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Real-time PCR quantification and diversity analysis of the functional genes *aprA* and *dsrA* of sulfate-reducing prokaryotes in marine sediments of the Peru continental margin and the Black Sea

***Anna Blazejak*[1†] *and Axel Schippers*[1,2*]**

[1] *Geomicrobiology, Federal Institute for Geosciences and Natural Resources (BGR), Hannover, Germany*
[2] *Faculty of Natural Sciences, Leibniz Universität Hannover, Hannover, Germany*

Edited by:
Andreas Teske, University of North Carolina at Chapel Hill, USA

Reviewed by:
Julie A. Huber, Marine Biological Laboratory, USA
Kasthuri Venkateswaran, NASA-Jet Propulsion Laboratory, USA

***Correspondence:**
Axel Schippers, Geomicrobiology, Federal Institute for Geosciences and Natural Resources (BGR), Stilleweg 2, 30655 Hannover, Germany.
e-mail: axel.schippers@bgr.de

[†]***Present address:***
Anna Blazejak, Max Planck Institute for Marine Microbiology, Bremen, Germany.

Sulfate-reducing prokaryotes (SRP) are ubiquitous and quantitatively important members in many ecosystems, especially in marine sediments. However their abundance and diversity in subsurface marine sediments is poorly understood. In this study, the abundance and diversity of the functional genes for the enzymes adenosine 5′-phosphosulfate reductase (*aprA*) and dissimilatory sulfite reductase (*dsrA*) of SRP in marine sediments of the Peru continental margin and the Black Sea were analyzed, including samples from the deep biosphere (ODP site 1227). For *aprA* quantification a Q-PCR assay was designed and evaluated. Depth profiles of the *aprA* and *dsrA* copy numbers were almost equal for all sites. Gene copy numbers decreased concomitantly with depth from around 10^8/g sediment close to the sediment surface to less than 10^5/g sediment at 5 mbsf. The 16S rRNA gene copy numbers of total bacteria were much higher than those of the functional genes at all sediment depths and used to calculate the proportion of SRP to the total *Bacteria*. The *aprA* and *dsrA* copy numbers comprised in average 0.5–1% of the 16S rRNA gene copy numbers of total bacteria in the sediments up to a depth of ca. 40 mbsf. In the zone without detectable sulfate in the pore water from about 40–121 mbsf (Peru margin ODP site 1227), only *dsrA* (but not *aprA*) was detected with copy numbers of less than 10^4/g sediment, comprising ca. 14% of the 16S rRNA gene copy numbers of total bacteria. In this zone, sulfate might be provided for SRP by anaerobic sulfide oxidation. Clone libraries of *aprA* showed that all isolated sequences originate from SRP showing a close relationship to *aprA* of characterized species or form a new cluster with only distant relation to *aprA* of isolated SRP. For *dsrA* a high diversity was detected, even up to 121 m sediment depth in the deep biosphere.

Keywords: deep biosphere, real-time PCR, subsurface, ODP, sulfate-reducing prokaryotes, *aprA*, *dsrA*

INTRODUCTION

Sulfate reduction plays a crucial role in the past and present global sulfur cycle, and may be regarded as one of the oldest metabolic pathways on Earth (Castresana and Moreira, 1999; Schen et al., 2001). Therefore, sulfate-reducing prokaryotes (SRP) are biogeochemically important organisms in the environment, especially for the degradation of organic matter in coastal but also in deeply buried marine sediments in the open ocean (Jørgensen, 1982; Ferdelman et al., 1997; Knoblauch et al., 1999; Sahm et al., 1999; Thamdrup et al., 2000; Jørgensen et al., 2001; D'Hondt et al., 2004; Parkes et al., 2005; Schippers et al., 2005, 2010). Despite their importance in subsurface marine sediments the abundance and diversity of SRP in this environment is poorly understood. Global surveys of SRP cell numbers and gene sequencing data are missing and thus, more primary data for particular sediment sites are necessary. This includes the development of new methods for the detection of SRP in environmental samples.

The abundance of SRP in marine sediments has been determined by a variety of methods including MPN-cultivation (Knoblauch et al., 1999), 16S rRNA slot-blot hybridization (Sahm et al., 1999), or FISH and CARD-FISH with 16S rRNA gene probes (Ravenschlag et al., 2000; Gittel et al., 2008). Since SRP are phylogenetically diverse (Stahl et al., 2002), 16S rRNA approaches require a comprehensive set of 16S rRNA probes for a full, quantitative coverage of all SRP in an environmental sample (Ravenschlag et al., 2000). The functional gene encoding for dissimilatory sulfite reductase (*dsrA*) of SRP shows a high similarity in different SRP (Wagner et al., 1998), thus a *dsrA* specific PCR primer set targeting both, Gram-positive and Gram-negative SRP species, was developed for competitive PCR quantification (Kondo et al., 2004). These primers were also used to design a quantitative, real-time PCR (Q-PCR) assay for *dsrA* for SRP quantification in subsurface marine sediments (Schippers and Neretin, 2006; Leloup et al., 2007, 2009; Nunoura et al., 2009; Webster et al., 2009; Schippers et al., 2010) and the Black Sea water column (Neretin et al., 2007).

Other Q-PCR assays for *dsrA* based on other primers (Wagner et al., 1998; Dhillon et al., 2003; Geets et al., 2006) were also applied to marine sediments (Wilms et al., 2007; Engelen et al., 2008), oil (Agrawal and Lal, 2009), and wastewater (Ben-Dov et al., 2007). Furthermore, RT-Q-PCR was applied to quantify mRNA of *dsrA* (Neretin et al., 2003).

Due to PCR bias or mismatches of the *dsrA* of not yet discovered SRP with the available *dsrA* primers, important SRP might have been overlooked in environmental samples. This might have happened in studies of deeply buried marine sediments (e.g., Peru continental margin, ODP Leg 201) in which sulfate reduction was identified as an important biogeochemical process, but *dsrA* or 16S rRNA genes of SRP were scarcely detected (D'Hondt et al., 2004; Parkes et al., 2005; Schippers et al., 2005; Inagaki et al., 2006; Schippers and Neretin, 2006; Teske, 2006; Webster et al., 2006, 2009; Fry et al., 2008; Nunoura et al., 2009). For this reason, another independent SRP quantification method is useful to reveal *dsrA* data and to confirm the full quantitative coverage of SRP in environmental sample analyses, especially for the deep biosphere.

A second functional gene of SRP is the adenosine 5′-phosphosulfate reductase gene *aprA*. In sulfate reducers, APS reductase catalyzes the two-electron reduction of APS to sulfite and AMP. APS reductase consists of an alpha and beta subunit, encoded by the genes *aprA* and *aprB*, respectively. The *aprA* gene has been thoroughly studied in SRP, and specific PCR and Q-PCR amplification of *aprA* was shown (Friedrich, 2002; Blazejak et al., 2005; Ben-Dov et al., 2007; Meyer and Kuever, 2007).

The objective of this study was a better understanding of the abundance and diversity of SRP in subsurface marine sediments. A Q-PCR assay specific for *aprA* of SRP was designed and applied to samples from different marine sediments together with the published Q-PCR assay for *dsrA* quantification (Schippers and Neretin, 2006). The diversity of SRP was analyzed based on cloning and sequencing of their functional genes *aprA* and *dsrA*. Marine sediments of the Peru continental margin, including samples from the deep biosphere (ODP site 1227), and the Black Sea were chosen because previous studies indicate that sulfate reduction is an important biogeochemical process in these sediments (Jørgensen et al., 2001; D'Hondt et al., 2004; Schippers et al., 2005). In addition, the abundance of sulfate reducers and other microorganisms was already determined using different assays, allowing comparisons with our newly developed method (Schippers et al., 2005; Inagaki et al., 2006; Schippers and Neretin, 2006; Leloup et al., 2007; Blazejak and Schippers, 2010).

MATERIALS AND METHODS

SAMPLE COLLECTION

Samples were collected from different sediment depths at three marine sites during three research vessel expeditions. Site 1227 (8°59.5′S, 79°57.4′W) at a water depth of 427 m on the Peru margin was sampled with advanced piston coring up to 121 mbsf during Ocean Drilling Program (ODP) Leg 201 in March 2002 (D'Hondt et al., 2003; Jørgensen et al., 2005). Site 2MC (11°35.0′S, 77°33.1′W) at a water depth of 86 m on the Peru continental margin was sampled with a multicorer up to 0.34 mbsf during the cruise SO147 of R/V Sonne in June 2000. Site 20 (43°57.25′N, 35°38.46′E) at a water depth of 2048 m in the Black Sea was sampled with a gravity corer up to 5.8 mbsf during cruise M72-5 of R/V Meteor in May 2007.

Samples for molecular analysis were taken aseptically from the center of the cores at all stations and were stored at −20°C until further processing in the laboratory. For the recovery of deeply buried sediments from site 1227 on the Peru margin seawater based drilling fluid was used. Thus a potential contamination with seawater microorganisms was routinely checked by application of fluorescent beads of prokaryotic cell size and a chemical tracer (D'Hondt et al., 2003). Only uncontaminated samples were used for further analysis.

DNA EXTRACTION

DNA was isolated from 0.5–4 g sediment of various depths using a FastDNA®Spin for Soil Kit (MP Biomedicals, Solon, OH, USA) with the following modification: to increase the yield of isolated DNA from clayish sediments 200 µg polyadenylic acid (Roche Diagnostics GmbH, Mannheim, Germany) dissolved in sterile water was added to the sample at the first step of the extraction procedure (Webster et al., 2003). DNA extracts from blank tubes (no sediment added) were used as procedural contamination control in later PCR analyses. Isolated DNA was stored in aliquots to avoid multiple defrosting and freezing and was thawed for Q-PCR measurements not more than twice.

Q-PCR MEASUREMENTS

Quantitative PCR measurements were run in triplicate on an ABI Prism 7000 detection system (Applied Biosystems, Foster City, CA, USA). Quantification of *Bacteria* in total was performed using a Q-PCR assay based on the detection of the 16S rRNA gene (Nadkarni et al., 2002). The dissimilatory sulfite reductase gene *dsrA* of SRP was quantified using a published protocol (Schippers and Neretin, 2006) and primers (Kondo et al., 2004). The size of the amplified fragments was 219 bp. To quantify the adenosine 5′-phosphosulfate reductase gene *aprA* of SRP, a novel Q-PCR assay was designed. For specific amplification of this gene the primers APS1F (5-TGGCAGATCATGATYMAYGG-3) and APS4R (5-GCGCCAACYGGRCCRTA-3) were used (Blazejak et al., 2005; Meyer and Kuever, 2007). The size of the amplified fragments was 384–396 bp. The Q-PCR assay was performed with Platinum® SYBR® Green Q-PCR SuperMix-UDG with ROX (Invitrogen, Carlsberg, CA, USA), a primer concentration of 300 nM, and the following amplification conditions: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Two microliters sample DNA were added to a PCR reaction assay with a total volume of 25 µL. Melting curve analyses were run after each assay to check PCR specificity. For amplification of standards, DNA was extracted, amplified, and purified from minipreps of cloned *aprA* gene sequences from sulfate-reducing endosymbiotic bacteria with the accession numbers AM234052 and AM234053.

Q-PCR DATA ANALYSIS

Relative standards were prepared by serial dilution (1:10) of the PCR product. For each standard, the concentration was plotted against the cycle number at which the fluorescence signal increased above the background or cycle threshold (C_t value). The

slope of each calibration curve was included into the following equation to determine the efficiency of the PCR reaction: efficiency $= 10^{(-1/\text{slope})} - 1$. According to this formula, an efficiency of 100% means a doubling of the product in each cycle. Data evaluation was performed with the software StepOne™ v2.0 (Applied Biosystems, Foster City, CA, USA).

PCR AMPLIFICATION, CLONING, AND SEQUENCING OF THE *dsrA* AND *aprA* GENES

DNA was isolated from sediment samples of the Peru margin from three depths, 3.6, 65.3, and 121.4 mbsf (site 1227, ODP Leg 201) and in the Black Sea from four depths, 0.15, 2.7, 4.5, and 5.8 mbsf (site 20 GC, M72-5). Except for the number of cycles, amplification of the *dsrA* and *aprA* genes was carried out at the same conditions as for the Q-PCR assays (see above). For amplification of the *dsrA* gene, 30 cycles of PCR were required for the sediment sample from 3.6 mbsf depth of the Peru margin, and 35 cycles for the other samples. To amplify the *aprA* gene, 25 cycles of PCR were applied to the sediment samples from 0.15 and 2.7 mbsf depth in the Black Sea, and up to 35 cycles for the remaining samples. Three parallel PCR products obtained from each depth were combined, purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and subsequently cloned using the pGEM®-T Easy vector system (Promega, Madison, WI, USA) and TOP10 chemically competent cells (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Because of the high number of PCR cycles also the yield of the negative controls, although no visible amplification was observed, was purified, and cloned. Clones were randomly picked, suspended in PCR grade water and selected for the correct insert size by PCR with vector primers. Approximately 50 positive clones per depth were sequenced with the vector primer M13 Forward. Sequencing reactions were run using ABI BigDye on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

PHYLOGENETIC ANALYSIS

For sequence alignment and phylogenetic tree reconstruction sequences were analyzed with the BioEdit program[1] and the software ARB[2]. Briefly, after removal of the vector sequence, sequences were aligned and clustered. Phylogenetic calculations for the partial *aprA* genes were generated from 128–132 deduced amino acids sequences using maximum-likelihood analyses with a 25% positional conservation filter. For the phylogenetic analysis of the partial *dsrA* sequences first a maximum-likelihood tree was generated from *dsrAB* sequences of full length (approximately 650 amino acids), than successively single partial *dsrA* sequences (73 amino acids) were added to the tree using a 25% positional conservation filter.

NUCLEOTIDE ACCESSION NUMBERS

The *dsrA* and *aprA* gene sequences obtained in this study were submitted to the DDBJ/EMBL/GenBank nucleotide databases under the accession numbers HE575209–HE575212 and HE575674–HE575681 for *aprA* sequences and HE575682–HE575732 for *dsrA* sequences.

[1] www.mbio.ncsu.edu/BioEdit/bioedit.html
[2] www.arb-home.de

RESULTS AND DISCUSSION

In this study the abundance and diversity of the functional genes for adenosine 5′-phosphosulfate reductase (*aprA*) and dissimilatory sulfite reductase (*dsrA*) of SRP were analyzed in marine sediments from the Black Sea, and the Peru continental margin, including deep biosphere sediments (ODP site 1227). For *aprA* quantification a Q-PCR assay was designed. The evaluation results for this assay are followed by data on the abundance and diversity of *aprA* and *dsrA* in sediments. For comparison and interpretation, 16S rRNA gene copy numbers of total bacteria from a previous study (Blazejak and Schippers, 2010) have been included here.

EVALUATION OF THE Q-PCR ASSAY FOR *aprA*

Amplification quantities of the standard ranged from 1.0×10^1 to 1.0×10^7 molecules with a correlation coefficient of 0.996. The efficiency of the PCR reactions was 96%. Detection of contaminant DNA in the negative control was not observed. In our experiments the detection limit was set to 1.0×10^2 molecules. This could be lowered to 1.0×10^1 still ensuring reliable detection values since no contaminant DNA in the negative controls was identified. Detection limits for gene quantification by PCR for functional genes can range up to 10 copies per reaction (Vaerman et al., 2004; Bustin et al., 2009). However one critical limitation of PCR-based methods is their sensitivity to compounds that are co-extracted with the DNA from environmental samples, in particular from sediments and soils, that may influence and inhibit the real-time PCR-process. For example humic acids can hamper the PCR reaction and impair fluorescence, and metal ions can inhibit DNA polymerases (Lindberg et al., 2007) whereby the detection limit is lowered. The maximum fluorescence signal of the melting curve occurred at a temperature of 87°C. Melting curves were analyzed after each assay and always showed a single peak, verifying the specificity of the PCR amplification.

QUANTIFICATION OF THE FUNCTIONAL GENES *aprA* AND *dsrA* OF SRP AND 16S rRNA OF TOTAL BACTERIA IN MARINE SEDIMENT SAMPLES

Depth profiles of DNA copy numbers of the functional genes *aprA* and *dsrA* as marker for sulfate-reducing prokaryotes (SRP) and the 16S rRNA gene of total *Bacteria* are shown in **Figure 1** for three sediment sites, surface (site 2MC, 0–0.35 mbsf) and deep (site 1227, 0–121.4 mbsf) sediments on the Peru margin, and in the Black Sea (site 20, 0–5.8 mbsf). The copy numbers of all genes decreased with sediment depth in different depth gradients. An important finding of this study was that the depth profiles of copy numbers of both functional genes, *aprA* and *dsrA*, were almost equal for all sediment sites expect for the ODP site 1227 below 40 mbsf. Congruent SRP quantification profiles based on independent Q-PCR analysis of two functional genes imply that no SRP have been overlooked, and that the results are close to the actual SRP gene density in the subsurface. Two independent Q-PCR assays with different primers are very unlikely to generate identical PCR biases and quantification profiles by chance.

In the Black Sea at site 20, all gene copy numbers decreased rapidly within 65 cm from the sediment surface. The *dsrA* and *aprA* copy numbers decreased from 10^7–10^8 copies/g at the sediment surface to less than 10^5 copies/g below 0.6 mbsf. They decreased

further to less than 10^4 copies/g below 3 mbsf. The *dsrA* copy numbers close to the sediment surface were similar to those for another sediment site of the Black Sea (Leloup et al., 2007). Downcore, the numbers in our study decreased toward lower counts than those in the previous study. Similar differences between these two sites were also found for the 16S rRNA gene copy numbers of total *Bacteria*. While site 20 was located in the central basin of the Black Sea southeast of the peninsula Crimea at 2048 m water depth, the site of the previous study was located west of the peninsula Crimea on the slope at 1024 m water depth. Thus, different organic matter availability may explain the different gene copy numbers in the two studies.

In the Peru continental margin near-surface sediments (site 2MC) the *dsrA* and *aprA* copy numbers were very close to each other and exhibited a more pronounced depth gradient than the 16S rRNA gene copy numbers of total *Bacteria* (**Figure 1**). The *dsrA* and *aprA* copy numbers decreased from more than 10^8 copies/g at the sediment surface to 10^6–10^7 copies/g between 0.18 and 0.34 mbsf. In a previous Q-PCR study of the same site (Schippers and Neretin, 2006), the *dsrA* and 16S rRNA gene copy numbers of total *Bacteria* copy numbers were similar to those of this new study.

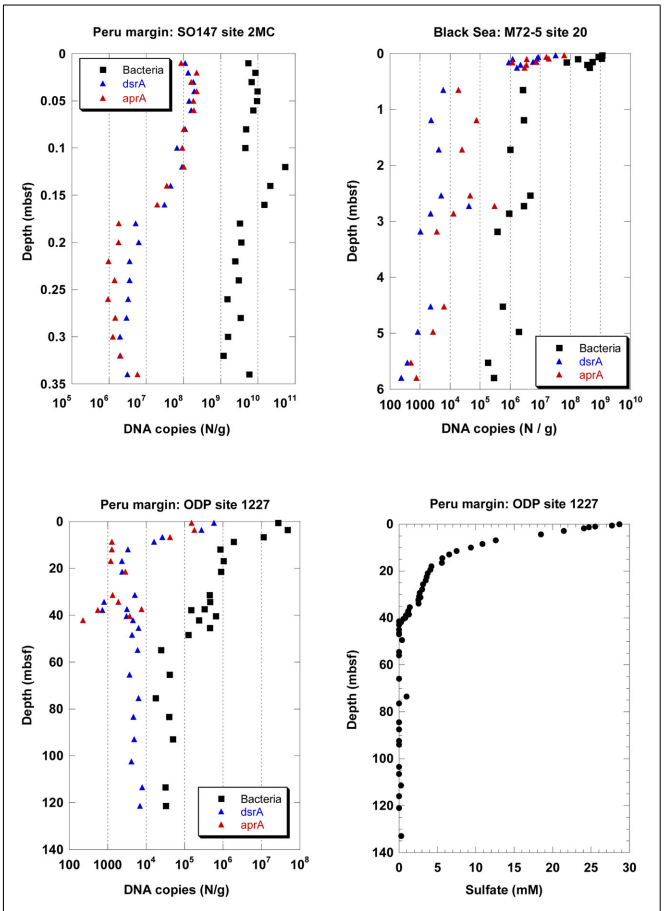

FIGURE 1 | Depth profiles of DNA copy numbers of the functional genes *aprA* and *dsrA* as marker for sulfate-reducing prokaryotes (SRP) and the 16S rRNA gene of total *Bacteria* at three sediment sites, surface (site 2MC, 0–0.35 mbsf) and deep (site 1227, 0–121.4 mbsf) sediments on the Peru margin, and in the Black Sea (site 20, 0–5.8 mbsf), and depth profile of pore water sulfate concentrations at site 1227 (0–135 mbsf, D'Hondt et al., 2004) on the Peru margin. ■, *Bacteria*; ▲, *dsrA*; ▲, *aprA*.

In the deeply buried Peru margin sediment (site 1227) the *dsrA* and *aprA* copy numbers decreased from 10^5–10^6/g sediment at the top of the core at 0.6 mbsf to less than 10^4/g sediment at 10 mbsf. These numbers for both genes stay steady up to 35 mbsf. Below 35 mbsf the run of the curves are different. After a slight increase of the *aprA* gene copy numbers between 37–40 mbsf they drop to less than 10^3/g sediment at 42 mbsf and are not more detectable underneath this depth. In contrast, *dsrA* copy numbers below 10^4 copies/g sediment are still observed up to the depth of 121 mbsf. For all samples between 10–121 mbsf, *dsrA* copy numbers remained consistent in this range. In contrast, *dsrA* was only patchily detected (5 out of 19 samples) in the previous study (Schippers and Neretin, 2006). The *dsrA* values in the deeper sediment are close to the detection limit of the Q-PCR method. Thus, slight differences in the efficiency of DNA extraction from the sediment or differences in the total amount of sediment used for DNA extraction may explain this discrepancy.

The 16S rRNA gene copy numbers of total *Bacteria* exceeded those of the functional genes at all sediment depths, and allowed to calculate the proportion of SRP to total *Bacteria*. The *aprA* and *dsrA* copy numbers comprised in average 0.5–1% of the 16S rRNA gene copy numbers of total *Bacteria* in the sediments of the Black Sea and those from the Peru continental margin up to a depth of ca. 40 mbsf. Below, only *dsrA* (but not *aprA*) was detected with copy numbers of less than 10^4/g sediment, comprising ca. 14% of the 16S rRNA gene copy numbers of total *Bacteria*. In other marine sediments sulfate reducers contributed to <1–30% to the prokaryotic community based on Q-PCR, FISH, or rRNA slot blot hybridization analyses (Sahm et al., 1999; Ravenschlag et al., 2000; Knittel et al., 2003; Schippers and Neretin, 2006; Leloup et al., 2007, 2009; Wilms et al., 2007; Gittel et al., 2008; Julies et al., 2010; Schippers et al., 2010). Overall our Q-PCR analysis of the functional genes revealed that SRP are a minor part of the prokaryotic community in the Peru margin sediments, in agreement with clone library data (Parkes et al., 2005; Inagaki et al., 2006; Webster et al., 2006). Based on Q-PCR analysis of the same sediment samples especially the bacterial groups *Chloroflexi* and/or candidate division JS-1 were shown to be dominant (Blazejak and Schippers, 2010), while *Archaea*, *Eukarya*, and the Fe(III)- and Mn(IV)-reducing bacteria of the family *Geobacteraceae* (Inagaki et al., 2006; Schippers and Neretin, 2006) were of minor abundance.

Active sulfate reduction for the two Peru margin sites up to a depth of ca. 40 mbsf was confirmed by pore water sulfate profiles and sulfate reduction rate measurements (Böning et al., 2004; D'Hondt et al., 2004; Schippers et al., 2005). At ca. 40 mbsf sulfate is reduced by methane oxidation (sulfate–methane transition zone) and a slight maximum of 16S rRNA genes was detected (Schippers et al., 2005; Schippers and Neretin, 2006; Sørensen and Teske, 2006; Teske and Sørensen, 2008). This maximum is not reflected by higher copy numbers of the functional genes *dsrA* or *aprA* of SRP indicating that sulfate-dependent anaerobic methane oxidation is not linked to a SRP population peak.

The detection of *dsrA* of SRP below ca. 40 mbsf was surprising because sulfate as the electron acceptor for active SRP was not detectable in the pore water from ca. 40–121 mbsf of site 1227 (**Figure 1**). There are three possibilities to explain this finding: 1. The detected *dsrA* was not extracted from living cells but is part of fossil DNA, persisting adsorbed to sediment particles over geological time scales as previously discussed (Inagaki et al., 2005; Schippers and Neretin, 2006; Schippers et al., 2010); 2. The *dsrA* originated from living SRP which use another electron acceptor than sulfate, e.g., Fe(III) as shown for several genera of SRP (Vandieken et al., 2006); 3. Low amounts of sulfate might be provided by anoxic oxidation of sulfides. The sulfate formed by this process is constantly consumed by SRP, thus it remained undetectable in the pore water.

We believe that the third possibility is most relevant. On the one hand very low rates of sulfate reduction have been measured with sulfate radiotracer for site 1227 even below 40 mbsf (Schippers et al., 2005). On the other hand, stable isotope data of oxygen and sulfate for sediment and experimental studies support a deep anoxic sulfur cycle. Sulfide oxidation occurs with reactive iron or manganese oxides as oxidant in deeply buried sediments (Bottrell et al., 2000, 2008; Schippers and Jørgensen, 2001; Riedinger et al., 2010; Holmkvist et al., 2011a,b).

DIVERSITY OF THE FUNCTIONAL GENES *aprA* AND *dsrA* IN SEDIMENT SAMPLES

To analyze not only the abundance but also the diversity of SRP, their metabolic key genes, *aprA* and *dsrA* genes, were cloned. Sediment samples of three depths at the Peru margin (site 1227), 3.6, 65.3, and 121.4 mbsf, and four depths in the Black Sea (site 20), 0.15, 2.7, 4.5, and 5.8 mbsf were selected for the study. Sequence analysis of the isolated *aprA* and *dsrA* sequences showed their relationship to *aprA* or *dsrA* genes from characterized SRP indicating that they also originate from SRP. Although high numbers of PCR cycles were required (up to 35 cycles for *dsrA* amplification), no visible amplification was observed in the negative controls. Sequences of a few clones obtained from the negative controls showed that only primer sequences were inserted into the cloning vectors. Thus, despite the high PCR cycle number no contamination was noted.

For *aprA* analysis, 50 clones from a Peru margin sediment at 3.6 mbsf, and 24–45 clones from each Black Sea sediment depth were sequenced. Sequences were grouped into distinct clone families based on their sequence similarities and their allocation within the phylogenetic tree after algorithmic calculations. Sequence similarities within a clone family as well as the similarity to the next relative sequence of a cultivated bacterium are shown in **Table 1**. For phylogenetic tree reconstruction all sequences were used; however only one representative sequence of each clone family is presented (**Figure 2**).

The *aprA* sequences isolated from 3.6 mbsf depth on the Peru margin were classified into four distinct clone families showing a close relationship to the sulfate-reducing bacteria (SRB) *Desulfococcus multivorans*, *Desulfomonile tiedjei*, *Desulfovibrio baarsii*, and a cluster including the genera *Desulfacinum*, *Desulforhabdus,* and *Syntrophobacter* (**Figure 2**; **Table 1**). In deeper sediment layers, at 65.3 and 121.4 mbsf, the *aprA* gene was not amplified although a high number of cycles (up to 35) was applied. This result corresponds to the absence of quantitative data of the *aprA* using the newly designed Q-PCR assay (**Figure 1**).

In the Black Sea sediment a slight decline of diversity with depth could be observed. Near-surface (0.15 mbsf) *aprA* sequences were assigned into four distinct clone families: *D. multivorans*, *D. tiedjei*, *Desulfobacterium anilini,* and a cluster including the genera *Desulfacinum*, *Desulforhabdus,* and *Syntrophobacter*. In contrast, sequences from 2.7 and 5.8 mbsf formed only two clone groups each, related to *Desulfonema magnum* and *Desulfobulbus elongatus,* and *Desulfomonile tiedjei* and *Desulfobacterium anilini,* respectively (**Figure 2**; **Table 1**).

Almost all isolated *aprA* sequences showed a close relationship to *aprA* sequences of cultivated, well characterized SRB of the *Deltaproteobacteria* indicating that they also originate from bacteria with a same metabolism. For two sequences (accession

Table 1 | Gene *aprA* clone library data for three sediment samples of the Peru margin and the Black Sea each.

Sampling site	No. of clones analyzed	Sequences classified as relatives to:						
		Desulfonema magnum	***Desulfococcus multivorans***	***Desulfobulbus elongatus***	***Desulfomonile tiedjei***	***Desulfovibrio baarsii***	***Desulfobacterium anilini***	***Desulfacinum, Desulforhabdus, Syntrophobacter***
		No. of clones (%)/sequence similarity within the group in %/sequence similarity to the next relative in %						
PERU MARGIN (SITE 1227)								
3.6 mbsf	50		22 (44)/100/90		5 (10)/81/74–77	6 (12)/100/85		17 (34)/100/66–69
65.3 mbsf	0							
121.4 mbsf	0							
BLACK SEA (SITE 20)								
0.15 mbsf	31		2 (6)/100/90		20 (65)/72–90/75–84		4 (13)/78/79–81	5 (16)/87/68–72
2.7 mbsf	45	1 (2)/100/91		44 (98)/97/87				
5.8 mbsf	24				20 (83)/98/83		4 (17)/83/81–84	

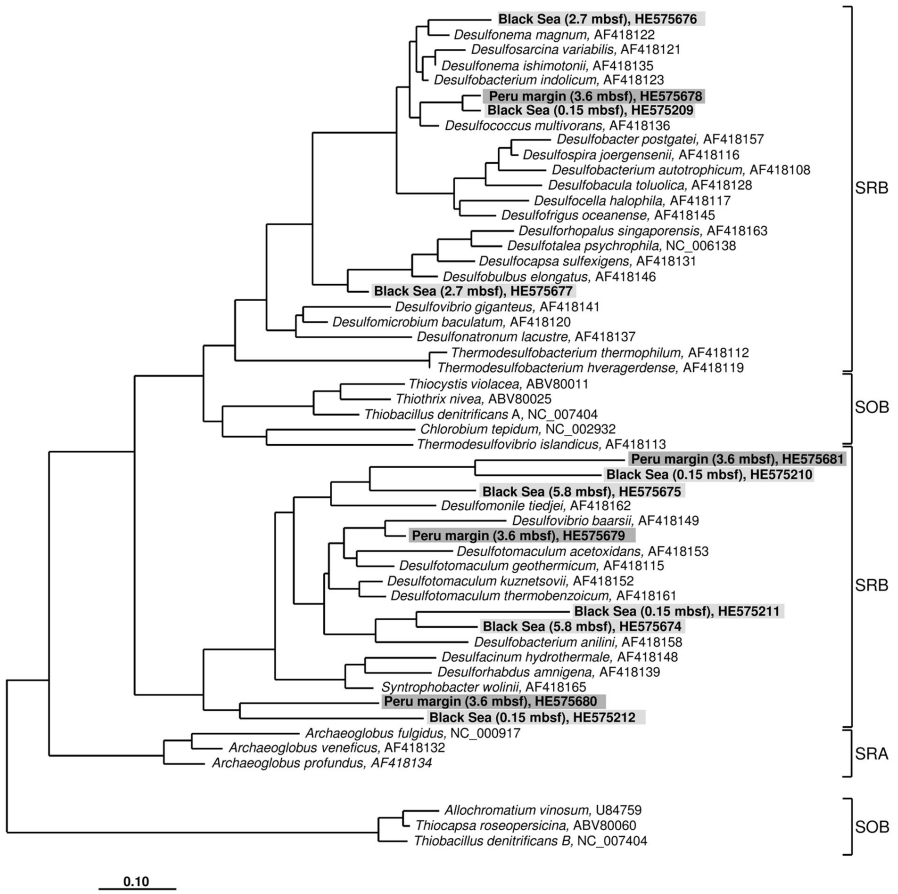

FIGURE 2 | Gene *aprA* phylogeny based on deduced amino acid sequences of the *aprA* gene coding for the alpha subunit of the adenosine 5′-phosphosulfate reductase. Sequences from this study are highlighted in light gray (Black Sea sediments) and dark gray (Peru margin sediments). Scale bar = 0.10 estimated substitutions per site. SOB, sulfide-oxidizing bacteria; SRB, sulfate-reducing bacteria; SRA, sulfate-reducing archaea.

numbers HE575680 and HE575212), their relationship to known organisms is difficult to predict because they form a separate branch and are only distantly related to *aprA* sequences of characterized SRB of the *Deltaproteobacteria* and Gram-positive SRB of the genus *Desulfotomaculum* (**Figure 2**; **Table 1**).

The *dsrA* sequences could be amplified from sediment samples of all analyzed depths from the Peru margin and the Black Sea (**Table 2**). Up to 51 clone sequences per depth were included in the phylogenetic analysis. Because of the high PCR cycle number of up to 35 cycles, the negative controls was also cloned although no PCR bands were observed. Sequences obtained from these negative controls showed that only primer sequences were inserted into the cloning vector, thus despite the high PCR cycle number no contamination was found. Phylogenetic analysis showed that all isolated *dsrA* sequences were closely related to the metabolic gene *dsrA*. The *dsrA* sequences isolated from the Peru margin and the Black Sea sediments were classified into eight clone families, showing overall a higher diversity than the isolated *aprA* sequences. Sequence similarities within a clone family as well as the similarity to the next relative sequence of cultivated prokaryote are shown in **Table 2**. For phylogenetic tree reconstruction all sequences were used however only one representative sequence of each clone family is presented in **Figure 3**. Except for three clone families, sequences belonging to all other groups are closely related to *dsrA* sequences isolated from SRP of *Deltaproteobacteria* showing for some sequences habitat specificity. For example: *dsrA* sequences related to *Desulfovibrio acrylicus*, *Desulfohalobium utahense*, and to the genera *Desulfobulbus*, *Desulfacinum*, and *Syntrophobacter* were only found in sediments from the Peru margin, whereas sequences related to the genus *Desulfomicrobium* and to *Desulfoarculus baarsii* were found exclusively in sediments from the Black Sea. A comparatively high proportion of *dsrA* sequences (20%) related to *D. acrylicus* were found in Peru margin deeply buried sediments at 121 mbsf. A specific feature of this anoxic, sulfate-reducing bacterium is the ability to switch from sulfate to acrylate reduction once this is energetically more favorable (van der Maarel et al., 1996). In contrast to *dsrA* sequences showing habitat specificity, sequences related to *Desulfococcus oleovorans*, *Desulfobacterium autotrophicum*, and *Desulfotalea psychrophila* were detected in sediments at both sites. Members of the genera *Desulfococcus* and *Desulfobacterium* belong

Table 2 | Gene *dsrA* clone library data for three sediment samples of the Peru margin and four sediment samples of the Black Sea.

Sampling site	No. of clones analyzed	Sequences classified as relatives to:						
		Desulfovibrio acrylicus	***Desulfo-microbium***	***Desulfohalo-bium utahense***	***Desufococcus oleovorans***	***Desulfobacterium autotrophicum***	***Desulfo-bulbus***	***Desulfotalea psychrophila***
		No. of clones (%)/sequence similarity within the group in %/sequence similarity to the next relative in %						
PERU MARGIN								
3.6 mbsf	32			1 (3)/100/87	5 (16)/100/77			1 (3)/100/76
65.3 mbsf	24				2 (8)/100/84	2 (8)/100/99		
121.4 mbsf	50	10 (20)/100/99		2 (4)/100/84		18 (36)/100/99	1 (2)/100/87	
BLACK SEA								
0.15 mbsf	31		1 (3)/100/84–87		15 (48)/78–96/84–85			
2.7 mbsf	51							51 (100)/100/69
4.5 mbsf	18				13 (72)/100/81	1 (6)/100/100		
5.8 mbsf	23		1 (4)/100/82		2 (9)/100/86	4 (18)/97/97–100		
Sampling site		***Desulfacinum/ Syntrophobacter***	***Desulfoarculus baarsii***	***Archaeoglobus fulgidus***	**Cluster A (see Fig. 3)**	**Cluster B (see Fig. 3)**		
		No. of clones (%)/sequence similarity within the group in %/sequence similarity to the next relative in %						
PERU MARGIN								
3.6 mbsf		6 (19)/100/79				19 (59)/74–76		
65.3 mbsf		2 (8)/100/79–80				18 (75)/71–87		
121.4 mbsf		1 (2)/100/82–83				18 (36)/74–93		
BLACK SEA								
0.15 mbsf			2 (7)/100/72	2 (7)/100/71	5 (16)/91	6 (19)/79		
2.7 mbsf								
4.5 mbsf						4 (22)/68–70		
5.8 mbsf			1 (4)/100/91		1 (4)/100	14 (61)/72–78		

to the family of *Desulfobacteraceae* that are known to be able to oxidize a great variety of different electron donors completely to CO_2. Thus, they successfully inhabit anoxic marine environments such as Black Sea and Peru margin sediments (Ravenschlag et al., 2000; Liu et al., 2003; Mußmann et al., 2005; Kondo et al., 2007; Leloup et al., 2007, 2009) or the anoxic water column of the Black Sea (Vetriani et al., 2003; Neretin et al., 2007) and other marine habitats (Kondo et al., 2007). Besides *dsrA* sequences affiliated to *Desulfobacteraceae*, also numerous *aprA* sequences belonging to this family were detected in samples from the Black Sea and Peru margin sediments indicating that bacteria of this community play an important role in sulfate reduction in these sediments.

Only two *dsrA* sequences, isolated from sediment of the Black Sea, showed a distant relationship (71% amino acid similarity) to the sulfate-reducing archaeon (SRA) *Archaeoglobus fulgidus*. Primarily SRA of the genus *Archaeoglobus* were isolated from marine hydrothermal systems, North Sea oil fields, and from petroleum hydrocarbon-rich Guaymas Basin sediments off the coast of Mexico (Hartzell and Reed, 2006). A few *dsrA* sequences, allocated within the same cluster, could be isolated from other habitats as from the Nankai Trough deep-sea and Black Sea sediments (Kaneko et al., 2007; Leloup et al., 2007), and showed however also only a distant relationship to *dsrA* sequences of the genus *Archaeoglobus*. Because of this distant relationship and the fact that Black Sea sediments are not a typical habitat for SRA, the affiliation of these sequences to the genus *Archaeoglobus* is questionable. SRA may play only a minor role in these sediments because of the low numbers of detected *dsrA* sequences, and the lack of *aprA* clones related to SRA.

The affiliation of *dsrA* sequences of two clone families, named cluster A and B, could not be clearly identified. Sequences within the cluster A form a clearly separated branch based on their unique sequence signature and showed only distant similarities to *aprA* of *A. fulgidus* (70–71% amino acid similarity). The cluster B was generated by 24 different *dsrA* sequences isolated from sediments of both habitats from each depth. Within this cluster numerous *dsrA* clone sequences isolated from different marine sediments as from deep-sea sediments from the Nankai Trough (Kaneko et al., 2007) and the Guaymas Basin (Dhillon et al., 2003), but also from salt marsh sediments (Bahr et al., 2005), and fen soil (Loy et al., 2004) are represented (data not showed). The closest described relatives based on amino acid similarity searches are *dsrA* sequences from the archaeon *A. fulgidus* (65–76% amino acid sequence similarity), the SRB of the genus *Thermodesulfovibrio* of the class *Nitrospira* (63–76% amino acid sequence similarity), and gram-positive SRB of the genus *Desulfotomaculum* (61–73%

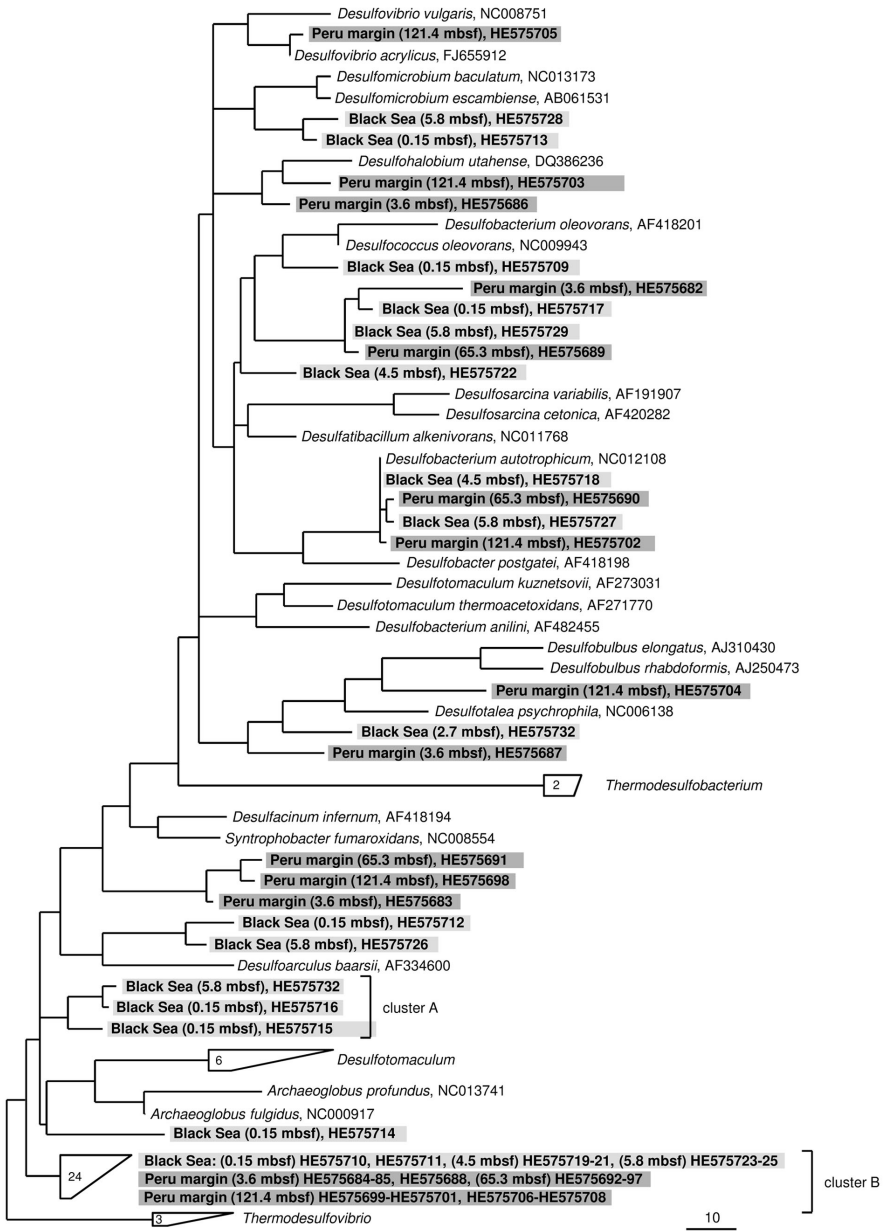

FIGURE 3 | Gene *dsrA* phylogeny based on deduced amino acid sequences of the *dsrA* gene coding for the alpha subunit of dissimilatory (bi)sulfite reductase. Sequences from this study are highlighted in light gray (Black Sea sediments) and dark gray (Peru margin sediments). Scale bar = 0.10 estimated substitutions per site.

amino acid sequence similarity). Because of the distant relationship to *dsrA* sequences from characterized microorganisms, the conclusion about the affiliation of the *dsrA* sequences of the cluster B to either SRA or SRB remains speculative. The common distribution of these *dsrA* sequences along the depth profiles of the two habitats and their high proportion within the clone libraries, up to 75%, argue for a significant role in sulfate/sulfite reduction of these microorganisms in marine sediments. They seem to be generalists and can adapt to a wide range of sulfate concentrations and electron donors, and it is tempting to suppose that they are a dominant group within the community of sulfate reducers in the anoxic sediments from the Black Sea and the Peru margin. Another unlike possibility could be that these *dsrA* sequences originate from microorganisms, which still contain this gene but have lost their ancestral ability of dissimilatory sulfate/sulfite reduction as shown for some members of *Desulfotomaculum* subcluster Ih (Imachi et al., 2006). This scenario would explain the unsuccessful cultivation of sulfate reducers related to the cluster B so far, because of incorrect selection of electron acceptors.

In summary of the *dsrA* diversity study, it can be concluded that this metabolic gene for the sulfate/sulfite reduction could

be detected in all analyzed sediment layers demonstrating a high diversity. Even in deeply buried sediments from the Peru margin in depths of 65 and 121 mbsf *dsrA* sequences were identified. Surprisingly, also in such an extreme habitat these sequences showed a high diversity belonging to well characterized genera as *Desulfobacterium* and *Desulfococcus* of the family *Desulfobacteraceae*, and *Desulfovibrio*, but also to novel deep-branching clusters A and B which phylogenetic affiliation and thus metabolism remain hidden. The presence of *aprA* and *dsrA* sequences affiliated to the same phylogenetic clusters, for example genera of the family *Desulfobacteraceae* or the genera *Syntrophobacter* and *Desulfacinum*, showed that sulfate reducers of equal phylogenesis were detected. This is particularly demonstrative for *aprA* and *dsrA* clone libraries from the Black Sea sediment of 2.7 mbsf depth showing that almost all *aprA* sequences (98%) and all *dsrA* sequences (100%) are affiliated either to *D. elongatus* or to *D. psychrophila*, respectively. The *aprA* and *dsrA* phylogeny of these two SRP of the family *Desulfobulbaceae* showed a close relationship to each other indicating that *aprA* and *dsrA* sequences detected in the Black Sea sediments could originate from the same bacterium.

Conclusively, although SRP do not belong to the most abundant prokaryotic groups, this study shows that they represent an inherent, diverse part of the microbial community in Black Sea sediments and the deep biosphere of Peru margin.

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