demonstrated the most active growth at Widdel medium with addition of $K_2Cr_2O_7$ (10 μM). According to the results of restriction analysis of the cloned 16S rRNA gene fragments of that culture, two groups were selected from 57 samples of the library (Fig. 2): with one restriction site for *Hae*III (12 clones) and without it (45 clones). Results of sequencing of three randomly selected samples of both groups showed that enrichment culture consists of SRB, according to nucleotide homology (97%) most close to *Desulfosporosinus* sp. 159 (AF295659), and of heterotrophic bacteria *Vibrio* sp. B234 (FN295777). Discovering of *Desulfosporosinus* spp. cells in the Black Sea is a remarkable fact in the research of marine SRB communities since that genus consists of spore-forming cells isolated from freshwater sediments, acidic mining wastewater sediments and various types of soil.

Obtained results on detection of sulfate-reducing bacteria using FISH and PCR, together with isolation of active enrichment cultures from the oxygen-containing water horizons, demonstrate that SRB abundance in the Black and the Baltic seas is not limited by deep anaerobic waters and bottom sediments as was assumed previously. Phylogenetic composition of SRB communities from oxygen-containing waters of continental slope of the Black Sea and from the Gdansk Deep of the Baltic Sea is rather similar on the whole. SRB of the genus *Desulfotomaculum* and *Desulfovibrio* were discovered in the upper aerobic waters. In the chemocline zone, mostly the members of *Desulfomicrobium* spp. and *Desulfovibrio* spp. were found. Heterogeneous distribution of various phylogenetic subgroups of SRB at depth of oxygen-containing waters of the Black and the Baltic seas might be caused by the presence of suspended organic particles in respective water layers (diatom frustules, pellet material, etc.) that provide the possibility of formation of inner anaerobic microzones optimal for SRB growth.

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