Assessing the Genetic Potential of Uncultivated Sulfate Reducing Bacteria

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When asked to move a mountain, do not look upon its size.

Merely move the first rock.

- David Gemmell

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ZUSAMMENFASSUNG

Mehr als 90% des in marinen Sedimenten gebildeten Methans wird durch die anaerobe Oxidation von Methan mit Sulfat als Elektronakzeptor (AOM) abgebaut. Der AOM Prozess wird von Konsortien aus nicht kultivierten anaeroben methanotrophen Archaeen (ANME) und sulfatreduzierenden Bakterien (SRB) katalysiert. Bisherige Studien lieferten nur ein begrenztes Verständins dieses Prozesses. Während die verschiedenen ANME Gruppen wiederholt im Bezug of Phylogenie, Schlüsselenzyme der Methanoxidation und ihr genetisches Potential untersucht wurden, gibt es bisher nur wenig Wissen über die assoziierten SRB. Ziel dieser Dissertation war es daher, die in den AOM Prozess involvierten SRB näher zu charakterisieren.

Zunächst wurde die Gruppe von SRB identifiziert, die mit Archaeen der ANME-2 Gruppe hauptsächlich assoziiert ist. Bakterielle 16S rRNA Gensequenzen, welche von ANME-2/SRB Anreicherungskulturen stammten, stützen eine frühere Hypothese, dass ANME-2 assoziierte SRB zur SEEP-SRB1 Gruppe innerhalb der *Desulfosarcina/Desulfococcus* Gruppe der *Deltaproteobakterien* gehören. Mit Hilfe der Fluoreszenz-*in-situ*-Hybridisierung (FISH) und Oligonukleotid-Sonden für neu definierte SEEP-SRB1 Untergruppen (a-f), wurden Bakterien der SEEP-SRB1a Untergruppe in sechs verschiedenen AOM Habitaten als dominante Partner von ANME-2 identifiziert. SEEP-SRB1a Einzelzellen wurden, mit Ausnahme einer Probe, dagegen sehr selten gefunden (<1%). Dies führte zu der Schlussfolgerung, dass SEEP-SRB1a Bakterien sehr stark an einen ANME-2 assoziierten Lebensstil angepasst sind. Zusätzlich wurden SEEP-SRB1a auch als alternative Partner von ANME-3 detektiert, welche vorher nur in Assoziation mit Bakterien des Genus *Desulfobulbus* beschrieben worden waren.

Im zweiten Teil dieser Dissertation wurde die Diversität von SRB an AOM Standorten basierend auf Schlüsselgenen der Sulfatreduktion, *aprA* bzw. *dsrAB,* untersucht. Proben von mikrobiellen Matten aus dem Schwarzen Meer sowie Anreicherungskulturen von Sedimenten über Gashydraten am Hydratrücken

(Cascardia Margin, NE Pazifik) wiesen eine geringe AprA- bzw DsrAB-Diversität auf, verglichen mit der Diversität in nicht angereichertem Hydratrückensediment. Die Klonbanken wurden von den *Desulfobacteraceae* zugeordneten Sequenzen dominiert, wiesen aber innerhalb der *Desulfobacteraceae* eine große Diversität auf. Die meisten der erhaltenen Sequenzen fielen in Gruppen die keinem kultivierten SRB zugeordnet werden konnten. Eine dieser Gruppen innerhalb des AprA-Phylogeniebaums konnte mit einer Kombination aus FISH und Durchflusszytometrie SEEP-SRB1a zugeordnet werden.

Im dritten Teil der Dissertation wurde versucht, einen Einblick in das genetische Potential von SEEP-SRB1a zu erhalten. Da es bisher keine Reinkulturen von SEEP-SRB1a gibt, wurde ein metagenomischer Ansatz verfolgt. Dafür wurde eine Fosmidklonbank aus DNA einer von ANME-2 und SEEP-SRB1a dominierten Anreicherungskultur hergestellt. Parallel dazu wurde ein Teil der DNA direkt durch „Pyrosequencing“ sequenziert. Insgesamt wurden 570 Mbp an Sequenzinformation generiert, die in größere Fragmente assembliert werden konnten. Von diesen Fragmenten wurden 9.075 aufgrund ihrer sehr großen Ähnlichkeiten mit Genomabschnitten von *Desulfococcus oleovorans* Hxd3, dem nächsten vollständig sequenzierten Verwandten von SEEP-SRB1a, SEEP-SRB1a zugeordnet. Zwei der Fragmente, die wahrscheinliche Apr bzw. Dsr Gene von SEEP-SRB1a trugen, wurden näher analysiert, um einen ersten Einblick in das genomische Potential von SEEP-SRB1a zu erhalten.

ABSTRACT

The anaerobic oxidation of methane with sulfate (AOM) removes more than 90% of the methane produced in marine sediments. The process is mediated by consortia of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB). Previous studies focusing on the archaeal part of ANME/SRB consortia yielded as yet only a fragmentary understanding of this process. Additionally, whereas ANME clades have been repeatedly studied with respect to phylogeny, key genes, and genomic capabilities, little is known about their sulfate-reducing partner. Thus, in order to change this situation, this thesis focused on SRB associated with AOM.

In the first part of this thesis, SRB associated with archaea from the ANME-2 clade were investigated. Sequences of bacterial 16S rRNA genes retrieved from ANME-2/SRB enrichment cultures supported a previous hypothesis that ANME-2 associated SRB belong to the SEEP-SRB1 group within the deltaproteobacterial *Desulfosarcina/Desulfococcus* (DSS) group. Using fluorescence *in situ* hybridization (FISH) and probes for newly defined SEEP-SRB1 subgroups (a-f), bacteria from the SEEP-SRB1a subgroup were identified as the dominant sulfate-reducing partners in ANME-2 consortia in samples from six different AOM sites. In contrast to their abundance as ANME-2 partners, single SEEP-SRB1a cells were very rare (<1%) in all but one of the examined samples. This suggested a highly adapted if not even obligate syntrophic lifestyle of the SEEP-SRB1a group in ANME-2 consortia. Additionally, SEEP-SRB1a was also detected as an alternative partner of archaea of the ANME-3 clade which was previously described to be predominantly associated with SRB of the *Desulfobulbus* group.

In the second part of this thesis, the diversity of SRB in AOM habitats was investigated using *aprA* and *dsrAB*, key genes of sulfate-reduction, as functional markers. AprA and DsrAB diversity in different samples from methanotrophic microbial mats from the Black Sea as well as in two enrichment cultures from sediment above gas hydrates at Hydrate Ridge was lower compared to not

enriched Hydrate Ridge sediment. Clone libraries were dominated by sequences affiliated with *Desulfobacteraceae*. Sequences within this group featured a considerable diversity. Most of the retrieved sequences affiliated with clusters that possessed no cultured representative. One AprA cluster was identified to represent SEEP-SRB1a by using a combination of FISH and fluorescence-activated cell sorting.

In the third part of this thesis, it was attempted to obtain knowledge about the genetic potential of SEEP-SRB1a. Since no pure cultures of SEEP-SRB1a existed, a metagenomic approach was used. For this, DNA from an enrichment culture dominated by ANME-2 and SEEP-SRB1a was used for constructing a large-insert fosmid library and for performing next-generation pyrosequencing. Altogether, 570 Mbp of sequence data was thus generated which could be assembled into longer contigs. In total, 9,075 contigs could be mapped onto the genome of *Desulfococcus oleovorans* Hxd3, the closest fully sequenced relative of SEEP-SRB1a, and thereby could be assigned to SEEP-SRB1a. Two contigs carrying putative SEEP-SRB1a *apr* and *dsr* genes, provided a first glimpse of the genetic potential of these bacteria.

ABBREVIATIONS

ANME	anaerobic methane-oxidizing archaea
AOM	anaerobic oxidation of methane
Apr	APS reductase
APS	adenosine-5'-phosphosulfate
BS	Black Sea
CARD-FISH	catalyzed reporter deposition fluorescence *in situ* hybridization
DNA	deoxyribonucleic acid
Dsr	dissimilatory sulfite reductase
DSS	*Desulfosarcina*/*Desulfococcus*
FACS	fluorescence-activated cell sorting
FISH	fluorescence *in situ* hybridization
HMMV	Haakon Mosby mud volcano
HR	Hydrate Ridge
kDa	kilodalton
LCM	laser-capture microdissection
MDA	multiple-displacement amplification
PCR	polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SRB	sulfate-reducing bacteria
SRP	sulfate-reducing prokaryotes
WGA	whole genome amplification

I. Introduction

I. INTRODUCTION

1. Methane in marine systems

Methane is the simplest and most reduced form of organic carbon. The methane molecule has a tetrahedral geometry, where the central carbon atom forms four equivalent C-H bonds (sp3-hybridization). Due to an unusually high C-H bond strength, a very high ionization potential, and a low stability of the methyl cation, methane is normally the least reactive of all alkanes (Crabtree, 1995). Methane forms a colorless, odorless, and non-toxic gas at room temperature and standard pressure. Methane is the most abundant hydrocarbon in the atmosphere and an important greenhouse gas. The main sources of atmospheric methane include animals (e.g. ruminants and termites), wetlands, and rice fields. Even though oceans also produce high amounts of methane, little of that methane ever reaches the atmosphere (Reeburgh, 2007). This discrepancy between methane production and emission is mainly caused by microbial methane consumption. It is estimated that more than 80% of the methane produced in the oceans is removed by anaerobic oxidation of methane with sulfate (AOM; Reeburgh, 2007).

Sources of methane in marine environments are of abiotic and biotic origin. Abiotically, methane is produced either by chemical transformation of buried organic carbon or by the interaction of CO_2 with H_2O and Fe(II) in the serpentinization process (Charlou et al., 1998). Microbially-mediated methanogenesis is the main biotic source of methane in marine environments (Reeburgh, 2007). All methanogenic microorganisms (methanogens) known so far are found in five orders within the Euryarchaeota: *Methanosarcinales*, *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, and *Methanopyrales*. Methanogens are strictly anaerobic and have an energy metabolism that is restricted to the formation of methane from CO_2 and H_2, formate, methanol, methylamines and/or acetate (Thauer et al., 2008). Methanogenesis is a process with only a low energy yield. Therefore methanogens are often outcompeted by microbes using more favorable electron acceptors such as nitrate or sulfate. Thus,

methanogenesis in marine sediments usually only occurs once other electron acceptors are depleted and only CO_2 is remaining.

2. Cold Seeps

Cold seeps are geological structures which are characterized by the expulsion of hydrocarbon-rich fluids. In contrast to hydrothermal vents, this expulsion is not associated with an appreciable temperature rise. Cold seeps are mainly formed by overpressuring of sediments due to rapid sedimentary loading or tectonic compression (Tunnicliffe et al., 2003). Since the first discovery of a cold seep in the Gulf of Mexico (Paull et al., 1984), numerous other cold seeps have been reported at a number of seafloor sites worldwide (Figure 1). Well studied seep sites include the Haakon Mosby Mud Volcano (N Atlantic), Hydrate Ridge (NE Pacific), seeps in the Gulf of Mexico, microbial reefs in the Black Sea, and mud volcanoes in the Eastern Mediterranean (Figure 1).

In the marine deserts of the deep-sea, cold seeps represent 'hotspots' of biological activity which harbor flourishing chemosynthetic communities. These communities are dominated by methane- and sulfide-oxidizing microorganisms as well as and tubeworms, mussels, and clams bearing methane- and/or sulfide-oxidizing symbionts (for a review see Levin, 2005). The community composition can vary considerably between seeps but also at small scales within a single seep, mostly depending on the ambient temperature, the advection speed of the up-transported pore fluids and their composition.

Most of the biological activity at cold seeps is fuelled directly or indirectly by upflow of methane through the sediment. At low flow rates most of the methane is consumed by AOM before it ever reaches the seabed. The so produced sulfide is transported upwards to the oxic seabed where it is consumed by mats of filamentous sulfur-oxidizing bacteria (*Beggiatoa*, *Thioploca*, *Arcobacter*, *Thiothrix*) or macrofauna bearing sulfur-oxidizing symbionts. At high flow rates, most of the methane reaches the seabed while only a fraction of it is being oxidized anaerobically. In this case, aerobic methane-oxidation can also occur,

being performed by aerobic methane-oxidizing bacteria and macrofauna with methanotrophic symbionts (for a review about cold seep ecology see Levin, 2005).

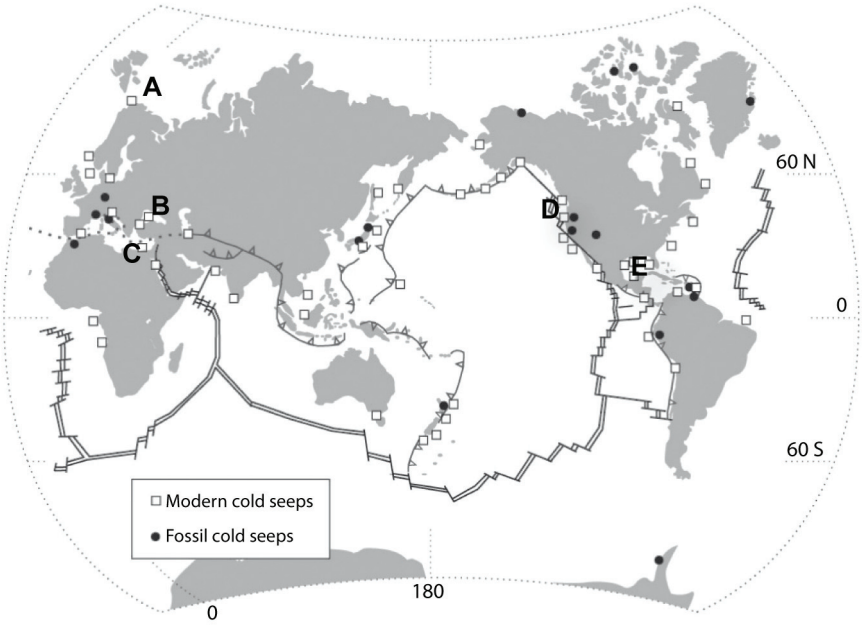

Figure 1 Distribution of modern and fossil cold seeps. Letters denote the well-studied seep sites at the Haakon Mosby Mud Volcano (A), microbial reefs in the Black Sea (B), mud volcanoes in the Eastern Mediterranean, Hydrate Ridge (D), and in the Gulf of Mexico (E). Modified from Levin, 2005.

3. Sulfate-reduction pathway

Microbially-mediated sulfate-reduction is a form of anaerobic respiration during which sulfate is reduced to sulfide in an eight electron step process:

$$SO_4^{2-} + 8\ e^- + 9\ H^+ \rightarrow HS^-\ (aq) + 4\ H_2O$$

Because all steps of the sulfate-reduction pathway are performed in the cytoplasm, sulfate ions need to be transported into the cell. This task is usually accomplished by symporters where sulfate is co-transported together with protons (mostly freshwater sulfate-reducing prokaryotes [SRP]) or sodium ions (marine SRP)

(Cypionka, 1995). The subsequent reduction of sulfate (Figure 2) involves three main steps: (1) the adenylation of sulfate to adenosine-5'-phosphosulfate (APS), (2) the reduction of APS to sulfite, and (3) the reduction of sulfite to hydrogen sulfide. Due to its low reactivity, sulfate needs to be activated prior to a subsequent reduction. This activation is performed by using ATP to adenylate sulfate to form APS. The activation is performed by the sulfate adenylyltransferase (Sat; E.C. 2.7.7.4). The two electron reduction of APS to form sulfite in the next step, is catalyzed by the dissimilatory APS reductase (Apr; E.C. 1.8.99.2). The Apr enzyme is a heterodimer which consists of an α-subunit (AprA; ≈75 kDa) which harbors the catalytic site and a β-subunit (AprB; ≈20 kDa) (Fritz et al., 2000; Fritz et al., 2002). The subunits of the Apr enzyme are encoded by the *aprBA* gene cluster. The final reduction of sulfite to sulfide is catalyzed by the dissimilatory sulfite reductase (Dsr; E.C. 1.8.99.1). Whether the reduction of sulfite occurs is a six electron transfer step or three two electron transfer steps is currently still a matter of debate. Based on studies on *Desulfovibrio*, the Dsr enzyme has a $(\alpha\beta)_2$ structure with the α- and β-subunit having approximate molecular masses of 50 and 45 kDa, respectively (Karkhoff-Schweizer et al., 1995; Steuber et al., 1995). The subunits of the Dsr enzyme are encoded by the *dsrAB* gene cluster.

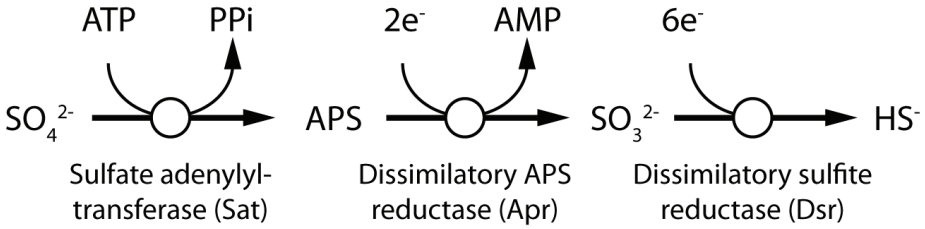

Figure 2 Schematic overview of the dissimilatory sulfate-reduction pathway. Shown are the main steps of the sulfate-reduction pathway and the catalyzing enzymes. PPi, pyrophosphate; AMP, adenosine monophosphate; APS, adenosine-5'-phosphosulfate; ATP, adenosine triphosphate

4. Sulfate-reducing prokaryotes

It is estimated that more than 50% of the carbon mineralization in marine sediments can be attributed to sulfate-reduction (Jørgensen, 1982; Canfield, 1989). Sulfate-reduction is performed by a phylogenetically very diverse guild, the sulfate-reducing prokaryotes (SRP), which comprises members from five bacterial and two archaeal lineages (Muyzer and Stams, 2008; Figure 3).

Within the *Bacteria*, most of the described SRP belong to the *Deltaproteobacteria* and the Firmicutes (*Desulfotomaculum*, *Desulfosporosinus*, *Desulfosporomusa,* and *Thermodesulfobium*) (Rabus et al., 2006). In addition, bacterial SRP are also found within the Nitrospirae (*Thermodesulfovibrio*) and the Thermodesulfobacteria (*Thermodesulfobacterium*) (Mori et al., 2003; Rabus et al., 2006). Within the *Archaea*, SRP belong to the genus *Archaeoglobus* in the Euryarchaeota, and to the genera *Thermocladium* (Itoh et al., 1998) and *Caldirvirga* (Itoh et al., 1999) in the Crenarchaeota.

Due to the phylogenetic diversity within the guild, different primer and probe sets must be used for characterizing sulfate-reducing communities when using 16S and 23S rRNA genes as genetic markers. In spite of the phylogenetic diversity of SRP,

only one dissimilatory sulfate-reduction pathway is known so far. Thus, a more targeted approach for detecting SRP is the use of sulfate-reduction key genes as functional markers. Genes often used for that purpose are genes coding for the dissimilatory sulfite reductase (Dsr) and genes coding for the dissimilatory adenosine-5'-phosphosulfate reductase (Apr). Those genes are highly conserved within the SRP and are therefore ideal candidates for phylogenetic analyses.

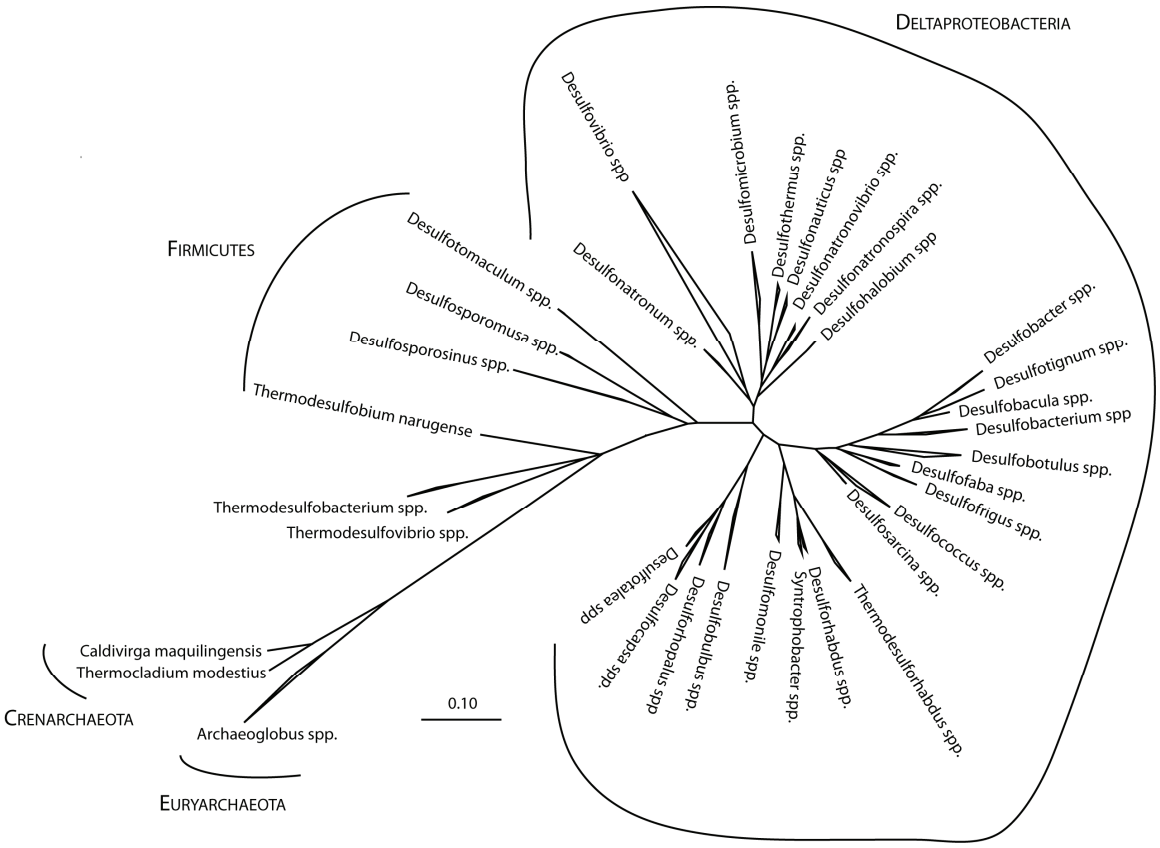

Figure 3 Overview of the phylogeny of SRP. Tree shows the phylogeny of selected SRP as inferred by neighbor joining analysis of 16S rRNA genes. Bar, 10% estimated sequence divergence.

Phylogenies inferred from Apr and Dsr genes are in general agreement with phylogeny inferred from the 16S rRNA gene; however, several cases of putative horizontal gene transfer (HGT) have also been reported (Klein et al., 2001;

Friedrich, 2002; Meyer and Kuever, 2007). Most notably members of the *Desulfotomaculum* and the *Archaeoglobus* genera seem to have been involved in events of HGT. In AprA and DsrAB-based phylogenies, *Desulfotomaculum* is not monophyletic. Instead, it splits into one "authentic" clade which is postulated not to have been affected by HGT, and one clade which due to its positioning most likely obtained sulfate-reduction genes from a donor lineage within the *Deltaproteobacteria* (Klein et al., 2001; Meyer and Kuever, 2007). Additionally, for the *Archaeoglobus* genus the deduced evolutionary distance between *Archaeoglobus* species and bacterial sulfate-reducers was reported to be much shorter in Dsr trees than compared to 16S rRNA trees (Klein et al., 2001). It is therefore speculated that sulfate-reduction genes of *Archaeoglobus* originated from a bacterial donor lineage (Klein et al., 2001). Interestingly, Apr and Dsr phylogenies are not congruent, which suggests independent acquisition of the corresponding genes by non-parallel HGT events (Meyer and Kuever, 2007). In contrast, the occurrence of HGT of the whole sulfate-reduction pathway seems also possible, because genomic islands of genes involved in sulfate-reduction have been reported (Mußmann et al., 2005).

Genes homologous to *apr* and *dsr* of SRP have also been detected in sulfur-oxidizing prokaryotes (SOP)(Schedel et al., 1979; Beller et al., 2006; Mußmann et al., 2007). In fact, it is even speculated that SRP obtained their sulfate-reduction genes from an ancestral sulfur-oxidizing bacterium (Meyer and Kuever, 2007). In SOP, the Apr enzyme is postulated to catalyze the reverse of the reaction it catalyzes in SRP and thus adds sulfite to adenosine monophosphate (AMP; see also Figure 2). The fact that *apr*-specific primer sets were shown to amplify both SRP and SOP-derived *apr* genes, indicates a surprising degree of conservation of the *apr* gene in both guilds. In contrast to the sulfate-reduction pathway where sulfite is postulated to be directly reduced to sulfide, the reverse of that pathway in SOP proceeds via the intermediate formation of sulfur. While the Dsr enzyme in SRP catalyzes the whole reduction of sulfite to sulfide, the reverse Dsr (rDsr) enzyme in SOP only catalyzes the oxidation of sulfur to sulfite. In addition, even

though homologous, both rDsr and Dsr are not highly conserved between SRP and SOP, and are phylogenetically clearly distinguishable (Molitor et al., 1998; Loy et al., 2009).

5. Ecology and physiology of sulfate-reducing prokaryotes

Due to the rapid depletion of other electron acceptors and high sulfate concentrations in seawater (up to 28 mM), SRP play an important role as terminal oxidizers of organic matter in marine systems. Here, they oxidize the low-molecular mass products from the primary fermentative breakdown of polysaccharides, proteins, lipids, and other substances of dead biomass to CO_2 (Widdel et al., 2007).

SRP utilize the oxidation of these low-molecular mass compounds as a source of energy and carbon. Common substrates of SRP include short-chain fatty acids, alcohols, alkanes (Rueter et al., 1994; So and Young, 1999), and aromatic compounds (Galushko et al., 1999). Several SRP are also capable of using H_2 as an energy source. Based on their ability to oxidize organic substrates to CO_2, complete (CO_2 as end-product) and incomplete (acetate as end-product) oxidizing SRP can be distinguished.

Even though SRP were named after their ability to use sulfate as a terminal electron acceptor, many can also use alternative electron acceptors, such as sulfur or nitrate, and can even perform fermentation (for an overview see Rabus et al., 2006). In fact, in freshwater (low-sulfate) environments, SRP also play an important role in degrading organic matter and may grow exclusively by fermentation (Muyzer and Stams, 2008). The presence of SRP is therefore no clear indication for sulfate-reduction.

6. Sulfate-reducing prokaryotes and the anaerobic oxidation of methane

The anaerobic oxidation of methane with sulfate (AOM) is the major sink of methane in marine sediments (Reeburgh, 2007). Studies suggest that the AOM process is present almost everywhere where sulfate meets methane in micromolar to millimolar concentrations (sulfate-methane transition zone; for an overview see Reeburgh, 2007). In addition to that, cold seeps, where high methane concentrations lead to increased AOM rates, are known to be AOM "hotspots". Well-studied seep sites include sediments above methane hydrates at Hydrate Ridge (NE Pacific), sediments in the Gulf of Mexico, and microbial reefs in the north-west of the Black Sea (see also section *Cold seeps* of this Introduction).

AOM is mediated by consortia of anaerobic methane-oxidizing archaea (ANME) and sulfate-reducing bacteria (SRB) (Boetius et al., 2000) which form cell-aggregates held together by a so far poorly characterized matrix. Currently three ANME clades have been identified (ANME-1 to ANME-3; Figure 4). Based on the 16S rRNA phylogeny, the three groups are not monophyletic and certainly represent different families or orders (Knittel and Boetius, 2009; Figure 4). The ANME-1 clade is distantly related to the orders *Methanosarcinales* and *Methanomicrobiales* (Hinrichs et al., 1999), while clades ANME-2 (Orphan et al., 2001) and ANME-3 (Niemann et al., 2006) belong to the *Methanosarcinales.* Within the ANME-2 clade, two main subgroups, ANME-2a/b and ANME-2c, can be distinguished (Orphan et al., 2001; Knittel et al., 2005).

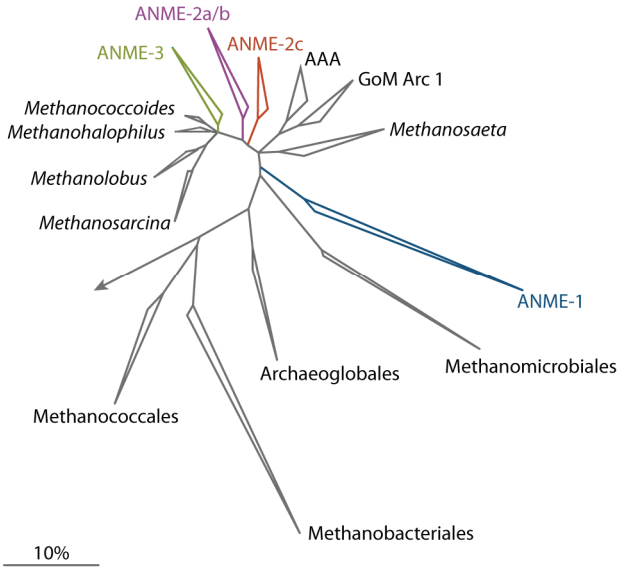

Figure 4 Phylogenetic tree showing the currently known ANME-clades. Tree is based on 16S rRNA sequences from ANME and selected reference sequences from the domain Archaea. Bar, 10% estimated sequence divergence. Modified from Knittel and Boetius, 2009.

Anaerobic methanotrophs of the ANME-3 clade were shown to be mainly associated with sulfate-reducing bacteria (SRB) closely related to the *Desulfobulbus*-genus (Niemann et al., 2006; Lösekann et al., 2007). However, a number of ANME-3 aggregates with an unknown bacterial partner have also been reported. The main partner of the ANME-1 and ANME-2 clades has been repeatedly shown to belong to the *Desulfosarcina/Desulfococcus* (DSS) branch of the *Deltaproteobacteria* (Boetius et al., 2000; Orphan et al., 2002; Knittel et al., 2005; Reitner et al., 2005; Knittel and Boetius, 2009). The DSS group comprises numerous phylogenetically and metabolically diverse genera of SRB including the well described *Desulfosarcina* spp. (Widdel and Hansen, 1992). The phylogenetic position of ANME-1 and ANME-2 associated SRB within the DSS-group is unfortunately still unclear, because these SRB have been identified by fluorescence *in situ* hybridization (FISH) using oligonucleotide probes targeting the whole DSS group (Knittel and Boetius, 2009).

Based on comparative 16S rRNA gene analysis, Knittel et al. (2003) defined altogether four groups of sulfate-reducing bacteria which were frequently detected in methane seep sediments, SEEP-SRB1 to SEEP-SRB4. It was hypothesized that SEEP-SRB1 (Figure 5) includes the ANME-2 partner, because this group is a subgroup of the DSS clade and SEEP-SRB1 sequences were always retrieved when ANME-2 aggregates were present (Knittel et al., 2003). An experimental proof of the association between ANME-2 and SEEP-SRB1, however, was still lacking. Additionally, a recent study reported ANME-2 associated with *Desulfobulbus*-related SRB and with partners from the *Alphaproteobacteria* and *Betaproteobacteria* (Pernthaler et al., 2008).

Consortia of ANME and SRB were reported with different morphologies (for a review see Knittel and Boetius, 2010). In the Black Sea, ANME-1 and its associated SRB were reported to possess a “mat-type” morphology (Figure 6a). The two main ANME-2 subgroups (ANME-2a and ANME-2c) principally feature different morphologies. ANME-2a/DSS consortia mainly feature the mixed-type form (Figure 6b). In contrast, ANME-2c consortia mainly feature the shell-type form where an inner core of ANME-2 is partially or fully surrounded by an outer shell of SRB (Figure 6c). Consortia of ANME-3 and its associated *Desulfobulbus*-related SRB are most often observed with a shell-type morphology (Figure 6d).

In addition to different aggregate morphologies, ANME-asscociated SRB cells were reported to have different morphologies. SRB associated with ANME-2 were reported to possess cocci-type (mostly associated with ANME-2c) and rod/vibrio-type (mostly associated with ANME-2a) morphologies (Knittel and Boetius, 2009). Due to the above mentioned identification with broad DSS-probes, it is currently not known if the different cell morphologies reflect phylogenetically different DSS-subgroups or are caused by environmental factors.

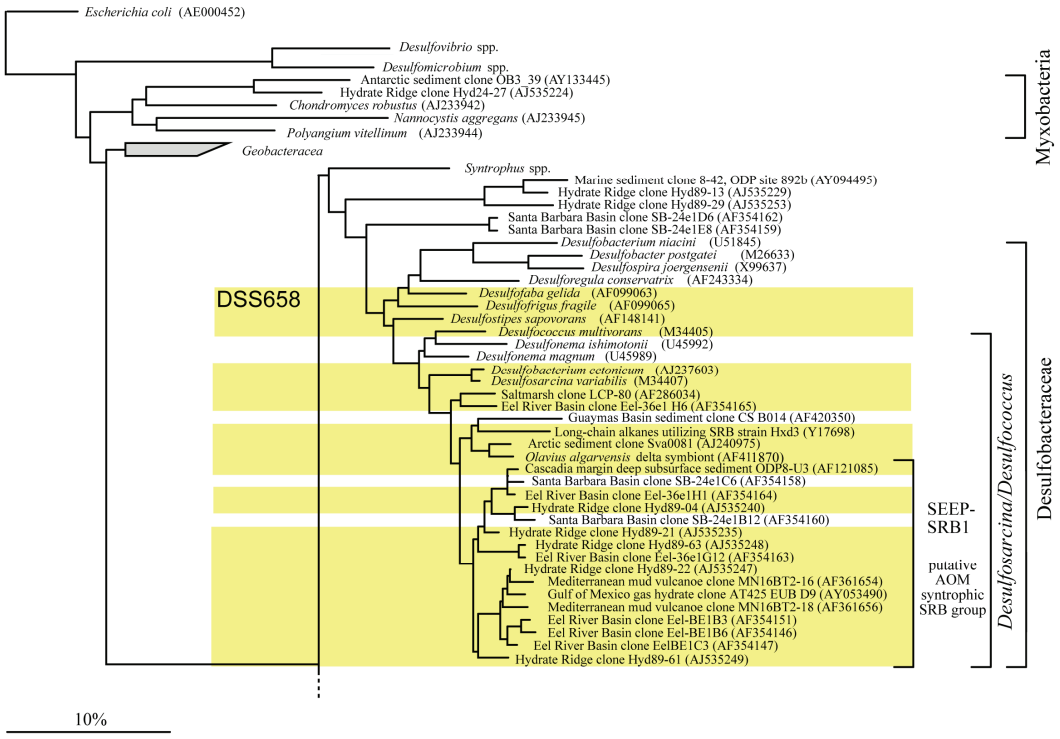

Figure 5 Phylogenetic tree showing the putative AOM syntrophic SRB group SEEP-SRB1. Shown are the SEEP-SRB1 group and selected reference sequences. The yellow colored box shows the probe specificity of the *Desulfosarcina*/*Desulfococcus*-specific probe DSS658 commonly used for identifying ANME-associated SRP. The bar represents 10% estimated phylogenetic divergence. Figure modified from Knittel et al., 2003.

Currently, the nature of the interaction between ANME and SRB, and the underlying biochemistry of AOM is still a matter of debate. During AOM, methane-oxidation to CO_2 is coupled to sulfate-reduction to sulfide:

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$$

$$\Delta G^{\circ\prime} = -16.6 \text{ kJ/mol}$$

In a well supported hypothesis ANME oxidize methane by a reversal of the methanogenesis pathway (Krüger et al., 2003; Hallam et al., 2004). However, the fate of reducing equivalents gained by this reaction and the coupling to the

reduction of sulfate is still unclear. In this respect two main scenarios can be distinguished (Widdel et al., 2007).

First, a syntrophic interaction may occur where reducing equivalents are transferred from ANME to SRB (Hoehler et al., 1994). Candidates which are common substrates of the complete-oxidizing DSS group as well as possible end-products of reverse methanogenesis are e.g. actetate, formate, methanol, or hydrogen. Previous studies, however, excluded these compounds as potential shuttles (Nauhaus et al., 2002; Nauhaus et al., 2005; Moran et al., 2008; Wegener et al., 2008). An alternative to an electron transfer via mobile intermediates would be a direct electron transfer with fixed structures such as the recently described nanowires (Reguera et al., 2005). The analysis of the draft genome of ANME-1 suggested such a direct electron transfer via *c*-type cytochromes (Meyerdierks et al., 2010).

The second possible scenario would be that methane oxidation as well as sulfate-reduction take place solely in the ANME-cells. In this case, the associated SRB would grow on scavenged metabolites from the ANME in a form of metabolic parasitism or commensalism (Widdel et al., 2007). This scenario is favored by the finding of monospecific ANME aggregates, i.e. aggregates solely consisting of ANME-cells (Orphan et al., 2001; Orphan et al., 2002; Knittel et al., 2005), and of microbial mats in the Black Sea almost exclusively consisting of ANME-1 (Arnds, 2009).

The quest to elucidate the interaction between ANME and SRB, and thereby the biochemistry of AOM, is hampered by the fact that currently neither ANME nor their sulfate-reducing partners have been obtained in pure culture. In fact both issues are closely connected: without pure cultures it is difficult to study the biochemistry of AOM, on the other hand it is difficult to obtain pure cultures without knowledge about the biochemistry.

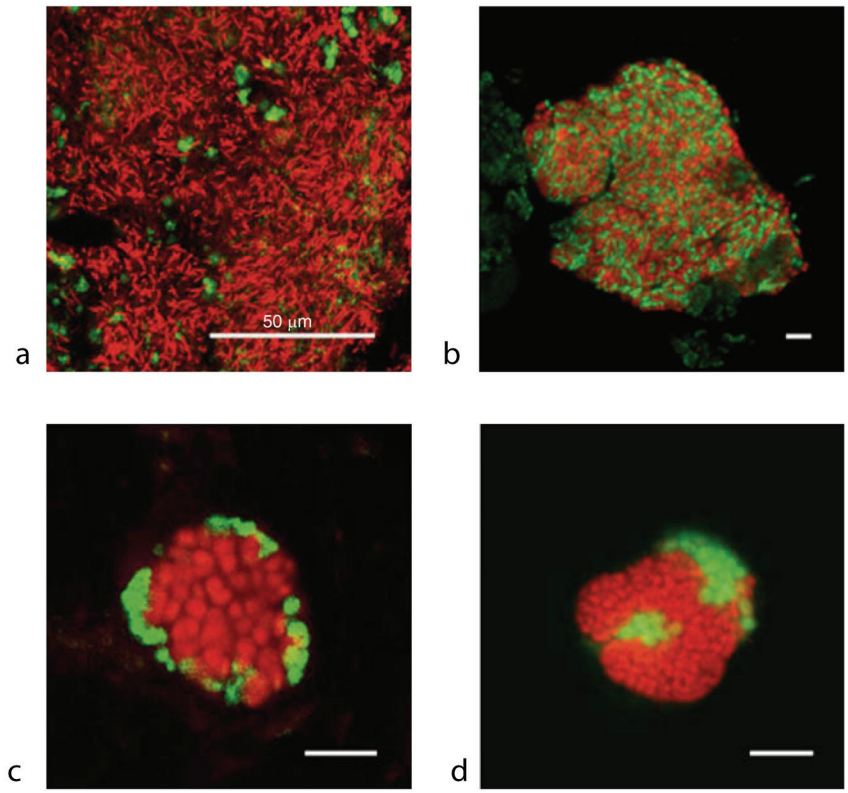

Figure 6 Different morphologies of ANME/SRB consortia. Shown are epifluorescence micrographs of different ANME consortia visualized by FISH or CARD-FISH. (a) Consortium of ANME-1 (red) and DSS cells (green) with "mat-type" morphology; (b) Consortium of ANME-2a (red) and DSS (green) with "mixed-type" morphology; (c) Consortium of ANME-2c (red) and DSS (green) cells with "shell-type" morphology; (d) ANME-3/Desulfobulbus consortium (archaea in red, bacteria in green). Unless indicated otherwise, scale bar 5 µm. Figure from Knittel and Boetius, 2010.

7. Metagenomics and single-cell techniques

Studies estimate that currently only about 1% of the biosphere's microbial diversity can be assessed by standard cultivation (Amann et al., 1995; Curtis et al., 2002). Thus, in order to assess the genetic potential of the remaining 99%, culture-independent approaches, such as metagenomics, must be employed. The term "metagenomics" was first introduced by Handelsman et al. (1998) and can now be defined as the "functional and sequenced-based analysis of the collective

microbial genomes contained in an environmental sample" (Riesenfeld et al., 2004). ‘Traditional’ metagenomics is based on the construction of small and large insert clone libraries from environmental DNA. The most impressive example for a study using small insert libraries is probably the global ccean sampling expedition by Rusch et al. (2007) that generated 6.3 billion base pairs of nonredundant sequence data. As an alternative, some studies cloned environmental DNA into large-insert vectors such as fosmids or BACs which can hold inserts with a size of *c*. 40 and 300 kbp, respectively (e.g Béjà et al., 2000; Hallam et al., 2003; Meyerdierks et al., 2005).

The large-insert approach often achieves a lower coverage of the community genome compared to small-insert libraries. However, the larger insert size allows a more reliable assignment of the cloned fragments to specific taxa via the presence of marker genes or more reliable inference of intrinsic sequence patterns, such as GC content (Hallam et al., 2004; Teeling et al., 2004; Meyerdierks et al., 2005). Large inserts can furthermore, also provide information about natural gene clusters, such as bacterial operons; something that can only be achieved with small insert libraries for habitats with low diversity.

With the advent of next-generation sequencing techniques, the field of metagenomics was revolutionized as it made metagenomics affordable even for small labs. Techniques such as 454 pyrosequencing circumvent the cloning step and generate millions of basepairs of sequence data per run (Margulies et al., 2005).

Metagenomics has proven to be a powerful tool for studying the microbial diversity of environmental samples (Venter et al., 2004; Martin-Cuadrado et al., 2007; Rusch et al., 2007). An alternative application of metagenomics is its use for reconstructing genomes of uncultured microbes. Such a task usually involves bioinformatic sequence binning based on intrinsic sequence patterns (Teeling et al., 2004; Tyson et al., 2004; Woyke et al., 2006) and strongly relies on the nature of the environmental sample. If the intraspecies diversity of the target is too high

or if the sample contains many species closely related to the target, such a reconstruction might become impossible. Thus, most studies succeeding in the genome reconstruction from a metagenome are based on sample material showing only a very low microbial diversity (Tyson et al., 2004; Erkel et al., 2006; Strous et al., 2006; Meyerdierks et al., 2010)

Single-cell techniques circumvent many of the challenges of metagenomics and provide an alternative approach to study the genomic potential of uncultured microorganisms. By studying genomes of single cells, the problem of microdiversity is not of importance and sequence binning becomes obsolete. The ability to do single-cell genomics has been made possible by the development of a protocol for whole genome amplification (WGA) by multiple displacement amplification (MDA) (Dean et al., 2001; Lasken and Egholm, 2003). Using MDA micrograms of amplified DNA can be obtained even from single bacterial cells (Raghunathan et al., 2005). This extreme sensitivity, however, also makes the MDA protocol very prone for even small amounts of contaminating DNA (Marcy et al., 2007). Two other problems of current MDA protocols are high amounts of randomly synthesized DNA (background amplification) at low template amounts and the formation of chimeric sequences (Raghunathan et al., 2005; Zhang et al., 2006). Despite the limitations of MDA however, several studies successfully amplified and sequenced DNA from single bacterial cells (Raghunathan et al., 2005; Marcy et al., 2007; Rodrigue et al., 2009; Woyke et al., 2009; Woyke et al., 2010). For isolation of single cells, a whole spectrum of different methods, including fluorescence-activated cell sorting (Raghunathan et al., 2005; Rodrigue et al., 2009; Woyke et al., 2009), micromanipulation (Woyke et al., 2010), and microfluidic devices (Marcy et al., 2007) have been reported.

With the introduction of next-generation sequencing platforms and single-cell techniques, the focus of the culture-independent genomics started to shift from methodological aspects to data analysis. One important aspect in that respect is the annotation of genomes from microorganisms that possess no cultured representatives. The usual annotation process involves predicting the function of a

gene based on similarities with genes whose functions were determined experimentally by using pure cultures. Therefore it is difficult to predict novel biochemical pathways or phenotype features for uncultured microorganisms. Instead, the corresponding genes can often only be labeled as "hypothetical" proteins (Roberts, 2004).

8. Aims of this work

This work focused on sulfate-reducing bacteria associated with AOM. AOM is the main methane-consuming process in marine environments and its H_2S end-product fuels whole communities of sulfur-oxidizing bacteria and symbiont-bearing macrofauna. The process was shown to be catalyzed by consortia of anaerobic methanotrophic archaea (ANME) and physically-associated sulfate-reducing *Deltaproteobcateria*. In spite of concentrated efforts for at least 10 years the underlying biochemistry of AOM is not well understood. Studies suggested that methane is oxidized by ANME by a reversal of the methanogenesis pathway (Hallam et al., 2004). However, how methane-oxidation is coupled to sulfate-reduction and the function of the associated sulfate-reducers are still unclear. This work focused on exploring SRP in AOM habitats to better understand their role in the AOM process and their ecology. More precisely this thesis focused on:

- ***Identification of ANME-2 associated SRP (Chapter II).*** The first part of this thesis focused on the identification of SRP associated with archaea of the ANME-2 clade. ANME-2 archaea were shown to be mainly associated with SRP from the phylogenetically and metabolically diverse *Desulfosarcina*/*Desulfococcus* (DSS) group (Boetius et al., 2000). However, the exact phylogenetic position of the ANME-2 associated SRB within the DSS group was unclear. A previous study suggested that ANME-2 associated SRP are positioned in the SEEP-SRB1 group, a group of DSS sequences exclusively retrieved from cold seep habitats (Knittel et al., 2003). The first aim of this study was to test this hypothesis by using

the full-cycle rRNA approach on two AOM enrichment cultures. In the next step, probes designed for the ANME-2 partner were then to be applied on environmental samples, to investigate the ecology of these SRP.

- ***Diversity of SRP at AOM site (Chapter III).*** Sulfate-reducing prokaryotes are an integral part of microbial communities in AOM habitats. Even though AOM habitats are often dominated by SRP directly involved in AOM, i.e. associated with ANME, previous studies have shown that besides that there is a high diversity of SRP in these habitats. The goal of this study was to study the diversity of SRP in different mat sections from the Black Sea and enrichment cultures from Hydrate Ridge sediment. This was to be achieved by using *aprA* and *dsrAB* as functional markers for SRP.

- ***Genomic potential of ANME-2 partner (Chapter IV).*** The third part of this thesis focused on the genomic potential of ANME-2 associated SEEP-SRB1a bacteria. Since currently no pure culture of these SRP exists, this goal was to be reached by using an enrichment culture dominated by ANME-2/SEEP-SRB1 consortia as sample material. DNA obtained from this enrichment was to be used for a metagenomic approach combining next-generation sequencing with insert end-sequencing of a fosmid-library. Based on the generated sequence data, it was aimed at reconstructing as much as possible of the genome of the ANME-2 associated SRP and thereby to gain knowledge about the genomic potential of these SRP.

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II. Identification of the dominant sulfate-reducing bacterial partner of anaerobic methanotrophs of the ANME-2 clade

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Identification of the dominant sulfate-reducing bacterial partner of anaerobic methanotrophs of the ANME-2 clade

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Summary

The anaerobic oxidation of methane (AOM) with sulfate as terminal electron acceptor is mediated by consortia of methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB). Whereas three clades of ANME have been repeatedly studied with respect to phylogeny, key genes and genomic capabilities, little is known about their sulfate-reducing partner. In order to identify the partner of anaerobic methanotrophs of the ANME-2 clade, bacterial 16S rRNA gene libraries were constructed from cultures highly enriched for ANME-2a and ANME-2c in consortia with *Deltaproteobacteria* of the *Desulfosarcina/Desulfococcus* group (DSS). Phylogenetic analysis of those and publicly available sequences from AOM sites supported the hypothesis by Knittel and colleagues that the DSS partner belongs to the diverse SEEP-SRB1 cluster. Six subclusters of SEEP-SRB1, SEEP-SRB1a to SEEP-SRB1f, were proposed and specific oligonucleotide probes were designed. Using fluorescence *in situ* hybridization on samples from six different AOM sites, SEEP-SRB1a was identified as sulfate-reducing partner in up to 95% of total ANME-2 consortia. SEEP-SRB1a cells exhibited a rod-shaped, vibrioid, or coccoid morphology and were found to be associated with subgroups ANME-2a and ANME-2c. Moreover, SEEP-SRB1a was also detected in 8% to 23% of ANME-3 consortia in Haakon Mosby Mud Volcano sediments, previously described to be predominantly associated with SRB of the *Desulfobulbus* group. SEEP-SRB1a contributed to only 0.3% to 0.7% of all single cells in almost all samples indicating that these bacteria are highly adapted to a symbiotic relationship with ANME-2.

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Introduction

The anaerobic oxidation of methane (AOM) coupled to sulfate-reduction is the major sink for methane escaping from marine sediments (for review: Hinrichs and Boetius, 2002; Reeburgh, 2007). AOM is mediated by consortia of anaerobic methane-oxidizing archaea (ANME) and sulfate-reducing bacteria (SRB). So far, neither the archaeal nor the bacterial members of AOM consortia have been obtained in pure culture. It is hypothesized that both operate in a syntrophic interaction (Hoehler *et al*., 1994).

Currently three ANME clades have been identified. These are either distantly related to the orders *Methanosarcinales* and *Methanomicrobiales* (ANME-1; Hinrichs *et al*., 1999) or belong to the *Methanosarcinales* (ANME-2 and ANME-3; Orphan *et al*., 2001; Niemann *et al*., 2006). In a well supported hypothesis ANME oxidize methane by a reversal of the methanogenesis pathway (Krüger *et al*., 2003; Hallam *et al*., 2004). However, the fate of reducing equivalents gained by this reaction is as yet unclear. It is assumed that these are shuttled to associated SRB and used for sulfate reduction (Hoehler *et al*., 1994). Shuttles from ANME to their sulfate-reducing partners are still unknown, even though a variety of candidates (e.g. acetate, hydrogen, formate, methylthiol) has been tested (Nauhaus *et al*., 2002; 2005; Moran *et al*., 2008; Wegener *et al*., 2008a). The analysis of the draft genome of ANME-1 suggested a direct electron transfer via *c*-type cytochromes (Meyerdierks *et al*., 2010).

Knowledge about the sulfate-reducing partners of ANME is even more limited. In general, anaerobic methanotrophs of the ANME-1 and ANME-2 clade have been repeatedly shown to be associated with SRB of the *Desulfosarcina/Desulfococcus* (DSS) branch of the *Deltaprotobacteria* (Boetius *et al*., 2000; Orphan *et al*., 2002; Knittel *et al*., 2005; Reitner *et al*., 2005; Knittel and Boetius, 2009). Additionally, a small fraction of ANME-2 consortia was found to be associated with *Desulfobulbus* (DBB)-related SRB but also with non-SRB partners such as *Alphaproteobacteria* and *Betaproteobacteria* (Pernthaler *et al*., 2008). ANME-3 was found to be predominantly associated with a small group of highly similar *Desulfobulbus*-related SRB (Niemann *et al*., 2006;

Lösekann *et al*., 2007). In contrast, DSS comprises numerous phylogenetically and metabolically diverse genera of SRB including the well-described *Desulfosarcina* spp. (Widdel and Hansen, 1992). The sulfate-reducing partners of ANME-2 have been assigned to this clade by fluorescence *in situ* hybridization (FISH) using phylogenetically broad oligonucleotide probes targeting the whole DSS group (Knittel and Boetius, 2009; Fig. 1). Based on comparative 16S rRNA gene analysis, Knittel and colleagues (2003) defined altogether four groups of SRB (SEEP-SRB1 to SEEP-SRB4) which were commonly detected in methane seep sediments. The SEEP-SRB1 group is a subgroup of the broad DSS clade. Sequences belonging to the SEEP-SRB1 cluster were always retrieved when ANME-2 aggregates were present, while other DSS sequences were rare. This led to the hypothesis that the SEEP-SRB1 group most likely includes the ANME-2 partner (Knittel *et al*., 2003). However, an experimental proof is as yet lacking.

In this study, we focused on the *in situ* identification and quantification of the dominant partner of ANME-2. Since the diversity of DSS-partners of ANME-2 is not well characterized, we tested the hypothesis whether these bacteria belong to the SEEP-SRB1 group, and whether it is possible to further narrow the affiliation down to specific SEEP-SRB1 subgroups. Second, we investigated whether ANME-2a and ANME-2c share partners of the same DSS subgroup. This was done because different morphologies for ANME-2a/DSS and ANME-2c/DSS consortia (mixed-type or shell-type consortia; Knittel *et al*., 2005) as well as different morphologies of ANME-2 associated DSS cells (rod-shaped or coccoid; Knittel and Boetius, 2009) were previously reported. Finally, the presence and abundance of single cells of SEEP-SRB1 were investigated, as it is still unclear whether or not the association between ANME-2 and their partners is obligate. This is of interest as all ANME types have already been observed as single cells or monospecific aggregates in environmental samples, challenging the hypothesis of an obligate syntrophy (Orphan *et al*., 2001; 2002; Knittel *et al*., 2005; Schubert *et al*., 2006; Lösekann *et al*., 2007; Treude *et al*., 2007).

Results and discussion

SEEP-SRB1: phylogeny, subclusters and probe design

In a first step to test the proposed association between SEEP-SRB1 bacteria and ANME-2 archaea, the diversity of bacterial 16S rRNA genes in two ANME-2 dominated AOM enrichment cultures was examined. One enrichment originated from the sediment above gas hydrates at Hydrate Ridge (named HR enrichment) and was previously described (Nauhaus *et al*., 2007; Holler *et al*., 2009). The other one was prepared from sediments of the Mediterranean Isis Mud Volcano (named Isis enrichment). Both enrichments were grown over years in the lab and showed similar microbial compositions based on FISH. They were dominated by ANME-2c cells, but also contained a significant population of ANME-2a. The HR enrichment contained 17% ANME-2a and 64% ANME-2c cells, while the Isis enrichment contained 20% ANME-2a and 49% ANME-2c cells. DSS associated with the two ANME-2 subgroups accounted for 18% of the cell population in the HR enrichment and for 26% in the Isis enrichment.

Bacterial 16S rRNA gene clone libraries were constructed from the AOM enrichments. Both clone libraries contained mostly 16S rRNA genes affiliating with *Deltaproteobacteria* (HR: 71%; Isis: 74%). In addition, the libraries contained sequences related to *Firmicutes* (HR: 11%; Isis: 2%), *Thermomicrobia* (HR: 5%; Isis: 6%), *Bacteroidetes/Chlorobi* (HR: 6%; Isis: 7%) and to a few other groups (for details see Supporting Information, Table S1). Within the deltaproteobacterial sequences, most sequences affiliated with the SEEP-SRB1 group (Knittel *et al*., 2003). They represented 56% (HR) and 65% (Isis) of the phylotypes in the two libraries, and were all closely related forming a single operational taxonomic unit at a 97% sequence-similarity cut-off.

In 2003, when Knittel and colleagues defined group SEEP-SRB1, the group comprised only 16 sequences (Knittel *et al*., 2003). In this study, a detailed phylogenetic analysis of currently available SEEP-SRB1 and related deltaproteobacterial sequences was performed. Altogether, more than 150 sequences could be assigned to SEEP-SRB1 (for a selection see Fig. 1). The SEEP-SRB1 group included sequences from well-investigated AOM habitats such as methane seeps and sulfate-methane transition zones, but also from, e.g. mangrove soils or hypersaline mats (Table S2). Cultured representatives did not affiliate with SEEP-SRB1. Based on sequences longer than 1200 bp, the sequence divergence within the SEEP-SRB1 group is currently up to 14%. Phylogenetic analysis with all of the used algorithms consistently yielded six well-supported subgroups within SEEP-SRB1. These subgroups are from hereon referred to as SEEP-SRB1a to SEEP-SRB1f (Fig. 1). The sequence similarities within subgroups SEEP-SRB1a, 1b, 1c, 1d and 1f ranged from $\geq$ 86% to $\geq$ 92%. Sequence similarities within SEEP-SRB1e were with $\geq$ 97% higher, suggesting a more coherent group at the level of a genus. The phylogenetic position of SEEP-SRB1c is still unresolved. The cluster branched only in some calculations together with the other SEEP-SRB1 groups, in other calculations SEEP-SRB1c showed a closer relationship to cultivated DSS microorganisms. The phylogenetic position is therefore shown as a multifurcation. The fact that SEEP-SRB1c sequences are not targeted by the general DSS probe

Fig. 1. Tree showing the phylogenetic positions of six SEEP-SRB1 subgroups compared with related reference sequences of the *Deltaproteobacteria*. Sequences within SEEP-SRB1 were selected in order to represent major habitats of the SEEP-SRB1 subgroups. Selected 16S rRNA sequences obtained from Hydrate Ridge and Isis enrichment cultures are shown in boldface type. Sequences of the SEEP-SRB1 group as described by Knittel and colleagues (2003) are marked with an asterisk. Probe coverage is indicated by coloured boxes: DSS-658, orange; SEEP1a-473, light green; SEEP1a-1441, dark green; SEEP1c-1309, blue; SEEP1f-152, yellow. Probe coverage was determined conservatively, i.e. sequences without information at the probe target site were considered as not targeted. The bar represents 10% estimated sequence changes.

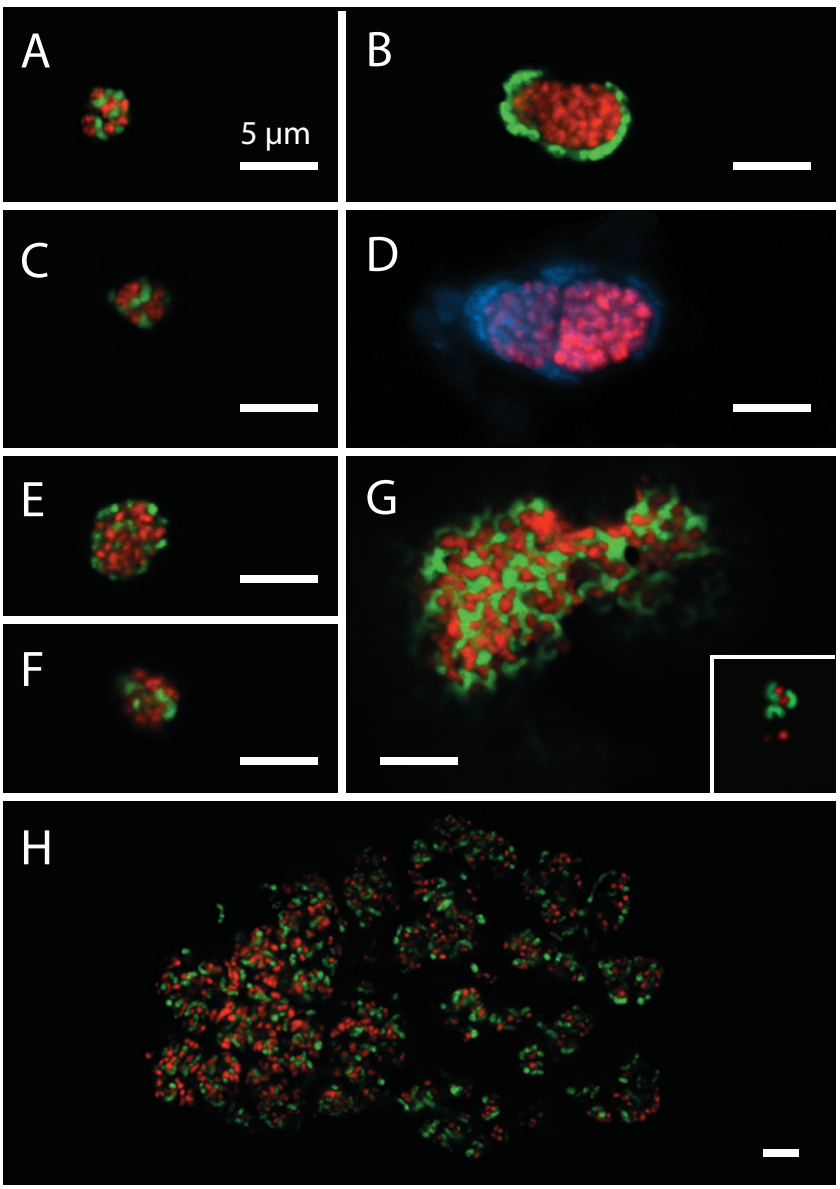

Fig. 2. Cell aggregates of ANME-2 and ANME-3 in AOM enrichments, a Black Sea mat, and sediments from Hydrate Ridge, the Gulf of Mexico, the Gullfaks oil field and the Haakon Mosby Mud Volcano, visualized by CARD-FISH.
A. ANME-2/SEEP-SRB1a aggregate as detected by using probe ANME2-538 (red) and probe SEEP1a-1441 (green) in the Isis enrichment.
B. ANME-2-aggregate (ANME2-538, red) surrounded by SEEP-SRB1a bacteria (SEEP1a-1441, green) as detected in Hydrate Ridge sediment (station 19-2).
C. ANME-2/SEEP-SRB1a-aggregate (ANME2-538, red; SEEP1a-473, green) detected in Gullfaks oil field sediment.
D. Association of ANME-2 with a bacterial partner not belonging to the SEEP-SRB1a group. The aggregate was observed in a Gulf of Mexico sediment sample after hybridization with probes ANME2-538 (red), SEEP1a-1441 (green, not present in micrograph) and a simultaneous DAPI-staining (blue).
E. ANME-2a-aggregate (ANME2a-647, red) with associated SEEP-SRB1a bacteria (SEEP1a-473, green) as detected in Hydrate Ridge sediment (station 19-2).
F. ANME-2c-aggregate (ANME2c-760, red) associated with SEEP-SRB1a bacteria (SEEP1a-473, green) as detected in Hydrate Ridge sediment (station 19-2).
G. ANME-3/SEEP-SRB1a aggregates, labelled with probe SEEP1a-1441 (green) and probe ANME3-1249 (Niemann *et al.*, 2006; Lösekann *et al.*, 2007; red) as observed in sediments from the Haakon Mosby Mud Volcano.
H. ANME-2/SEEP-SRB1a aggregate (ANME2-538, red; SEEP1a-473, green) as observed in a Black Sea microbial mat sample. All scale bars = 5 µm.

DSS658 (one mismatch next to last position of the probe) is an indication that the dominant partner of ANME-2 is most likely not from the SEEP-SRB1c group.

Almost all of the SEEP-SRB1 sequences obtained from the two enrichments affiliated with subgroup SEEP-SRB1a (HR: 100%, Isis: 95%) (Table S1). Intriguingly, other SEEP-SRB1a sequences exclusively originated from AOM habitats. Moreover, in 16S rRNA gene libraries constructed for other ANME-2 dominated habitats, a fraction of the SEEP-SRB1 sequences often affiliated with the SEEP-SRB1a subgroup. The only exceptions were observed for the Tommeliten and Gullfaks oil field from which only sequences affiliated with SEEP-SRB1d and 1e were obtained (Table S2). However, CARD-FISH confirmed the presence of SEEP-SRB1a bacteria at least in sediments from the Gullfaks oil field (Fig. 2C, Table 3). Thus, SEEP-SRB1a sequences at these sites were possibly missed due to biased clone libraries or an insufficient number of clones analysed.

Oligonucleotide probes were designed and tested for each of the six SEEP-SRB1 subgroups (Table S3). Two probes were designed for SEEP-SRB1a, probe SEEP1a-473 and probe SEEP1a-1441 (Table 1). Probe SEEP1a-1441 targeted 98% of all SEEP-SRB1a sequences. Besides SEEP-SRB1a sequences, the probe also targeted a few sequences from *Acidobacteria* and *Desulfobacterales* not affiliated with the SEEP-SRB1 group (Table 1). The second probe designed for subgroup SEEP-SRB1a, probe SEEP1a-473, targeted 77% of the sequences within group SEEP-SRB1a. SEEP-SRB1a

Table 1. SEEP-SRB1 specific oligonucleotide probes.

Probe	Specificity	Sequence (5′ to 3′)	Position	FA conc. (%, vol/vol)	Hits in target group[a]	Outgroup hits[b]	
SEEP1a-473 (S-*-SP1a-0473-a-A-23)	SEEP-SRB1a group	TTC AGT GAT ACC GTC AGT ATC CC	473–495	30	79/102 (77%)	–	
HSEEP1a-453 (S-*-SP1a-0453-a-A-21)	Helper 1 for SEEP1a-473	RCG RTA TTR RCG CGG RAT AGG	453–472	n/a	n/a	n/a	
HSEEP1a-496 (S-*-SP1a-0496-a-A-21)	Helper 2 for SEEP1a-473	ACG GAG TTA GCC GGT GCT TCC	496–516	n/a	n/a	n/a	
SEEP1a-1441 (S-*-SP1a-1441-a-A-18)	SEEP-SRB1a group	CCC CTT GCG GGT TGG TCC	1441–1470	45	85/87 (98%)	*Desulfobacterales*	19
						Acidobacteria	15
						Chloroflexi	3
						Betaproteobacteria	2
SEEP1c-1309 (S-*-SP1c-1309-a-A-21)	SEEP-SRB1c group	ATG GAG TCG AAT TGC AGA CTC	1309–1329	30	22/24 (92%)	*Fibrobacteria*	592
						Deltaproteobacteria	147
						Bacteroidetes	69
						Planctomycetes	67
						Firmicutes	66
SEEP1f-153 (S-*-SP1f-0153-a-A-18)	SEEP-SRB1f group	AGC ATC GCT TTC GCG GTG	153–170	35	9/10 (90%)	*Acidobacteria*	25
						Chloroflexi	12
						Nitrospirae	10
						Desulfobacterales	8

a. Only sequences which possessed sequence information at the probe binding site were considered.
b. Based on ARB/SILVA SSU Ref dataset Release 100 (Pruesse *et al.*, 2007).

sequences not targeted by SEEP1a-473 exhibited 1–4 mismatches to the probe sequence. SEEP1a-473 is currently not targeting any non-SEEP-SRB1a sequence. The probe was used in combination with two helper probes (Fuchs *et al.*, 2000), HSEEP1a-453 and HSEEP1a-491 (Table 1), to increase signal intensity. Probe SEEP1c-1309 was designed for the SEEP-SRB1c subcluster. It targeted 92% of all SEEP-SRB1c affiliated sequences. Non-SEEP-SRB1c sequences targeted by the probe included sequences from *Fibrobacteres*, *Bacteroidetes/Chlorobi* and non-SEEP-SRB1 *Deltaproteobacteria* (Table 1). Furthermore, probe SEEP1f-153 was designed for group SEEP-SRB1f. The probe targeted 90% of all SEEP-SRB1f affiliated sequences. Besides that, the probe also targeted a few sequences from *Acidobacteria*, *Chloroflexi* and *Deltaproteobacteria* not affiliated with the SEEP-SRB1 group (Table 1). Probes designed for subgroups SEEP-SRB1b, 1d and 1e (Table S3) did not show sufficient signal intensity or specificity during probe testing, even when used in combination with unlabelled helper or competitor oligonucleotides respectively. However, they may be used for other molecular techniques in future studies, e.g. as primers for polymerase chain reactions.

SEEP-SRB1a is the dominant partner of ANME-2 in AOM enrichment cultures

The SEEP-SRB1a probes were first applied to enrichment cultures in double FISH experiments combining either of the two newly designed SEEP-SRB1a-specific probes with an ANME-2 specific probe, probe ANME2-538 (Treude *et al.*, 2005; Table S4). In the HR enrichment 97% (SEEP1a-1441) and 93% (SEEP1a-473) of the ANME-2-aggregates were targeted by the SEEP-SRB1a specific probes. In the Isis enrichment, probe SEEP1a-1441 hybridized to all ANME-2-aggregates (Fig. 2A), while probe SEEP1a-473 labelled the partners of 76% of the ANME-2-aggregates. This was consistent with the fact that probe SEEP1a-473 was only covering 77% of all known SEEP-SRB1a 16S rRNA sequences leading to an underestimation of the percentage of SEEP-SRB1a/ANME-2 consortia. It also proved that in the Isis enrichment there were at least two SEEP-SRB1a partners of ANME-2 consortia, one hybridizing with SEEP1a-1441 and SEEP1a-473, and one only hybridizing to probe SEEP1a-1441. The results indicated that the dominant partners of ANME-2 in both enrichments were from the SEEP-SRB1a group. Knowing about the ratio of ANME-2a to ANME-2c cells in the enrichments (HR: ANME-2a 17%, ANME-2c 64%; Isis: ANME-2a 20%, ANME-2c 49%) it was also evident that both, ANME-2a and ANME-2c, associate with bacteria of the SEEP-SRB1a group.

Distribution and ecology of SEEP-SRB1a bacteria in various methane seep systems

To test if the association between ANME-2 and SEEP-SRB1a is of general nature and not only induced by the enrichment procedure, six ANME-2 dominated environmental samples were examined by multi-colour catalysed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) (Pernthaler *et al*., 2004). The samples originated from a methanotrophic microbial mat growing in the Black Sea and sediments at gas seeps at the Hydrate Ridge (NE Pacific), in the Gulf of Mexico (W Atlantic), at the Isis Mud Volcano (Eastern Mediterranean Sea) and the Gullfaks oil field (N Atlantic) (for a detailed description of the sampling sites see Table 2). In double hybridizations with the ANME-2 specific probe ANME2-538, altogether between 92% (Gulf of Mexico) and 99% (HR) of all ANME-2 consortia were targeted by DSS658 (Manz *et al*., 1998; Fig. 3). Subsequent application of the newly designed probes showed the presence of SEEP-SRB1a and their association with ANME-2 in all examined samples. In sediment samples from Hydrate Ridge, the Isis Mud Volcano, and the Gulf of Mexico at least 75% and up to 95% of the ANME-2 aggregates exhibited a partner from the SEEP-SRB1a group (Figs 2B and 3). In addition, SEEP-SRB1a was also frequently observed as the partner of ANME-2 in mat samples from the Black Sea (Fig. 2H) and a sediment sample from the Gullfaks oil field (Fig. 2C). Due to the complex structure of the microbial mat and very low aggregate abundances in the Gullfaks sediment, it was, however, not possible to quantify these associations. Altogether, our data from geographically distant AOM habitats indicate that SEEP-SRB1a is the dominant partner of ANME-2 at methane seeps.

The association of ANME-2 with SEEP-SRB1a seemed to be independent of the ANME-2 subgroup. A previous report (Knittel *et al*., 2005) showed that the two Hydrate Ridge samples examined in this study (Table 2) were dominated by different ANME-2 subgroups (station 19-2, 80% ANME-2a vs. 16% ANME-2c aggregates; station 38: 20% ANME-2a vs. 75% ANME-2c aggregates; Knittel *et al*., 2005). In spite of this difference, at least 87% (station 19-2) and 85% (station 38) of the ANME-2 aggregates in both samples exhibited a SEEP-SRB1a partner (Fig. 3) suggesting that both, ANME-2a and ANME-2c, were predominantly associated with bacteria of the SEEP-SRB1a group. This was confirmed by CARD-FISH hybridizations with probes specific for ANME-2a (ANME2a-647; Knittel *et al*., 2005) and ANME-2c (ANME2c-760; Knittel *et al*., 2005) (Fig. 2E and F). Quantification of the association of SEEP-SRB1a with the ANME-2 subgroups, yielded numbers in the same range as those obtained with the general ANME-2 probe (for details see Table S5).

ANME-2 associated SEEP-SRB1a were observed as coccoid cells (Fig. 2B) but also as rod/vibrio-shaped morphotypes (Fig. 2C, E and F). This morphological variability likely reflects the genomic variations within the SEEP-SRB1a group (92% 16S rRNA sequence similarity) which might be at the level of genera. Different ANME-clades, species within a particular clade, or environmental parameters seem to select for different strains within the SEEP-SRB1a group. FISH studies involving probes of a higher resolution, e.g. by targeting ITS sequences, might be useful to gain further insight into the diversity within SEEP-SRB1a.

In addition to ANME-2 dominated AOM samples, one ANME-3 dominated sediment sample from the Haakon Mosby Mud Volcano (HMMV; Table 2) was screened for the presence of SEEP-SRB1a bacteria. Sediments from this site were previously described by Lösekann and colleagues (2007) who showed that the majority of ANME-3 aggregates was associated with bacteria related to the genus *Desulfobulbus.* In addition, however, a small number of ANME-3 aggregates was also detected which possessed an unknown bacterial partner. In the present study, 8% and 23% of the examined ANME-3-aggregates were found to be associated with partners detected by probe SEEP1a-1441 or SEEP1a-473 respectively (Fig. 2G). This suggests that at least a fraction of the unknown bacterial partner belongs to the SEEP-SRB1a group. Most of the SEEP-SRB1a-positive aggregates (43 of 48 aggregates) consisted of only 1–3 SEEP-SRB1a and 1–3 ANME-3 cells (Fig. 2G). However, some bigger mixed-type aggregates (150–300 total cells) were also detected (Fig. 2G).

Diversity of the bacterial partners of ANME-2

The majority of the bacterial partners of ANME-2 belonged to the SEEP-SRB1a cluster within the DSS branch. However, the abundance of ANME-2/SEEP-SRB1a consortia was significantly lower than those of ANME-2/DSS consortia (Figs 2D and 3). This discrepancy might have been caused by an insufficient coverage of the developed SEEP-SRB1a-probes or microdiversity of SEEP-SRB1a microorganisms within a sample, as shown for the analysed AOM enrichments (Fig. 1). Another possible explanation is an affiliation of these DSS cells with another SEEP-SRB1 subgroup. The SEEP-SRB1f probe was used to test for the discrepancy between DSS658 and SEEP-SRB1a targeted cells. None of the examined ANME-2 aggregates featured a partner targeted by the SEEP-SRB1f probe. An association of the remaining SEEP-SRB1 subgroups (SEEP-SRB1b, 1d and 1e) with ANME-2 could not be tested as probes designed for these groups showed either no signals or insufficient specificity when evaluated (see above). However, the presence of

Table 2. Environmental samples used in this study.

Sample	Cruise	Site description	Position	Depth (m)	Date	Sediment layer depth	References
Black Sea microbial mat, P822 top	P317/3 (RV POSEIDON)	Microbial mat sample 822 from top of microbial reef, Dnieper area, northwestern Black Sea	44° 46.542′ N, 31° 58.978′ E	190	Oct 2004	n/a	Rossel *et al.* (2008)
Hydrate Ridge St. 19-2	SO148-1 (RV SONNE)	Crest of southern Hydrate Ridge, coast of Oregon; gas hydrate bearing sediment covered by *Beggiatoa* mat	44° 34.104′ N, 125° 08.807′ W	777	July 2000	0–1 cm	Linke and Suess (2001), Knittel *et al.* (2003)
Hydrate Ridge St. 38	SO148-1 (RV SONNE)	Crest of southern Hydrate Ridge, coast of Oregon; gas hydrate bearing sediment covered by clam fields of *Calyptogena* spp.	44° 34.186′ N, 125° 08.847′ W	787	July 2000	2–3 cm	Linke and Suess (2001), Knittel *et al.* (2003)
Isis Mud Volcano, St. 812	M70-2 (RV METEOR)	Isis mud volcano, Eastern Mediterranean; mud volcano sediment covered by *Arcobacter* mat	32° 21.669′ N, 031° 23.387′ E	992	November 2006	0–1 cm	This study, Felden and Boetius (2009)
Gulf of Mexico St. 156	SO174/OTEGA II (RV SONNE)	White sulfide-oxidizing bacteria occurring as a 'mat' on the surface of the sediment, oily hydrate	27° 46.95′ N, 91° 30.47′ W	546	October/ November 2003	0–2 cm	Bohrmann and Schenck (2004), Orcutt *et al.* (2008)
Haakon Mosby Mud Volcano, ATL19-27	AWI (RV L'ATALANTE)	Mud Volcano at Norwegian-Barents-Svalbard continental margin; *Beggiatoa* mat site	72° 00.19′ N, 14° 43.67′ E	1250	August 2001	1–2 cm	Lösekann *et al.* (2007)
Gullfaks oil field, St. 771; Heincke seep	HE 208 (RV HEINCKE)	Coarse sand sediment densely covered with bacterial mats, gas ebullition observed	61° 10.40′ °N, 02° 14.50′ °E	150	May 2004	0–10 cm	Wegener *et al.* (2008b)

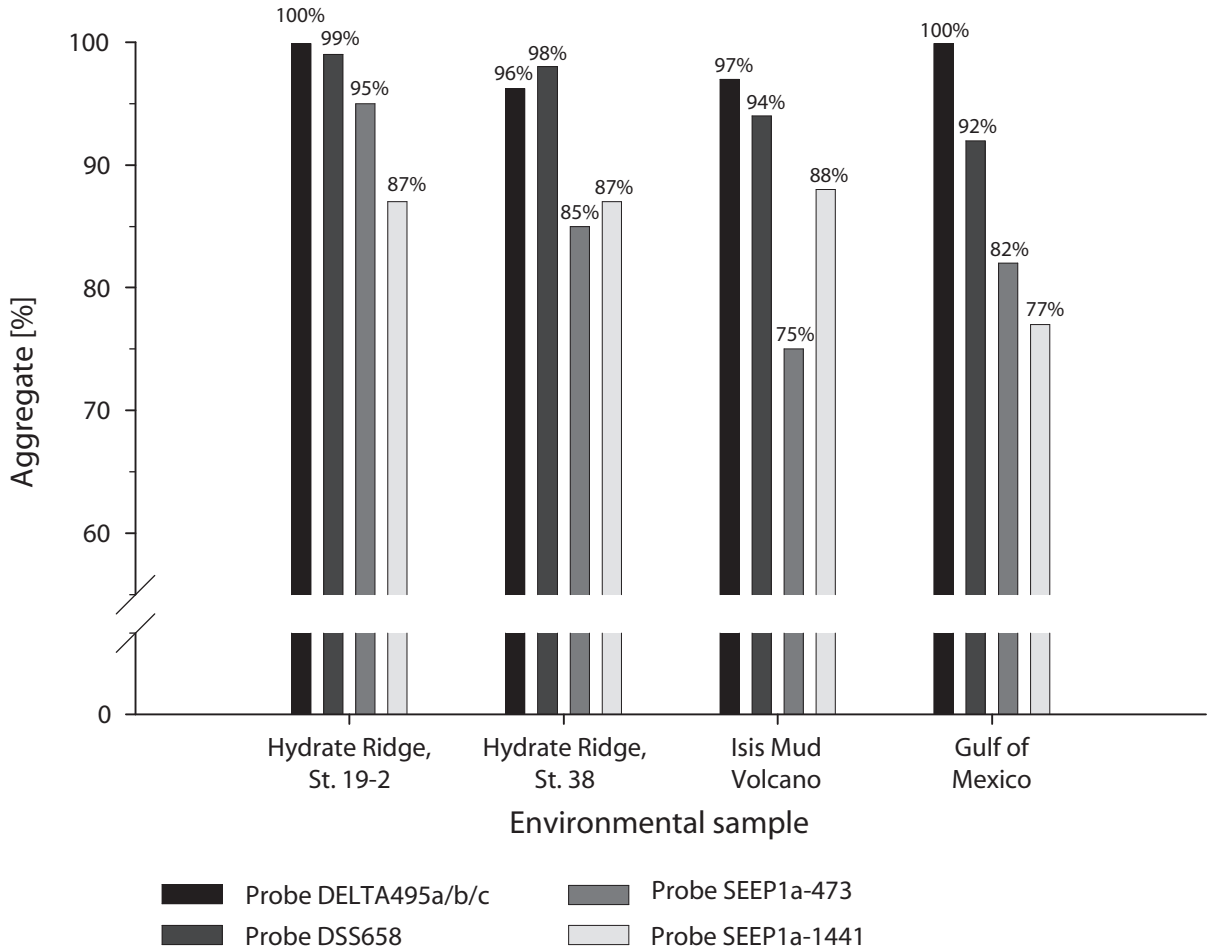

Fig. 3. ANME-2-partners in the environment. The graph shows the percentages of ANME-2/*Deltaproteobacteria* (probes: ANME2-538, DELTA495a/b/c), and ANME-2/DSS (probes: ANME2-538, DSS658) aggregates (Table S4) as well as of ANME-2/SEEP-SRB1a detected with probe SEEP1a-473, or probe SEEP1a-1441 (Table 1). For each sample and probe at least 110 ANME-2-aggregates were counted (for details see SI Table S5). Only ANME-2 aggregates featuring a partner were considered.

sequences from ANME-2 dominated habitats within these subgroups suggests that the other SEEP-SRB1 subgroups either interact with ANME-2 or use short-chain alkanes (Kniemeyer *et al*., 2007) or other hydrocarbons occurring in the habitat.

In all examined sediments, the proportion of Bacteria/ANME-2-aggregates – as quantified using the EUB338I-III probe mix (Amann *et al*., 1990; Daims *et al*., 1999; Table S4) targeting most of the *Bacteria* – ranged from 96% for the Isis Mud Volcano, over 98% for the Gulf of Mexico and station 38 at Hydrate Ridge, to 100% for station 19-2 at Hydrate Ridge (Table S5). Results with a general probe mix targeting *Deltaproteobacteria*, Delta495a/b/c (Loy *et al*., 2002; Macalady *et al*., 2006; Lücker *et al*., 2007; Table S4), were in the same range (Table S5). The percentage of ANME-2/DSS positive aggregates was, in contrast, lower for the Isis Mud Volcano (97% versus 94%) and especially for the Gulf of Mexico (100% versus 92%) sample (Fig. 3). The SEEP-SRB1c probe was used to test for the discrepancy between Delta495a/b/c and DSS658 targeted cells (as SEEP-SRB1c sequences are likely not targeted by DSS658). None of the examined ANME-2 aggregates featured a partner of that group. This indicates that ANME-2 may have partners distinctly different from the DSS group. Similar observations were previously described for other ANME-2 habitats such as the Eel River Basin (Pernthaler *et al*., 2008) or Mud Volcanoes in the Eastern Mediterranean (Omoregie *et al*., 2009). In sediments from the Eel River Basin, Pernthaler and colleagues (2008) identified *Alphaproteobacteria* and *Betaproteobacteria* associated with ANME-2. This finding could not be confirmed by the present study, because even though a small number of single *Alphaproteobacteria* and *Betaproteobacteria* were detected in the examined sediments, none of these bacteria showed an association with ANME-2 (data not shown). In addition, Pernthaler and colleagues (2008) also showed that ANME-2 can, similar

to ANME-3 (Lösekann *et al*., 2007), have a bacterial partner related to *Desulfobulbus* spp. This observation could also not be confirmed, suggesting that the association between ANME-2 and *Alphaproteobacteria*, *Betaproteobacteria,* or *Desulfobulbus*-related bacteria might be restricted to certain AOM habitats.

Presence of single SEEP-SRB1a cells

Cells of SEEP-SRB1a were not only found to be associated with ANME-2, but also as single cells. In sediment samples from Hydrate Ridge, the Isis Mud Volcano and the Gulf of Mexico, DSS cells accounted for 3–6% of all DAPI-stained single cells. Out of these, 8–17% were labelled with probe SEEP1a-1441. This translated into relative abundances of single SEEP-SRB1a cells of 0.3% to 0.7% (Table 3). Contrastingly, in a sediment sample from the Gullfaks oil field, DSS cells accounted for 18% and SEEP-SRB1a for 9% of all single cells. This sediment sample also featured an unusually high abundance of single ANME-2 cells and only very few ANME-2/DSS aggregates in comparison with other AOM habitats (Table 3, Knittel *et al*., 2005; Wegener *et al*., 2008b; Omoregie *et al*., 2009). Considering also the nature of the sample (Wegener *et al*., 2008b), it is likely that the high number of single ANME-2 and SEEP-SRB1a cells were an artifact of sample preparation. Here, harsher sonication was required to remove the microorganisms from coarse sand prior to CARD-FISH analysis. This procedure most likely disrupted part of the aggregates, releasing single cells. This conclusion was supported by the analysis of a Gullfaks oil field enrichment culture from the same sample in which SEEP-SRB1a was almost exclusively observed in aggregates together with ANME-2 (data not shown).

Apart from this exception, the generally low number of single SEEP-SRB1a cells in the environment is in line with results for the *Desulfobulbus*-related partner of ANME-3 in sediments of the Haakon Mosby Mud Volcano. An overall low percentage of less than 0.5% of single *Desulfobulbus*-related cells indicated that the partner, if at all, accounts for a very low fraction of the single cells, whereas single ANME-3 cells accounted for about 25% of DAPI-stained single cells (Lösekann *et al*., 2007). Due to the specificity of the probes and the diversity within the SEEP-SRB1a group, it cannot conclusively be answered whether single and ANME-2 associated SEEP-SRB1a bacteria are identical. Thus, further phylogenetic analyses targeting genomic regions with a higher variability (e.g. ITS) are necessary to address this question. Finally, it may also be possible that the detected single cells are inactive without ANME partner. Altogether the results indicate that SEEP-SRB1a is highly adapted to or even depending on life in ANME-2-consortia. This is also supported by failed ^{13}C-

Table 3. Abundances of single SEEP-SRB1a and ANME-2 cells in sediments at different AOM sites.

	Hydrate ridge, St. 19-2[a]	Hydrate ridge, St. 38[a]	Isis mud volcano	Gulf of Mexico	Gullfaks oil field[b]
Total number of single cells (cm^{-3})	2.9×10^9	3.5×10^9	1.5×10^9	3.6×10^9	6.7×10^9
Single cells showing a signal with probe DSS658	3%	3%	5%	6%	19%
	8.7×10^7 cm^{-3}	1.1×10^8 cm^{-3}	7.5×10^7 cm^{-3}	2.2×10^8 cm^{-3}	1.3×10^9 cm^{-3}
SEEP1a-1441 labelled cells relative to DSS658 labelled cells	17%	9%	13%	8%	n.d.
Calculated single SEEP-SRB1a cells	0.5%	0.3%	0.7%	0.5%	9%[c]
	1.5×10^7 cm^{-3}	1.1×10^7 cm^{-3}	1.1×10^7 cm^{-3}	1.8×10^7 cm^{-3}	6.0×10^8 cm^{-3}
Single cells showing a signal with probe ANME2-538	1%	1%	1%	1%	10%
	2.9×10^7 cm^{-3}	3.5×10^7 cm^{-3}	1.5×10^7 cm^{-3}	3.6×10^7 cm^{-3}	6.7×10^8 cm^{-3}

a. Total and absolute cell numbers based on Knittel and colleagues (2005).
b. Total and absolute cell numbers based on Wegener and colleagues (2008b).
c. SEEP-SRB1a cells counted directly and not determined semi-quantitatively. n.d., not determined.

labelled bicarbonate uptake in lipids of the SRB from AOM sediments in the absence of methane (Wegener *et al*., 2008a), and the failure to stimulate sulfate reduction in AOM samples with common substrates for SRB (Nauhaus *et al*., 2002).

Conclusion

In this study, we narrowed the phylogenetic affiliation of the dominant partner of ANME-2a and ANME-2c down to a single, well-supported subgroup (SEEP-SRB1a) within the broad DSS group. An important target group for further studies of the biochemical pathways underlying AOM, and for isolation approaches is herewith well defined. We could not confirm earlier studies reporting the association of *Alphaproteobacteria*, *Betaproteobacteria* or *Desulfobulbus*-related SRB with ANME-2 (Pernthaler *et al*., 2008). However, we also observed ANME-2 partners not from the SEEP-SRB1a group. Therefore, in the investigated habitats interactions of ANME-2 with alternative partners cannot be ruled out. The idea of ANMEs associating with diverse bacterial partners is in line with our identification of SEEP-SRB1a as an alternative partner of ANME-3, which was previously only described to be associated with *Desulfobulbus*-related SRB (Lösekann *et al*., 2007). The *Desulfobulbus*-related partner of ANME-3 could only rarely be observed as single cells in a previous study. This almost exclusive association with ANME was also observed for SEEP-SRB1a. This points towards a highly adapted if not even obligate syntrophic lifestyle of the bacterial partners in AOM aggregates.

Metagenomic studies focusing on the archaeal part of the supposed syntrophy yielded as yet only a fragmentary understanding of AOM (Hallam *et al*., 2003; Krüger *et al*., 2003; Hallam *et al*., 2004; Meyerdierks *et al*., 2005; 2010). Genomic or proteomic data of ANME-associated SRB are currently limited to a single metagenomic study of AOM consortia providing only little information on the associated SRB (Pernthaler *et al*., 2008). Knowing the partners of ANME allows the application of metagenomic or single cell techniques in order to access at least a snapshot of the metabolic capabilities of the partners. Comparative genome analysis of the different ANME-partners and of closely related cultivated, free-living relatives, such as the recently sequenced *Desulfococcus oleovorans* Hxd3 (accession number CP000859), might reveal common features of the bacterial partners, leading to a far better understanding of biochemical processes in AOM aggregates.

Experimental procedures

Description of AOM enrichments

The enrichment cultures originated from sediment from Hydrate Ridge (NE Pacific, 044°34.2′ N, 125°08.7′ W, taken during RV Sonne cruise SO-148/1 in August 2000) and the Isis Mud Volcano (Eastern Mediterranean Sea, 031°23.4′ N, 032°21.7′ E, taken during RV L'Atalante cruise NAUTINIL in September 2003). Methane-dependent sulfide formation was observed for both types of samples when incubated in artificial seawater medium (Nauhaus *et al*., 2002) at 12°C for the Hydrate Ridge (HR) and at 20°C for the Isis Mud Volcano (Isis) enrichment respectively. The AOM rate of the HR (Nauhaus *et al*., 2007) and Isis samples increased gradually. Consecutive sub-incubations over long periods (HR, 84 months; Isis, 49 months) resulted in detritus-free enrichments of loose flocks essentially composed of microbial cells. Background methanogenesis in the absence of methane was below the detection limit and thus must be below 0.05% of the AOM rate.

DNA extraction, PCR amplification and clone library construction

DNA was extracted from 5 ml of enrichment culture according to the SDS-based DNA extraction protocol by Zhou and colleagues (1996). The protocol encompassed three cycles of chemical lysis in a high-salt extraction buffer (1.5 M NaCl) by heating of the suspension in the presence of SDS and hexadecyltrimethylammonium bromide (CTAB), and a proteinase K step. Prior to the first cycle a lysozyme step was performed. Additionally, prior to the third cycle a freeze and thaw step was added.

The DNA was directly used to amplify almost full-length 16S rRNA genes with the general bacterial primers GM3F and GM4R (Muyzer *et al*., 1995) by PCR. The PCR was performed in a Mastercycler Gradient (Eppendorf, Germany) in a 50 μl reaction volume. Each PCR reaction contained: 0.5 μM of each primer, 200 μM of each deoxyribonucleoside triphosphate, 15 μg bovine serum albumin, 1 × PCR buffer (5Prime, Germany), 1 × PCR Enhancer (5Prime), 1.25 U *Taq* DNA Polymerase (5Prime) and 5–60 ng of template DNA. The following cycling conditions were applied: one initial step at 95°C for 4 min; 20 cycles at 95°C for 1 min, 42°C for 1 min and 72°C for 3 min; and final step at 60°C for 60 min.

After PCR, the DNA of 10 reactions was pooled and purified by using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's recommendations. The DNA was then ligated to the pCR4 TOPO vector and transformed into *Escherichia coli* TOP10 cells by using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Germany) according to the manufacturer's recommendations. Taq cycle sequencing was performed using ABI BigDye Terminator chemistry and an ABI377 sequencer (Applied Biosystems, USA).

Phylogenetic analysis and probe design

The phylogenetic affiliation was inferred with the ARB software package (Ludwig *et al*., 2004) based on Release 90 of the SILVA database (Pruesse *et al*., 2007). All phylogenetic analyses were performed with representative sequences from two AOM enrichments together with sequences of related *Deltaproteobacteria* found in public databases. In total, 265 nearly full-length sequences (> 1200 bp) were used for tree construction. Phylogenetic trees were calculated by

maximum likelihood analysis (RAxML, PHYML) and the ARB neighbour-joining algorithm. A 50% base frequency filter was used for each tree calculation to exclude highly variable positions. The resulting phylogenetic trees were compared manually and a consensus tree was constructed. Relevant partial sequences were subsequently added to the tree according to maximum parsimony criteria, without allowing changes in the overall tree topology. RAxML maximum likelihood (Stamatakis *et al*., 2005) analysis was performed via the ARB tree building tool (Ludwig *et al*., 2004). Maximum likelihood tree reconstruction with the PHYML 3.0 algorithm was performed via the PHYML web server (Guindon *et al*., 2005).

Oligonucleotide probes were designed using the ARB probe tool (Ludwig *et al*., 2004). Specificity of the probes was evaluated by Clone-FISH (Schramm *et al*., 2002). In short, 16S rRNA sequences having no, one, or two mismatches to the designed probes were ligated to the pCR4-TOPO vector and transformed into chemically competent *E. coli* JM109 (DE3) cells. Recombinant cells were grown at 37°C in 1 × Luria–Bertani medium. Isopropyl-β-D-thiogalactopyranosid (IPTG) was added to a final concentration of 0.1 mM to induce transcription of the introduced 16S rRNA gene. After growth for 1 h, cell division was inhibited by adding chloramphenicol to a final concentration of 170 μg ml^{-1}. Subsequently, cells were incubated for an additional 4 h at 37°C, before being fixed in PBS containing 1% formaldehyde. Clones used for Clone-FISH in this study are listed in Table S6. To generate melting curves, probes were hybridized to clones at formamide concentrations of 0%, 10%, 20%, 30%, 40%, 50%, 60% and 70%. Probes showing insufficient sensitivity or specificity during Clone-FISH were re-evaluated in combination with helper or competitor oligonucleotides respectively.

In addition to Clone-FISH, the probes were tested for sensitivity (target group hits) and specificity (outgroup hits) *in silico* with the ARB probe match tool (Ludwig *et al*., 2004). For evaluation of probe sensitivity, only sequences which possessed sequence information at the probe binding site were considered. Probe specificity was based on 362 515 prokaryotic sequences of the ARB/SILVA SSU Ref dataset Release 100 (Pruesse *et al*., 2007).

Fluorescence in situ *hybridization (FISH) on AOM enrichment cultures*

Subsamples of the HR and Isis enrichment cultures were fixed for 1 h in 1% formaldehyde, washed with 1 × phosphate buffered saline (130 mM NaCl, 10 mM sodium phosphate; pH 7.4), and finally stored in 1 × phosphate-buffered saline-ethanol (1:1) at –20°C. Fixed samples were treated by mild sonication for 40 s with a MS73 probe (Sonopuls HD70, Bandelin, Germany) at an amplitude of 42 μm < 10 W. An aliquot was filtered onto 0.2 μm GTTP polycarbonate filters (Millipore, Germany). FISH was performed as described previously (Snaidr *et al*., 1997). Oligonucleotide probes were either labelled with 6-FAM or Cy3 and were purchased from Biomers (Germany). Probe sequences are shown in Table 1 and Table S4. For double hybridization experiments the following probe combinations were used: (i) ANME2-538 [6-FAM] and DSS-658 [Cy3] at 50% formamide (FA); (ii) ANME2-538 [6-FAM] and SEEP1a-473 [Cy3] at 30% FA; (iii) ANME2-538 [6-FAM] and SEEP1a-1441 [Cy3] at 45% FA.

Preparation of environmental samples

Sediment samples from the Hydrate Ridge, the Haakon Mosby Mud Volcano and the Gulf of Mexico were prepared as described in the references given in Table 2. Of the Isis Mud Volcano sample 0.5 ml of sediment were fixed by adding 2 ml ethanol. The resulting suspension was diluted 1:10 with a PBS/ethanol solution (1:1, v/v). All samples were treated by mild sonication with a type MS73 probe (Sonopuls HD70; Bandelin, Germany) at a setting of 20 s, an amplitude of 42 mm and < 10 W prior to filtration.

The Gullfaks oil field sediment sample consisted of coarse sand and was not suitable for direct microscopic analysis. Therefore, a protocol was used to separate sand particles from the cells. First, 1 ml PBS/ethanol (1:1, v/v) was added to 100 mg sediment. Cells were dislodged from sediment grains by sonicating the sample on ice with a type MS73 probe at a setting of 100 s, an amplitude of 42 mm and 50 W. The sediment was allowed to settle and the supernatant was transferred to a fresh tube. This procedure was repeated four times and in total 5 ml of supernatant was obtained (Wegener *et al*., 2008b). The combined supernatant was directly used for filtration.

For quantification of total cell numbers, the following aliquots of the sediment samples were filtered onto an area of ≈ 227 mm^2 on 0.2 μm GTTP polycarbonate filters (Millipore, Germany): 5 μl of a 1:50 dilution (Isis MV) and 10 μl of a 1:40 dilution (Gulf of Mexico). For aggregate quantification the following aliquots were filtered: 10 μl of a 1:40 dilution (HR19-2, HR 38),10 μl of a 1:50 dilution (Isis MV), 25 μl of a 1:40 dilution (Gulf of Mexico), 25 μl of a 1:80 dilution (Haakon Mosby MV), 40 μl of a 1:50 dilution (Gullfaks oil field).

Multi-colour CARD-FISH

Multi-colour catalysed reporter deposition (CARD)-FISH was performed as described previously (Pernthaler *et al*., 2004) with the following modifications: Sediment samples were filtered onto 0.2 μm GTTP polycarbonate filters. For cell wall permeabilization and inactivation of endogenous peroxidases, filters were sequentially incubated in lysozyme solution (10 mg ml^{-1}, 0.1 M Tris-HCl, 0.05 M EDTA, pH 8) for 60 min at 37°C, 0.01 M HCl (10 min) and 0.5% SDS solution (10 min). In between sequential hybridizations, peroxidases of previous hybridizations were inactivated by a 30 min incubation in 0.1% H_2O_2 in methanol as described previously (Ishii *et al*., 2004). After the multi-colour CARD-FISH procedure samples were stained with 4′,6′-diamidino-2-phenylindole (DAPI). Catalysed reporter deposition was performed using the fluorochromes Alexa Fluor 488 and Alexa Fluor 594. Oligonucleotide probes were purchased from Biomers (Germany). Hybridized samples were examined with an epifluorescence microscope (Axiophot II; Carl Zeiss Germany). Micrographs were obtained by confocal laser scanning microscopy (LSM510; Carl Zeiss, Germany).

Non-DSS partners of ANME-2 were attempted to be identified by multi-colour CARD-FISH (Pernthaler *et al*., 2004) as described above. The ANME-2 specific probe ANME-2-538 was combined with probe ALF968 (Neef, 1997) for the detection of *Alphaproteobacteria*, BET42a (Manz *et al*., 1992) in combination with competitor GAM42a (Manz *et al*., 1992) for

the detection of *Betaproteobacteria*, or with probe 660 (Devereux *et al*., 1992) for the detection of *Desulfobulbus*-related bacteria. Sequences of the used probes are listed in Table S4.

Quantification of single SEEP-SRB1a cells

Total cell numbers were determined after staining sediment aliquots on GTTP filters with DAPI. SEEP-SRB1a-cell numbers were determined by a semi-quantitative method due to very low abundances of single SEEP-SRB1a cells: First the percentage of single DSS cells belonging to the SEEP-SRB1a-group was determined by performing multi-colour CARD-FISH with probes DSS658 and SEEP1a-1441. In a second step, the percentage of single DSS cells in relation to the total number of single cells was determined. Based on those counts and the determined total numbers of single cells, the number of single SEEP-SRB1a cells was calculated.

Sequence accession numbers

The nucleotide sequence data reported in this paper have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers FN549918 to FN550094.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Phylogenetic affiliation of 16S rRNA gene sequences obtained from Hydrate Ridge and Isis AOM enrichments.
Table S2. Retrieval of SEEP-SRB1 subgroup sequences from different habitats.
Table S3. Oligonucleotide probes designed for SEEP-SRB1 subgroups.
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Supporting Information

Supporting Tables

Table S1 Phylogenetic affiliation of 16S rRNA gene sequences obtained from Hydrate Ridge and Isis AOM enrichments

Phylogenetic group	HR enrichment	Isis enrichment
Proteobacteria		
Gammaproteobacteria	0	1
Deltaproteobacteria		
SEEP-SRB1	44	57
(SEEP-SRB1a)	(44)	(55)
*Desulfuromonadacea*e	6	1
Other	6	7
Epsilonproteobacteria	2	0
Bacteroidetes/Chlorobi	5	6
Firmicutes	9	2
Thermomicrobia	4	5
Nitrospirae	1	1
Other (WS3, OD1, OP11, WS6, TM7)	2	8
Total number of clones in library	79	88

Table S2 Retrieval of SEEP-SRB1 subgroup sequences from different habitats. The presented numbers indicate the number of SEEP-SRB1 16S rRNA gene sequences detected in the respective studies. DB Rel. = database release

Site	SEEP-SRB1a	SEEP-SRB1b	SEEP-SRB1c	SEEP-SRB1d	SEEP-SRB1e	SEEP-SRB1f	Reference	AOM activity
AOM Enrichments								
HR Enrichment	44/79	-	-	-	-	-	This study	[7]
Isis Enrichment	54/88	-	2/88	-	1/88	-	This study	This study
Eel River Basin Sediment	1/19[a]	3/19[a]	2/19[a]	-	1/19[a]	-	[28]	[28]
Gulf of Mexico sediments	3/36[a]	-	-	1/36[a]	-	-	[21]	[9]
	-	-	9/196	2/196	-	1/196	[22]	
	-	1/21[a]	-	-	-	-	[12]	
	-	-	-	-	1/26[a]	-	DB Rel., Orcutt	
Haakon Mosby Mud Volcano sediments	3/73	-	-	4/73	-	-	[14]	[14]
	12[b]	1[b]	-	1[b]	5[b]	1[b]	Lösekann, unpubl.	
Hydrate Ridge Sediments	10/145	4/145	-	1/145	1/145		[11]	[11]
Santa Barbara Basin sediments	2/5[a]	-	-	-	-	-	[28]	[28]
	-	-	5/91	-	-	-	[5]	
Black Sea microbial mat	1/7[a]	-	1/7[a]	-	1/7[a]	-	DB Rel., Schumann	[20]
	12/74	2/74	8/74	-	2/74	-	Knittel, unpubl.	
Eastern Mediterranean sediments								
Amon Mud Volcano	4/79	1/79	-	-	-	-	[27]	[27]
	1[b]	1[a]	-	-	7[a]	-	Knittel, unpubl.	
Amsterdam Mud Volcano	1/126[a]	-	1/126[a]	3/126[a]	-	1/126[a]	DB Rel., Heijs	[29]
Isis Mud Volcano	2/64	1/64	1/64	-	1/64	-	[27]	[27]
Kazan Mud Volcano	4/152	-	1/152	-	4/152	8/152	[6]	[6]
Wadden Sea Sediment	-	-	1/55[a]	6/55[a]	-	-	[24]	-
Mangrove Soil	-	-	2/255[a]	3/255[a]	-	-	DB Rel., Yan	-
Antarctic Continental Shelf	-	-	2/86[a]	-	-	-	[2]	-
Gullfaks Oil Field	-	-	-	-	10/70	-	[31]	[31]
Tommeliten Oil Field	-	-	-	1/102	1/102	-	[31]	[31]
	-	6[b]	2[b]	-	-	3[b]	Boetius, unpubl.	
Peru Methane Hydrate-bearing Continental Margin	-	1/232	1/232	-	1/232	-	[8]	-
Guerrero Negro Hypersaline Mat	-	-	2/158	-	-	-	[13]	-
Benzene-degrading Enrichment	-	-	-	3/15[a]	-	-	[23]	-

[a] Studies where the total number of analyzed clones was not provided. In those cases, the shown ratio is based on the number of sequences submitted to public databases.

[b] Size of 16S rRNA library unknown

Table S3 Oligonucleotide probes designed for SEEP-SRB1 subgroups

Probe	Specificity	Sequence (5' to 3')	Position in *E. coli*	FA conc. (%, vol/vol)	Comments
SEEP1a-473 (S-*-SP1a-0473-a-A-23)	SEEP-SRB1a group	TTC AGT GAT ACC GTC AGT ATC CC	473-495	30	
HSEEP1a-453 (S-*-SP1a-0453-a-A-21)	Helper 1 for SEEP1a-473	RCG RTA TTR RCG CGG RAT AGG	453-472	-	
HSEEP1a-496 (S-*-SP1a-0496-a-A-21)	Helper 2 for SEEP1a-473	ACG GAG TTA GCC GGT GCT TCC	496-516	-	
SEEP1a-1441 (S-*-SP1a-1441-a-A-18)	SEEP-SRB1a group	CCC CTT GCG GGT TGG TCC	1441-1470	45	
SEEP1b-168 (S-*-SP1b-0168-a-A-23)	SEEP-SRB1b group	CTG AAA TAT TAT CCG GTA TTA GC	168-184	-	No signal as probe even when used with helper probes; possible use as primer
HSEEP1b-152 (S-*-SP1b-0152-a-A-21)	Helper 1 for SEEP1b-168	TTA GCC CCG CTT TCG CGG AGT	152-172	-	
HSEEP1b-184 (S-*-SP1b-0184-a-A-21)	Helper 2 for SEEP1b-168	TGA TTT CAA AAC CCG AAG ATT	184-195	-	
SEEP1b-468 (S-*-SP1b-0468-a-A-24)	SEEP-SRB1b group	TCA TTT TTT ACT GAC GGT ACC ACT	468-491	-	No signal as probe; possible use as primer
SEEP1c-1309 (S-*-SP1c-1309-a-A-21)	SEEP-SRB1c group	ATG GAG TCG AAT TGC AGA CTC	1309-1329	30	
SEEP1d-1420 (S-*-SP1d-1420-a-A-18)	SEEP-SRB1d group	CAA CTT CTG GTA CAG CCA	1420-1437	-	Even in combination with competitor probe no distinction between match and single mismatch clone possible
cSEEP1d-1420 (S-*-cS1d-1420-a-A-18)	Competitor for SEEP1d-1420	CAA CTT CTG GTA CA**A** CCA	1420-1437	-	
SEEP1e-203 (S-*-SP1e-203-a-A-24)	SEEP-SRB1e group	CAA ACA ACA GCT TAC ATG TAG AGG	203-224	-	No signal as probe even when used with helper probes; possible use as primer
HSEEP1e-193 (S-*-SP1e-193-a-A-21)	Helper 1 for SEEP1e-203	AGG CCA TTT TTG ATC TCA AAA	193-206	-	
HSEEP1e-222 (S-*-SP1e-222-a-A-21)	Helper 2 for SEEP1e-203	GGT ACG CGG GCT CAT CTC CAA	222-242	-	
SEEP1e-632 (S-*-SP1e-0632-a-A-24)	SEEP-SRB1e group	CTC CCA TAC TCA AGC CCT TTA GTT	632-655	-	Even in combination with competitor probe no distinction between match and single mismatch clone possible
cSEEP1e-632 (S-*-cS1e-0632-a-A-24)	Competitor for SEEP1e-632	CTC CCA TAC TCA AG**T** CC**C** TTA GTT	632-655	-	
SEEP1f-153 (S-*-SP1f-0153-a-A-18)	SEEP-SRB1f group	AGC ATC GCT TTC GCG GTG	153-170	35	

Table S4 Non-SEEP-SRB1 oligonucleotide probes used in the present study

Probe	Specificity	Sequence (5' to 3')	Position	FA conc. (%, vol/vol)	Reference
ALF-968	*Alphaproteobacteria*	GGT AAG GTT CTG CGC GTT	968-985	35	[25]
BET42a (L-C-bProt-1027-a-A-17)	*Betaproteobacteria*	GCC TTC CCA CTT CGT TT	1027-1043	35	[19]
DELTA495a (S-C-dProt-0495-a-A-18)	Most *Deltaproteobacteria* and most *Gemmatimonadetes*	AGT TAG CCG GTG CTT CCT	495-512	25	[15]
cDELTA495a	Competitor for DELTA495a	AGT TAG CCG GTG CTT C**T**T	495-512	-	[17]
DELTA495b (S-*-dProt-0495-b-A-18)	Some *Deltaproteobacteria*	AGT TAG CCG GCG CTT CCT	495-512		[15]
cDELTA495b	Competitor for DELTA495b	AGT TAG CCG GCG CTT C**K**T	495-512	-	[16]
DELTA495c (S-*-dProt-0495-c-A-18)	Some *Deltaproteobacteria*	AAT TAG CCG GTG CTT CCT	495-512		[15]
cDELTA495c	Competitor for DELTA495c	AAT TAG CCG GTG CTT C**T**T	495-512	-	[16]
Eub338-I (S-D-Bact-0338-a-A-18)	Most Bacteria	GCT GCC TCC CGT AGG AGT	338-355	35	[1]
Eub338-II (S-*-BactP-0338-a-A-18)	*Planctomycetales*	GCA GCC ACC CGT AGG TGT	338-355	35	[3]
Eub338-III (S-*-BactV-0338-a-A-18)	*Verrucomicrobiales*	GCT GCC ACC CGT AGG TGT	338-355	35	[3]
GAM42a (L-C-gProt-1027-a-A-17)	*Gammaproteobacteria* Competitor in combination with BET42a	GCC TTC CCA CAT CGT TT	1027-1043	35	[19]
660 (S-G-Dsbb-0660-a-A-20)	*Desulfobulbus*	GAA TTC CAC TTT CCC CTC TG	660-679	50	[4]
DSS658 (S-*-Dsb-0658-a-A-18)	*Desulfosarcina-Desulfococcus*	TCC ACT TCC CTC TCC CAT	658-685	50	[18]
ANME-2-538	ANME-2 (incl. *Methanolobus tindarius*)	GGC TAC CAC TCG GGC CGC	538-555	40	[30]
ANME2a-647	ANME-2 subgroup a	TCT TCC GGT CCC AAG CCT	647-664	35	[10]
ANME2c-760	ANME-2 subgroup c	CGC CCC CAG CTT TCG TCC	760-777	50	[10]
ANME3-1249	ANME-3	TCG GAG TAG GGA CCC ATT	1250-1267	40	[26]
ANME3-1249H3	Helper 1 for ANME3-1249	GTC CCA ATC ATT GTA GCC GGC	1229-1249	-	[14]
ANME3-1249H5	Helper 2 for ANME3-1249	TTA TGA GAT TAC CAT CTC CTT	1268-1288	-	[14]

Table S5 Average cell and aggregates sizes and aggregate percentages in sediments at different AOM sites

	HR, St. 19-2, 0-1 cm	HR, St. 38, 2-3 cm	Isis, 0-1 cm	GoM #156, 0-2 cm	HMMV ATL19, 1-2 cm
Average aggregate size	3.6 ± 1.7 µm (n=390)	3.5 ± 1.5 µm (n=396)	2.6 ± 0.8 µm (n=440)	3.9 ± 1.8 µm (n=374)	4.1 ± 2.1 µm (n=305)
Average size of SRB	0.5 ± 0.1 µm (n=58)	0.5 ± 0.1 µm (n=70)	0.4 ± 0.1 µm (n=44)	0.5 ± 0.1 µm (n=61)	0.5 µm[a]
Average diameter of ANME-2	0.6 ± 0.1 µm (n=51)	0.6 ± 0.1 µm (n=44)	0.5 ± 0.1 µm (n=41)	0.7 ± 0.1 µm (n=46)	0.72 µm[b]
ANME2-538 & Eub338-I to III[d]	100% (n=136)	98% (n=127)	96% (n=141)	98% (n=143)	96%[c] (n=83)
ANME2-538 & DELTA495a/b/c[d]	100% (n=129)	96% (n=113)	97% (n=138)	100% (n=140)	100%[c] (n=80)
ANME2-538 & DSS658[d]	99% (n=248)	98% (n=234)	94% (n=265)	92% (n=240)	25%[c] (n=162)
ANME2-538 & SEEP1a-473[d]	95% (n=141)	85% (n=133)	75% (n=150)	82% (n=130)	23%[c] (n=157)
ANME2-538 & SEEP1a-1441[d]	87% (n=238)	87% (n=258)	88% (n=267)	77% (n=248)	8%[c] (n=148)
ANME2a-647 & SEEP1a-473[d]	95% (n=81)	100% (n=19)	88% (n=125)	100% (n=28)	n.d.
ANME2a-647 & SEEP1a-1441[d]	95% (n=57)	89% (n=27)	96% (n=122)	80% (n=25)	n.d.
ANME2c-760 & SEEP1a-473[d]	94% (n=48)	88% (n=92)	70% (n=50)	79% (n=126)	n.d.
ANME2c-760 & SEEP1a-1441[d]	91% (n=46)	91% (n=108)	63% (n=52)	71% (n=150)	n.d.

[a] Estimated value
[b] Diameter of ANME-3, value taken from Lösekann and coworkers [14]
[c] Based on aggregates labeled with probe ANME3-1249
[d] Aggregates labeled with the respective ANME-2 probe with a partner labeled with the respective secondary probe. Aggregates without partner were neglected
n.d. not determined

Table S6 Clones used for Clone-FISH

Probe	Match clone	Mismatch clone	Position of mismatch(es) (5'-3')	Remarks
SEEP1a-473	LARHR_58-01B08 (Acc. #: FN549970)	LARHR_26-01B04 (Acc #: FN549936)	7	No signals for match or mismatch clone!; HR enrichment culture used for determination of most stringent hybridization conditions
SEEP1a-1441	LARHR_26-01B04 (Acc #: FN549936)	*Actinosynema mirum* (DSMZ No.: 43827)	3	No mismatch clone available, therefore pure culture of *A .mirum* used as a mismatch control
SEEP1b-168	HydGH-Bac44 (unpublished)	-	-	No mismatch clone available; no signals for match clone, not even with helper probes
SEEP1b-468	HydGH-Bac44 (unpublished)	Hyd89-63 (Acc. # AJ535248)	1, 9	Only clone with 2 mismatches available as a control; no signals for match or mismatch clone
SEEP1c-1309	Black Sea BS-SR-B4 (unpublished)	LARHR_26-01B04 (Acc #: FN549936)	11	
SEEP1d-1420	LARIS_58-01B08 (Acc #: FN550053)	LARHR_58-01B08 (Acc. #: FN549970)	4	Good signals, but no clear distinction between match and mismatch clone, not even when adding a competitor probe
SEEP1e-203	Hyd89-63 (Acc #: AJ535248)	-	-	No mismatch clone available; no signals for match clone, not even with helper probes
SEEP1e-632	Hyd89-63 (Acc #: AJ535248)	LARHR_26-01B04 (Acc #: FN549936)	7, 10	Only clone with 2 mismatches available as a control; Good signals, but no clear distinction between match and mismatch clone, not even when adding a competitor probe
SEEP1f-153	HyGH-Bac5 (unpublished)	-	-	No mismatch clone available; stringency determined based on melting curve of match clone

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III. Diversity of adenosine-5'-phosphosulfate reductase and dissimilatory sulfite reductase in microbial communities mediating the anaerobic oxidation of methane

Contributions to this chapter:

Lars Schreiber, Friedrich Reinhard, Thomas Holler, Martin Krüger, Jan Küver, Marc Mussmann, Anke Meyerdierks, Rudolf Amann. Diversity of adenosine-5'-phosphosulfate reductase and dissimilatory sulfite reductase in microbial communities mediating the anaerobic oxidation of methane.

L.S.: developed the concept, constructed aprA and dsrAB clone libraries of samples HREnrLT and BSExt, analyzed aprA and dsrAB data set, conceived and wrote the manuscript; F.R.: developed the concept, constructed aprA and dsrAB clone libraries of samples BSBlack, BSPink, HRSed, and HREnrPr; T.H.: provided AOM enrichment cultures; M.K.: developed the concept; J.K.: developed the concept; M.M.: developed the concept; performed initial analysis of dsrAB sequences; A.M.: developed the concept, conceived and edited the manuscript; R.A.: developed the concept, conceived and edited the manuscript.

Diversity of adenosine-5'-phosphosulfate reductase and dissimilatory sulfite reductase in microbial communities mediating the anaerobic oxidation of methane

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ABSTRACT

Sulfate reducing bacteria (SRB) are associated with anaerobic methanotrophic archaea in consortia mediating the anaerobic oxidation of methane (AOM). Furthermore, a significant fraction of SRB is present as free-living cells at AOM sites. In this study, the diversity of SRB in AOM habitats, more precisely different microbial mats from the Black Sea, and enrichment cultures from the sediment above gas hydrates at Hydrate Ridge, was characterized by using the AprA and DsrAB genes as functional markers. The diversity of SRB was well covered by comparative analysis of 37-71 clones (≥ 92% coverage for Black Sea samples and Hydrate Ridge subjected to intermediate high, 15 mM, methane pressure; 79-86% coverage for Hydrate Ridge sediment kept at low, 1.5 mM, methane partial pressure). Within the Black Sea mat sections the diversity of SRB (2-8 retrieved OTUs) was comparable to the diversity in Hydrate Ridge enrichment cultures after high methane pressure incubation and considerably lower compared to Hydrate Ridge sediment kept at low methane concentration (14-19 OTUs). Clone libraries from Black Sea mats were dominated by sequences assigned to *Desulfobacteraceae*. Nonetheless, several different *Desulfobacteraceae* groups were detected in these mats and the SRB communities of the mat sections were heterogeneous with similarities scores of only 17-30%. Clone libraries from Hydrate Ridge enrichment cultures after high methane pressure incubation were also dominated by sequences related to *Desulfobacteraceae* (98-100% of the analyzed clones); also here a considerable diversity within this family was detected. Finally, many *Desulfobacteraceae* sequences were also retrieved from Hydrate Ridge sediment kept at low methane concentration. However, a *dsrAB* clone library from that sample was dominated by a group of deep-branching sequences (63% of analyzed clones) previously retrieved from other marine habitats and a salt marsh. With a fluorescence-activated cell sorting approach, the AprA of the yet uncultivated dominant partner of ANME-2, SEEP-SRB1a, was tentatively assigned to a sequence cluster within the *Desulfobacteraceae*.

INTRODUCTION

The anaerobic oxidation of methane (AOM) is the major sink for methane in marine sediments. It is proposed that AOM is present almost everywhere where sulfate meets methane in micromolar to millimolar amounts (Reeburgh, 2007). Sites where AOM has been observed range from diffusion controlled sulfate-methane transition zones (SMTZ) to cold seeps with high methane flux (for review see Knittel and Boetius, 2009). AOM is proposed to be mediated by microbial consortia of anaerobic methanotrophic archaea (ANME) and sulfate reducing bacteria (SRB) (Hoehler et al., 1994). The archaea involved in this process have been intensively studied within the past years in order to understand the biochemical pathways underlying AOM (e.g. Orphan et al., 2001a; Hallam et al., 2004; Meyerdierks et al., 2010). This led to the well supported hypothesis that methane is oxidized in a reversal of the methanogenesis pathway. However, the fate of the electrons derived from this reaction is still unknown. A range of intermediates for shuttling electrons to the SRB have been proposed but none could as yet be confirmed. SRB detected in association with ANME (ANME-1, ANME-2, or ANME-3) affiliate with the *Desulfosarcina/Desulfococcus* (DSS) or the *Desulfobulbus* group within the *Deltaproteobacteria* (Boetius et al., 2000; Knittel et al., 2005; Niemann et al., 2006). Recently, the SEEP-SRB1a group within the DSS has been identified to harbour the dominant partner of ANME-2 (Schreiber et al., 2010). Fluorescence *in situ* hybridization revealed for almost all analysed AOM samples that ANME associated SRB-lineages are only rarely present as single cells, pointing towards a high adaptation of these SRB groups to life in AOM mediating consortia (Lösekann et al., 2007; Schreiber et al., 2010). In addition to ANME-associated SRB a significant fraction of free-living SRB have been detected at AOM sites and several other, possibly methane seep-endemic, SRB clades have been defined (Orphan et al., 2001b; Knittel et al., 2003).

Sulfate reduction is one of the most important processes in the mineralisation of organic compounds, estimated to account for more than 50% of organic carbon

mineralization in anoxic marine habitats (Jørgensen, 1982; Canfield, 1989). SRB are a phylogenetically and metabolically very diverse guild. Most of the cultivated bacterial SRB affiliate with the class *Deltaproteobacteria*, but they have also been detected within other bacterial lineages and within the *Archaea* (e.g. Stetter, 1988; Itoh et al., 1998; Itoh et al., 1999). SRB grow on a variety of substrates (Rueter et al., 1994; Galushko et al., 1999; So and Young, 1999). They all have the capability to use sulfate as a terminal electron acceptor, but they do not necessarily depend on this electron acceptor for growth, and can e.g. grow by fermentation (for an overview see Rabus et al., 2006). Most of the SRB are free-living, sometimes growing in syntrophy with other microorganisms (Bryant et al., 1977). Because of their phylogenetic diversity the detection of SRB by comparative rRNA analysis is restricted to the groups of already known SRB. Moreover, this approach implies the extrapolation of metabolic capabilites from rRNA phylogeny, which can be misleading. Despite the phylogenetic diversity of SRB, only one dissimilatory sulfate-reduction pathway is as yet known. Thus, a more targeted approach for the detection of SRB is the study of key genes of the sulfate-reduction pathway as functional markers. Two genes often used for that purpose are *dsrAB*, coding for the dissimilatory sulfite reductase (Dsr), and the *aprA* gene, coding for the α-subunit of the dissimilatory adenosine-5'-phosphosulfate reductase (Apr). Both genes are highly conserved within the SRB and inferred phylogenies are, except for few cases of known horizontal gene transfer (Klein et al., 2001; Friedrich, 2002; Meyer and Kuever, 2007) congruent with 16S rRNA-based phylogenies. Within phylogenetic trees of DsrAB and AprA, groups of sequences such as deep branching DsrAB sequences still await an assignment to taxonomic groups (Thomsen et al., 2001; Dhillon et al., 2003). As yet few AOM samples from SMTZ were investigated with respect to the diversity of the functional marker DsrAB (Thomsen et al., 2001; Lloyd et al., 2006; Leloup et al., 2007; Harrison et al., 2009; Lloyd et al., 2010). No study to our knowledge targeted the AprA diversity in AOM samples.

In this study, SRB communities in AOM samples from different methane seep habitats were explored by assessing the diversity of AprA and DsrAB genes. Three samples from different areas within Black Sea microbial mats were investigated, one reflecting an ANME-1 hotspot. Moreover, ANME-2 dominated enrichment cultures from sediment above gas hydrates at Hydrate Ridge were analysed. The aim of the study was to expand our knowledge of SRB diversity associated with AOM based on functional markers instead of indirect evidence deduced from rRNA diversity. We conducted comparative sequence analysis to identify AOM specific SRB groups. Moreover, we attempted to identify groups within the AprA and DsrAB trees that harbour the AprA and DsrAB, respectively, of the dominant partner of ANME-2, SEEP-SRB1a. This was done by an approach combining fluorescence *in situ* hybridization (FISH), fluorescence-activated cell sorting (FACS), and multiplex PCR.

RESULTS

Construction of clone libraries and phylogenetic analysis

Black Sea microbial mat samples originated from two microbial reefs from the Lower Crimean shelf in the Black Sea. One was retrieved from the exterior (BSExt) of a microbial mat which was frozen upon sample retrieval (Arnds, 2009). Two represent the outer black (BSBlack) and the inner pink (BSPink) part, respectively, of a different reef. These samples were stored under anoxic conditions with 1.5 mM partial pressure of methane at 12°C prior to DNA extraction. Hydrate Ridge sediment samples (HRSed, HREnrPr and HREnrLT) represent enrichment cultures from surface sediment sampled above gas hydrates. HRSed (*H*ydrate *R*idge *sed*iment) represents the sediment sample stored at a methane partial pressure of 1.5 mM prior to DNA extraction. In contrast to that, sample HREnrPr (*H*ydrate *R*idge *enr*ichment, *pr*essure-incubated) was stored for *c*. 50 months at increased methane partial pressure of 15 mM to enrich for microorganisms involved in AOM (Nauhaus et al., 2002). Finally, HREnrLT (*H*ydrate *R*idge *enr*ichment, *l*ong-*t*erm) represents the same enrichment after being subjected to reduced pressures and a total incubation time of 84 months (Holler et al., 2009). A detailed overview of the samples used in this study is found in Table 1.

In total six *aprA* and six *dsrAB* clone libraries were established for the three Hydrate Ridge and the three Black Sea samples (for an overview see Table 2). For that, two different primer sets were used to amplify *aprA*. Forty five to 71 clones of the *aprA* libraries and 37 to 57 clones of the *dsrAB* libraries were analyzed (for more detail see Figure 1). Prior to phylogentic analysis deduced amino acid sequences, AprA and DsrAB, were grouped in operational taxonomic units (OTUs) based on a 90% amino acid identity threshold (species level; Kjeldsen et al., 2007). Statistical evaluation by rarefaction analysis of the OTUs (Sanders, 1968) and calculation of the homologous coverage rates (Singleton et al., 2001) showed that,

apart from HRSed, sufficient clones were analyzed to cover ≥92% of the AprA and DsrAB diversity in the libraries (Figure S1). For HRSed 86% and 79% of the AprA and DsrAB, respectively, diversity had been covered. An overview of the phylogenetic affiliation of the retrieved AprA and DsrAB sequences according to the groups proposed by Kaneko et al. (2007) for DsrAB is given in Figure 2. A more detailed phylogenetic analysis showed that OTUs fell into 22 AprA and 18 DsrAB groups which possessed a bootstrap support of more than 75% and shared inter-group identities of more than 70% (Figure 2 & 3).

Diversity of AprA and DsrAB sequences in Black Sea samples

The SRB diversity determined by AprA and DsrAB analysis revealed 6 and 8 OTUs, respectively, for BSBlack. Besides that, BSPink and BSExt exhibited a similar diversity for one of the functional markers investigated (AprA for BSExt: 7 OTU; DsrAB for BSPink: 5 OTU). However, the other functional marker pointed for both samples towards an extremely low diversity with only 1 or 2 OTUs (Table 2).

Most of the AprA sequences from the Black Sea libraries (158 of 160) affiliated with groups devoid of any reference sequence from cultured species (Figure 3). Of these, groups C, K, M, and R were the most prominent ones. Group K was the only one detected in all three samples and formed the dominant fraction in the libraries of sample BSBlack (65%) and BSPink (100%). Sequences affiliating with group C were detected in two samples (BSBlack: 5%; BSExt: 48%). The unaffiliated groups M and R were only detected among AprA from BSBlack or BSExt, respectively. There, they made up more than 20% of the clones.

All Black Sea samples contained DsrAB sequences affiliating with group I-a (2% to 70% clone frequency) within the *Desulfobacteraceae* (Figure 2). Additonal DsrAB sequences in this group originate from the Black Sea and Aarhus bay sediment (Figure 2). Moreover group I-b was shared between BSBlack (51%) and BSExt (92%). Both groups were assigned to the *Desulfobacteraceae* but featured no sequence from a cultured representative. Additionally, BSBlack and BSPink

shared sequences related to *Desulfobacterium anilinii* (group IV; BSBlack: 30%, BSPink: 2%) and the unaffiliated group X (BSBlack: 5%, BSPink: 2%). Other DsrA sequences retrieved from BSBlack fell within the *Desulfoarculaceae* (group IX; 12%). BSPink additionally contained sequences affiliating with *Desulfobacterium postgatei* (group I-g; 23%) within the *Desulfobacteraceae* and sequences affiliating with group VI (2%), a group with an unknown affiliation.

Table 1 Detailed description of samples n/a, not applicable

Sample	Sample description	Partial pressure of methane	Storage temp. / time	Average AOM rates (µmol gdw-1 d-1	Original source	Reference
HRSed	Sediment sample (1-3 cm depth), ANME-2 dominated	1.5 mM, 1 bar	12°C / -	8	Sediment in area of active gas seepage; Southern Hydrate Ridge; NE Pacific	Linke and Suess, 2001; Nauhaus et al., 2002
HREnrPr	Pressure incubated enrichment of the HRSed sample, ANME-2 dominated	15 mM, 80 bar	12°C / 50 months	230	*see HRSed*	Nauhaus et al., 2002
HREnrLT	Long-term incubated enrichment of the HRSed sample, ANME-2 dominated	3 mM, 3 bar	12°C / 84 months	300	*see HRSed*	Holler et al., 2009
BSExt	Exterior microbial mat; ANME-1 dominated	n/a	n/a	330	Microbial reef structure (reef 822) at cold, anoxic methane seep; Paleo-Dnepr area, Black Sea	Arnds, 2009
BSPink	Pink, interior microbial mat, ANME-1 dominated	1.5 mM, 1 bar	12°C / -	31	Microbial reef structure at cold, anoxic methane seep; Lower Crimean shelf, Black Sea	Michaelis et al., 2002
BSBlack	Grey-black, exterior microbial mat, ANME-1 dominated	1.5 mM, 1 bar	12°C / -	45	*see BSPink*	

Table 2 Description of clone libraries AA, amino acid based

Clone library	Source	Amplicon primers	No. of analyzed clones	OTUs / Chao1 (AA, 90% cut-off)
AprA libraries				
HRSed	Hydrate Ridge sediment	AprA-1-FW / AprA-5-RV	49	14 / 22
HREnrPr	Pressure incubated enrichment of HRSed sample	AprA-1-FW / AprA-5-RV	71	2 / 2
HREnrLT	Long-term Hydrate Ridge enrichment	AprA-1-FW / AprA-10-RV-deg	45	6 / 7
BSExt	Exterior of Black Sea microbial mat, ANME-1 dominated	AprA-1-FW / AprA-10-RV-deg	48	7 / 7
BSPink	Interior of Black Sea microbial mat, pink fraction	AprA-1-FW / AprA-5-RV	46	1 / 1
BSBlack	Exterior of Black Sea microbial mat, grey-black fraction	AprA-1-FW / AprA-5-RV	66	6 / 6
DsrAB libraries				
HRSeI-D	Hydrate Ridge sediment	DSR1F / DSR4R	51	19 / 36
HREnrPr-D	Pressure incubated enrichment of HRSed sample	DSR1F / DSR4R	37	5 / 8
HREnrLT-D	Long-term Hydrate Ridge enrichment	DSR1F / DSR4R	45	7 / 8
BSExt-D	Exterior of Black Sea microbial mat, ANME-1 dominated	DSR1F / DSR4R	48	2 / 2
BSPink-D	Interior of Black Sea microbial mat, pink fraction	DSR1F / DSR4R	48	5 / 8
BSBlack-D	Exterior of Black Sea microbial mat, grey-black fraction	DSR1F / DSR4R	57	8 / 8

Diversity of DsrAB and AprA sequences in the Hydrate Ridge samples

The AprA and DsrAB diversity was always highest for the Hydrate Ridge sediment sample kept at 1.5 mM methane (Table 2), with 14 (AprA) and 19 (DsrAB) predicted OTUs for HRSed compared to 2-7 OTUs for the enrichment cultures HREnrPr and HREnrLT at or after incubation at high methane concentration. The diversity within HREnrPr and HREnrLT was in the same range (5-7 OTU) except for HREnrPr AprA diversity (2 OTUs).

All three Hydrate Ridge samples contained AprA sequences affiliating with the to the *Desulfobacteraceae* assigned group J (33% to 99% clones frequency). No other group comprised sequences of all three Hydrate Ridge samples. Whereas both enrichment samples exclusively contained *Desulfobacteraceae*-affiliated AprA sequences, a significant part of the sequences amplified from HRSed affiliated with those of sulfur-oxidizing bacteria (18%) or were assigned to completely unaffiliated groups (group R, S, and T; 39%) (Figure 1).

Regarding DsrAB diversity, sequences from all samples were present in group I-e (2% to 7% of clones). *DsrAB* sequences amplified from HREnrPr and HREnrLT contained, except for one *Desulfobulbaceae*-assigned sequence (group VII), only sequences of *Desulfobacteraceae* affiliated groups (group I). *Desulfobacteraceae* affiliated DsrAB sequences of sample HREnrPr were either closely related to *Desulfobacter postgatei* (group I-g, 57%) or grouped together with sequences, to our knowledge exclusively detected in the present study (group I-b; 35%). *D. postgatei* related sequences and sequences of group I-b were also retrieved from sample HREnrLT. Here they made up 30% and 2% of the total number of analyzed clones, respectively. The dominant fraction of DsrAB sequences of sample HREnrLT (42%) affiliated with sequences of the genus *Desulfosarcina* (group I-c). In contrast, the majority of DsrAB sequences of HRSed (69%) affiliated with deep-branching sequences of group V. Sediment sequences within

this group, fell into three different clusters which indicated an origin from different phylotypes.

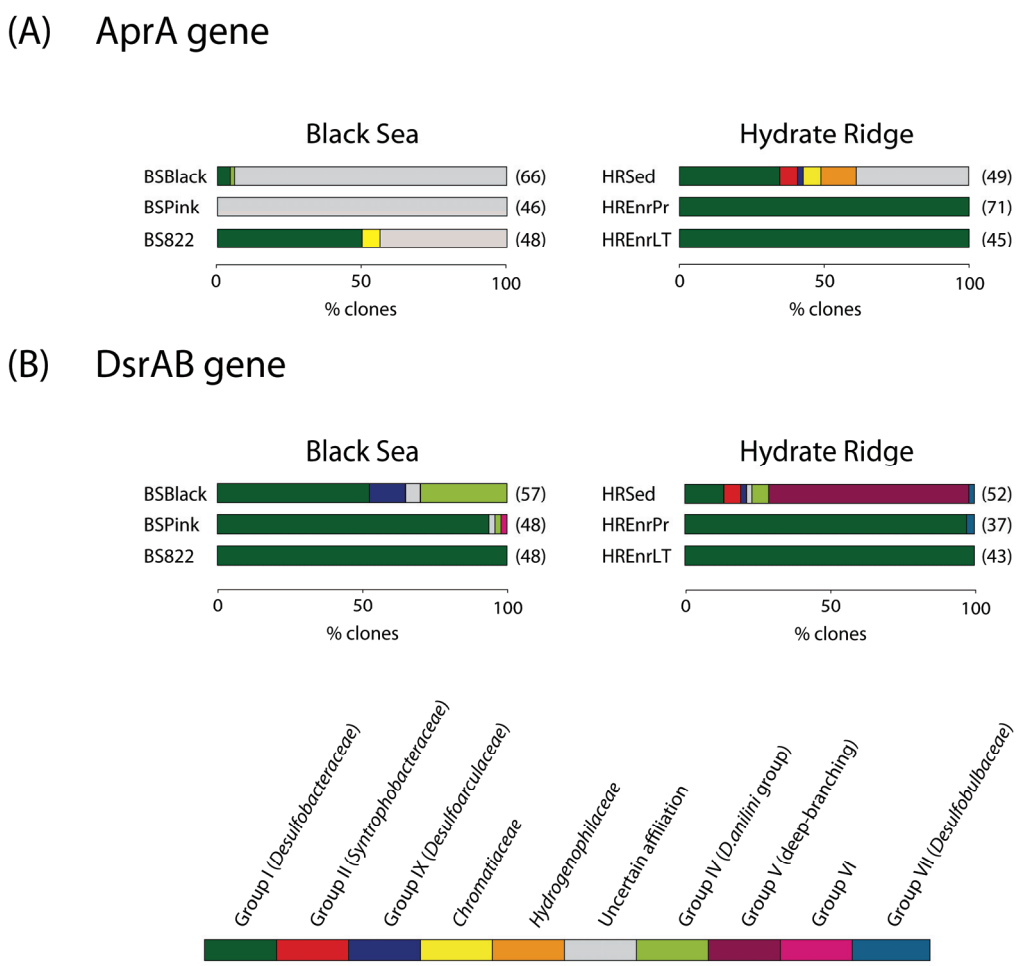

Figure 1 Frequencies and phylogenetic affiliation of AprA und DsrAB sequences retrieved from different AOM habitats. Shown is the percentage of clones obtained from each group in the color coded bar graph legend. Numbers in parentheses are the total number of clones analyzed per library. Groupings are based on those proposed by Kaneko et al. (2007) for DsrAB and are depicted in Figure 2 and 3

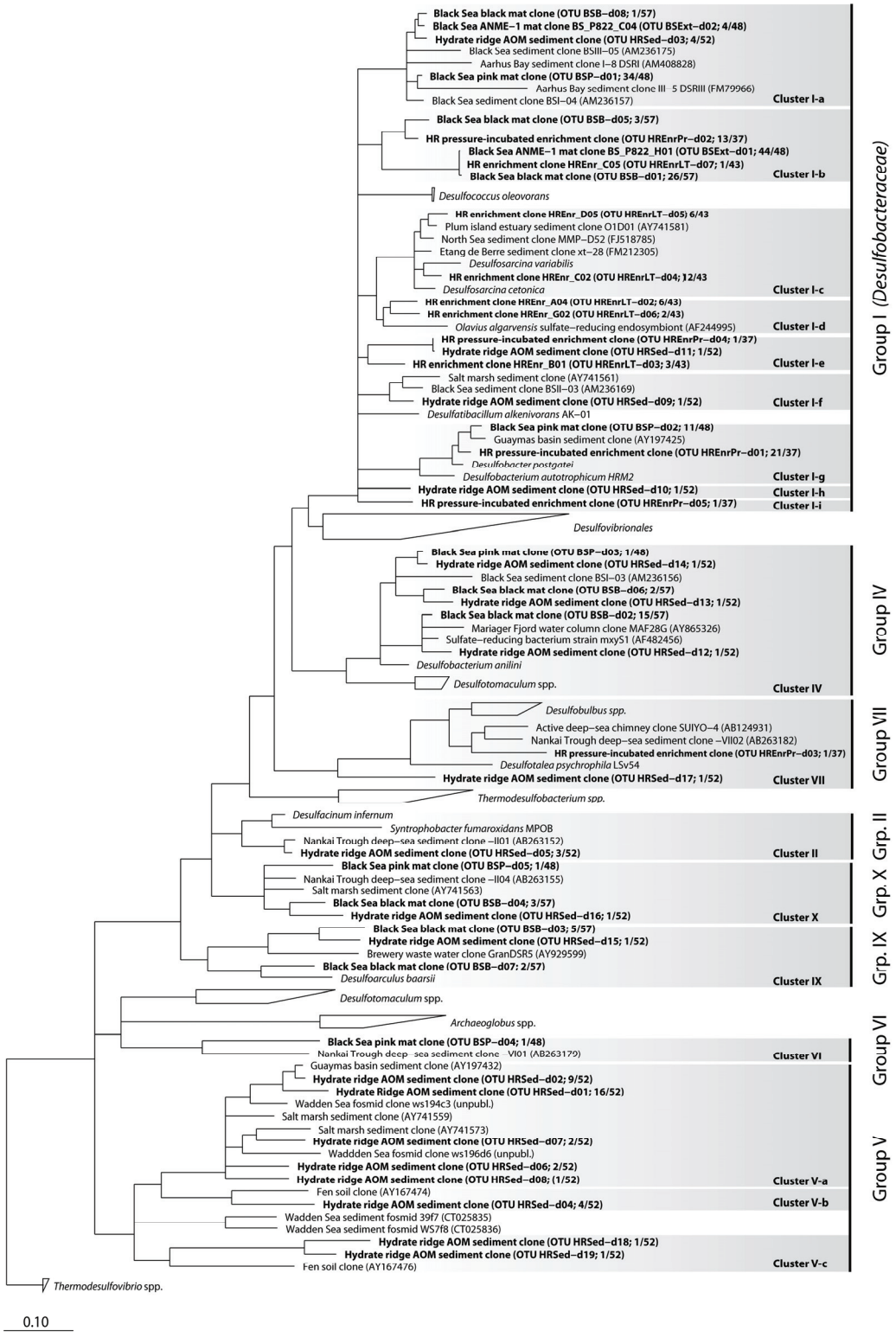

Figure 2 Phylogenetic consensus tree showing the affiliation of DsrAB sequences retrieved from different AOM samples. Shown is the affiliation of OTUs (90% amino acids identity cut-off) from Hydrate Ridge sediment, and Black Sea mat samples (boldface type) to selected reference sequences. Nodes not observed in all of the treeing methods (distance matrix, maximum parsimony, and maximum likelihood) are shown as multifurcations. Groups of OTUs …

Figure 2 continued. ... with bootstrap support >75% and inter-group DsrAB identities of >70% were categorized in different phylogenetic groups (shaded in grey). Labels to the right indicate the phylogenetic affiliation of the groups. Bar: 10% estimated sequence divergence as inferred from the maximum likelihood method.

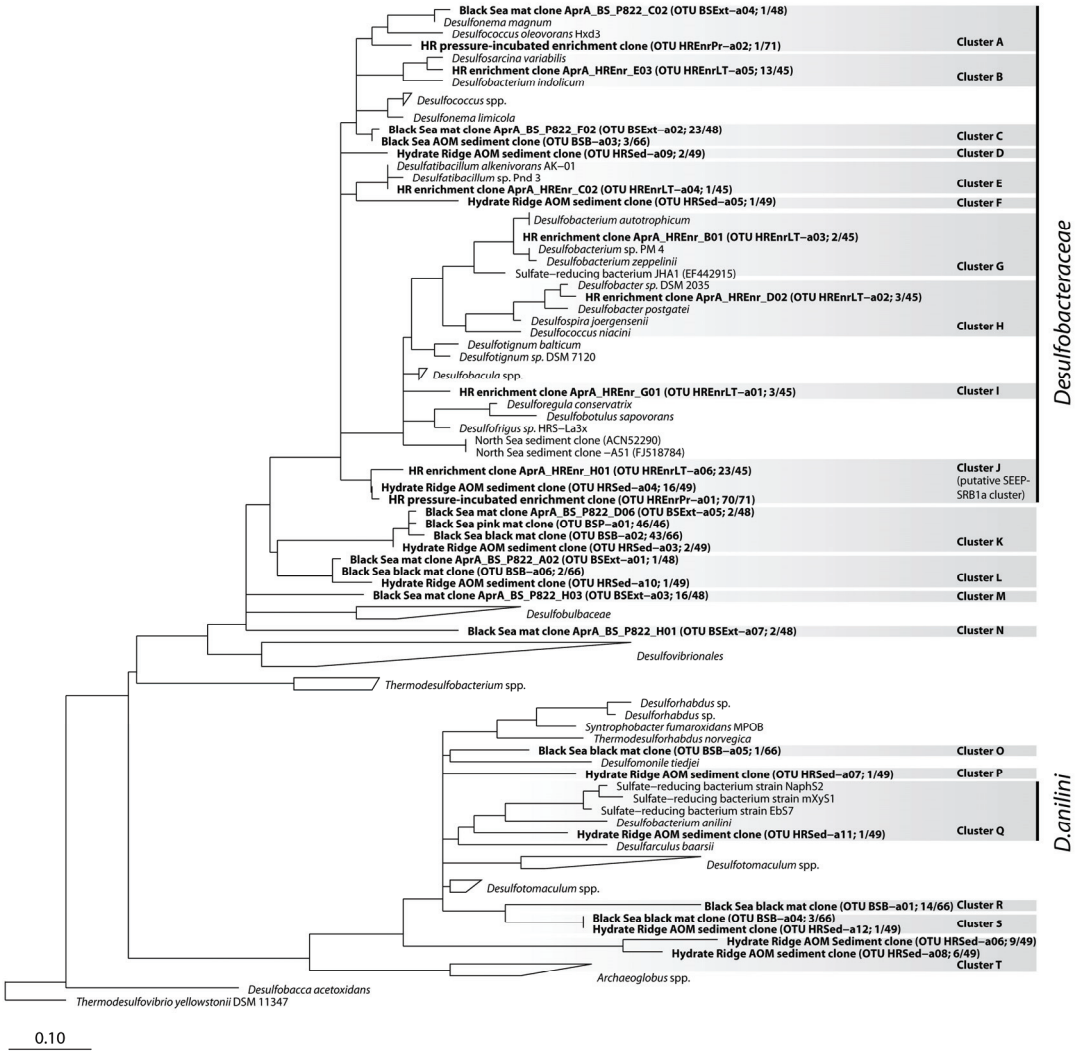

Figure 3 Phylogenetic consensus showing the affiliation of AprA sequences retrieved from different AOM samples. Shown are relationships of OTUs (90% amino acids identity cut-off) from Hydrate Ridge sediment, and Black Sea mat samples (boldface) to selected reference sequences. Nodes not observed in all of the treeing methods (distance matrix, maximum parsimony, and maximum likelihood) are shown as multifurcations. Groups of OTUs with bootstrap support >75% and inter-group AprA identities of >70% were categorized in different phylogenetic groups (shaded in grey). Labels to the right indicate the phylogenetic affiliation of the groups. Groups without a label have not been assigned to any phylogenetic group. Bar: 10% estimated sequence divergence as inferred from the maximum likelihood method.

Similarity between AOM samples

The different AOM samples were compared based on the presence or absence of AprA and DsrAB sequence clusters (Figure 2 & 4; Figure S1 & S2). Cluster analysis (Table S6, Figure S4) showed that the highest similarities were found between the pink (BSPink) and black (BSBlack) mat samples from the Black Sea as well as between the two Hydrate Ridge enrichment samples that had been subjected intermediately to 15 mM methane (HREnrPr and HREnrLT). None of the AprA or DsrAB sequence clusters were detected in all samples. Moreover, only 5 out of 22 AprA groups and 6 out of 18 DsrAB groups contained sequences from Hydrate Ridge and Black Sea mats. In each case, four of these matches could be attributed to sequences from the more diverse HRSed sample, covering a large set of phylogenetic groups. DsrAB affiliating with deep branching groups (group V) were exclusively retrieved from HRSed.

Identification of AprA and DsrA genes of SEEP-SRB1a

A combination of catalyzed-reporter deposition fluorescence *in situ* hybridization (CARD-FISH; Pernthaler et al., 2002), fluorescence-activated cell sorting (FACS), and multiplex PCR was employed to identify the *aprA* and *dsrA* sequences of SEEP-SRB1a, the dominant deltaproteobacterial partner of ANME-2 (Schreiber et al., 2010). CARD-FISH was performed using a SEEP-SRB1a specific probe on samples of the Hydrate Ridge long term enrichment (HREnrLT). In total *c*. 1,000,000 SEEP-SRB1a cells were isolated from HREnrLT. Visual inspection of the sorted cells by fluorescence microscopy revealed ≥95% cells with a fluorescent label. A mixture of primers targeting bacterial 16S rRNA genes as well as AprA and DsrA genes was used for multiplex PCR on aliquots of *c*. 150,000 SEEP-SRB1a cells. Whereas no DsrA amplification product could be obtained, 16S rRNA and AprA gene fragments could be successfully amplified. Control reactions using FACS sheath fluid only yielded 16S rRNA amplification products. Amplification products were cloned and 18 to 23 of the obtained clones were sequenced. All retrieved AprA sequences fell into cluster J within the

Desulfobacteraceae. Phylogenetic analysis of 16S rRNA clones originating from sorted cells did not yield a single SEEP-SRB1a sequence. The obtained sequences were similar to those amplified from the sheath fluid negative control (see Appendix Table S7).

DISCUSSION

SRB in microbial mats from the Black Sea

This study is the first to investigate the diversity of SRB in microbial mats in the Black Sea based on functional marker genes, *aprA* and *dsrAB*. Mat samples investigated in this study included an exterior, ANME-1 dominated part (BSExt) of a microbial mat from one reef. Two represent the outer black (BSBlack) and the inner pink (BSPink) part, respectively, of a microbial mat on a different reef. These samples had not been investigated for 16S rRNA diversity and microbial abundance in parallel. In previous studies, black mat sections were shown to be mostly dominated by anaerobic methanotrophs of the ANME-2 clade (A. Meyerdierks, personal communication), while pink mat sections were dominated mostly by archaea from the ANME-1 clade (Krüger et al., 2003; Meyerdierks et al., 2005). However, mat sections differ considerably in respect to their archaeal composition and this assignment does not always hold true (Knittel et al., 2005; Arnds, 2009).

The currently only other study to investigate SRB communities in microbial mats of the Black Sea used a set of oligonucleotide probes to assess the presence of SRB groups by fluorescence *in situ* hybridization (Treude, 2003). This previous study showed that also SRB communities in these microbial mats can be highly heterogeneous and strongly depend on what section of the mat is sampled. Although the sampling size in the present study was low and different DNA extraction protocols and primer sets targeting the AprA gene were used to assess SRB diversity in the Black Sea mat samples, a statistical analysis was attempted. Corresponding to the study by Treude (2003), the similarity between the SRB communities of the three studied mat sections was, with 17-30%, low (Table S6). In spite of the low similarity (30%), cluster analysis indicated a clustering of the black and pink mat sections. The supposedly ANME-1 dominated pink mat section and the ANME-1 dominated exterior mat section showed a similarity of only 17% (Table S5); the lowest similarity score for the Black Sea samples. This

result suggests that the heterogeneity between different microbial reefs in the Black Sea has a bigger influence on the SRB community than zonation within a mat at a given site.

DsrAB clone libraries indicated a dominance of *Deltaproteobacteria* from within the *Desulfobacteraceae* in all mat samples. Even though most of the corresponding AprA sequences showed an uncertain affiliation, the phylogenetic position of these sequences also suggests a position within the *Desulfobacteraceae.* Only the *dsrAB* clone library of the black mat sample contained notable clone numbers of other SRB-groups. In this library, a significant number of sequences related to *Desulfobacterium anilini* as well as sequences from within the *Desulfoarculaceae* were detected. Since both groups are also positioned within *Deltaproteobacteria*, our data suggest that bacteria from within the *Deltaproteobacteria* dominate the sulfate-reducing communities of the studied Black Sea mats.

The detection of *Desulfoarculaceae* sequences confirms an earlier study which also reported the presence of *Desulfoarculus*-related bacteria in the black section of a microbial mat from the Black Sea (Treude, 2003). Sequences from the *D. anilini* group (group IV) were previously also retrieved from the anoxic water column and sediments from the Black Sea (Vetriani et al., 2003; Leloup et al., 2007) as well as from other marine AOM habitats (Kaneko et al., 2007; Harrison et al., 2009; Lloyd et al., 2010; this study). Cultivated members of the *D. anilini* group are characterized by their ability to couple dissimilatory sulfate reduction to the oxidation of aromatic hydrocarbons and other organic substrates (Harms et al., 1999; Kniemeyer et al., 2003). Thus, based on these cultivated representatives, bacteria from the *D. anilini* group are hypothesized to utilize complex sources of carbon associated with cold seeps (Harrison et al., 2009). Interestingly, *Desulfobacteraceae*, *Desulfoarculaceae*, and the *D. anilini* group all share the ability to oxidize their substrates completely to CO_2, suggesting that this capability is of advantage when thriving in the Black Sea mats.

SRB in Hydrate Ridge samples

The second set of samples investigated in this study included different enrichment cultures from the surface layer of sediment sampled above gas hydrates at Hydrate Ridge. The samples were enriched in ANME-2, with a dominance of ANME-2c over ANME-2a consortia (Nauhaus et al., 2002; Holler et al., 2009).

Of these samples, Hydrate Ridge sediment sample kept at low methane concentration (HRSed) featured a diversity of SRB more than twice as high as those of the enrichment cultures after high methane pressure (HREnrPr and HREnrLT). This indicates a selection for specific SRB during the enrichment. More precisely, while Hydrate Ridge sediment kept at low methane concentration still featured SRB from many different clades, both enrichment cultures were dominated by *Desulfobacteraceae* sequences. Since the *Desulfobacteraceae* also harbor the DSS-partner of ANME-2, this result confirms studies which reported a dominance of ANME-2/DSS aggregates in the used enrichments cultures (Nauhaus et al., 2002; Holler et al., 2009). Even though, mostly *Desulfobacteraceae* sequences were retrieved from the enrichment cultures HREnrPr and HREnrLT, we detected a surprising diversity of sequences within this group. Even after an incubation time of 84 months, 6 AprA and 5 DsrAB clusters within the *Desulfobacteraceae* were detected in the long-term enrichment HREnrLT. This observed diversity is in agreement with an earlier study which also detected a diversity of 16S rRNA gene sequences from within the *Desulfobacteraceae* in the long-term enrichment (HREnrLT; Schreiber et al., 2010). We speculate that this diversity was present, either because the enrichment procedure does not apply sufficient selection pressure to purge all but one *Desulfobacteraceae* cluster or because the slow growth of microorganisms in the enrichment (*c*. 6 months generation time) did not allow for the emergence of a single winning genotype yet. The SRB communities of the two enrichment cultures HREnrPr and HREnrLT were remarkably different and only shared a similarity of 29% (Table S6). This low similarity may be attributed to technical variations during clone library construction. However, another possible

explanation for this observation are different growth conditions for the enrichment cultures. While the HREnrPr enrichment culture was incubated with methane under high pressure, the other (HREnrLT) was incubated under reduced pressure combined with a shaking protocol. Even though the different enrichment procedures seemingly did not have an effect on the dominance of ANME-2/DSS aggregates in the enrichments (Nauhaus et al., 2002; Holler et al., 2009), their effect on the sulfate-reducing community is not clear.

Although a previous study confirmed the presence of *Desulfobulbus*-related 16S rRNA sequences in the long term enrichment (Schreiber et al., 2010), AprA or DsrA sequences affiliated with the *Desulfobulbaceae* group, were only detected within Hydrate Ridge sample HRSed, and the pressure-incubated Hydrate Ridge enrichment culture. Whether such sequences remain to be detected in the long term enrichment, or belong to *Desulfobulbus*-species with AprA or DsrA from a group as yet not assigned to *Desulfobulbus* remains to be determined. *Desulfobulbus*-related bacteria were also reported as being an alternative partner of ANME-2 archaea which could explain their presence in the three Hydrate Ridge samples. A future study employing FISH or even GeneFISH (Moraru et al., 2010) might be useful to test this hypothesis.

The majority of DsrAB sequences retrieved from Hydrate Ridge sediment affiliated with a cluster of deep-branching sequences that have been previously retrieved from other marine environments such as Guaymas Basin (Dhillon et al., 2003), salt marsh (Bahr et al., 2005), and the Wadden Sea (Mußmann et al., 2005). Due to different tree topologies we were not able to identify a corresponding AprA cluster. The whole group of deep-branching sequences does not contain a single cultivated representative so its phylogenetic affiliation remains unclear. The function of the corresponding microorganisms in the Hydrate Ridge sediment is currently similarly unclear.

Comparison of SRB diversity in Hydrate Ridge and Black Sea samples

A comparison between the studied AOM samples showed that the diversity of SRB in the Black Sea mats was in the same range as the diversity within the Hydrate Ridge enrichment cultures after high methane partial pressure (Table 2). AprA and DsrAB sequences from these samples only contained 1-8 OTUs. In contrast to that, the diversity of SRB within Hydrate Ridge sediment which was kept at low methane concentration was much higher (14-19 OTUs, Table 2). Possible explanations for this observation are the limited availability of potential substrates for SRB within the microbial mats as well as the selection for specific SRB groups by high methane partial pressure. Sediments at Hydrate Ridge experience an input of diverse pelagically-derived organic matter that likely supports growth of a variety of SRB not directly or indirectly involved in AOM, such as those of deep-branching DsrAB groups. Likely, some of these groups did not grow at or even survive high methane concentrations in culture. Whether this was due to the high methane concentration itself or due to other parameters that changed during this enrichment step, e.g. sulfide concentrations or the dilution of organic matter, remains to be determined. In contrast, Black Sea microbial mats seem to be entirely fed by the AOM process. Thus, SRB within the inner mats and the enrichments cultures are hypothesized to be either directly involved in AOM or to be specialized in degrading AOM-derived biomass.

AprA and DsrAB of ANME associated sulfate-reducing bacteria

Bacteria related to the *Desulfosarcina/Desulfococcus* (DSS) group were shown to form aggregates with anaerobic methanotrophs of the ANME-1 and ANME-2 clades (Boetius et al., 2000; Michaelis et al., 2002). In addition, a recent study narrowed the identity of the main ANME-2 partner down to the SEEP-SRB1a group, a subgroup within the DSS group (Schreiber et al., 2010). Since ANME/DSS aggregates catalyze the AOM process, DSS-related bacteria usually form the dominant sulfate-reducing fraction in most AOM habitats (Michaelis et

al., 2002; Knittel et al., 2003; Omoregie et al., 2009). Unfortunately, due to the abundance and ubiquitous presence of free-living DSS-related bacteria in AOM habitats (for a review see Knittel and Boetius, 2009), it was so far not possible to assign sulfate-reduction genes to the DSS-partners of ANME-1 or ANME-2.

An analysis of the *aprA* clone libraries of Hydrate Ridge sediment and the Hydrate Ridge enrichment cultures showed a dominance of clones from AprA group J in all three libraries. Since ANME-2/DSS aggregates dominated the two Hydrate Ridge enrichment cultures (Nauhaus et al., 2002; Holler et al., 2009), this suggested that AprA group J harbours sequences from SEEP-SRB1a, the main DSS-partner of ANME-2. This conclusion was confirmed by retrieving AprA sequences from cluster J from SEEP-SRB1a cells isolated by fluorescence-activated cell sorting (FACS).

In case of the DsrAB gene, no clear picture emerged. Even though, one DsrAB cluster was detected in all three Hydrate Ridge datasets, the low abundance of sequences affiliating with this cluster makes an origin from the SEEP-SRB1a group unlikely. In addition, unfortunately no *dsrAB* amplicons were obtained from FACS-isolated SEEP-SRB1a cells. We assume that this failure was caused by DNA damage of the sorted cells induced by the applied formaldehyde fixation (Chang and Loew, 1994; Bucklin and Allen, 2004) and by radicals generated during the CARD-FISH procedure (Pernthaler et al., 2002; Demple and Harrison, 2003). This may have compromised the amplification of long fragments (DsrAB: 1,000 bp; 16S rRNA: 585 bp) whereas the amplification of the shorter AprA fragment (395 bp) was still successful. In this respect, the inability to amplify 16S rRNA sequences of SEEP-SRB1a from the sorted cells may be attributed to the preferred amplification of contaminating DNA present in the sampling line, over formaldehyde fixed SEEP-SRB1a DNA. Future studies employing similar approaches should employ alternative fixation methods and a different method of cell identification. In this respect, a recently reported fixation-free FISH protocol which does without the CARD amplification step (Yilmaz et al., 2010) provides a good starting for future method development.

Based on the analysis of lipid biomarkers, Niemann and Elvert (2008) concluded a significant difference between the ANME-1 and ANME-2 associated DSS-partners. This hypothesis is supported by the absence of the putative AprA cluster of the ANME-2 partner SEEP-SRB1a in the ANME-1 dominated Black Sea sample (BSExt). Based on clone abundances, the AprA of the DSS-partner of ANME-1 may belong to cluster K; the dominant AprA cluster in clone libraries from the black and pink mat sections as well as the only one present in all Black Sea samples.

CONCLUSION

We certainly realize the limitations of this PCR-based study. Clone frequency information should at best be treated semi-quantitatively. In addition, the samples of this study were very different in texture and we used different DNA extraction protocols. Yet, this study is an important step to increase the knowledge about SRB in AOM habitats as it provides the first assessment of sulfate-reducing communities in AOM-active Black Sea mats and Hydrate Ridge sediment based on AprA and DsrAB genes. Some of the detected groups were retrieved from multiple samples which suggests an important role of the corresponding SRB in the studied AOM habitats. However, similar to previous studies of SRB in AOM habitats (Orphan et al., 2001b; Thomsen et al., 2001; Knittel et al., 2005; Harrison et al., 2009), many of the phylogenetic branches contained no cultivated representatives and therefore may represent new, still uncharacterized species or even genera with unknown physiological properties. Thus, in order to better understand the ecology of SRB in AOM habitats, future studies should focus on the characterization and cultivation of these groups of SRB. Additionally, the detection of multiple *Desulfobacteraceae* groups in AOM enrichment cultures showed that the AOM process on its own is capable of sustaining a diverse SRB community even without the input of external organic matter.

EXPERIMENTAL PROCEDURES

Sample description

Black Sea microbial mat samples originated from microbial reef structures at cold anoxic methane seeps on the lower Crimean shelf. Sample BSExt was collected during a research cruise of RV POSEIDON (POS317-3) at reef 822. The studied site was located at 191 m water depth at the position 44°47′N, 31°59′E. The sample represents the exterior, black part of a microbial mat (Arnds, 2009), which was frozen upon retrieval until further processing. Sample BSBlack and BSPink were collected during a research cruise of RV Prof. LOGACHEV. The study site was located at 230 m water depth at the position 44°46′N, 31°60′E and is described in more detail by Michaelis et al. (Michaelis et al., 2002). Samples were transferred into glass bottles, sealed with butyl-rubber stoppers and stored at 12°C under an atmosphere of methane or nitrogen. In the home laboratory they were stored in anoxic artificial seawater (Widdel and Bak, 1992) under partial pressure of methane (1.5 mM).

Hydrate Ridge sediment was collected during a research cruise of RV SONNE (SO148-1) at the Crest of southern Hydrate Ridge off the coast of Oregon in July 2000 (Linke and Suess, 2001). The study site was located at 777 m water depth at the position 44°34′N, 125°09′W. The sample represents the surface layer (1-3 cm) of *Beggiatoa* mat covered sediments above gas hydrates (Nauhaus et al., 2002). The sample was stored anoxically in glass bottles (250 ml) without headspace at 5°C until further processed in the laboratory. While HRSed was kept at 1.5 mM methane concentration, HREnrPr represents the enrichment culture during the high pressure incubation stage (15 mM methane, 80 bar, 50 months incubation time) as described by Nauhaus et al. (2002) HREnrLT represents the HREnrPr enrichment after being subjected to reduced pressures and an incubation time of 84 months (Holler et al., 2009).

DNA extraction, PCR amplification, clone library construction, and sequencing

Two-hundred milligrams of Hydrate Ridge sediment (samples HRSed and HREnrPr) and 450 mg of Black Sea microbial mat (BSPink and BSBlack) were extracted using the FastDNA Spin Kit for soil (Q-Biogene, Carlsbad, USA). DNA of sample HREnrLT and BSExt was extracted as described by Schreiber et al. (2010) and Arnds (2009), respectively. The extracted material of samples HRSed, HREnrPr, and BSBlack, was subjected to a second round of extraction to obtain DNA suited for PCR amplification.

Primer sequences used for PCR amplification of *dsrAB* and *aprA* fragments are shown in Table S2. The primer set AprA-1-FW/AprA-5-RV (Meyer and Kuever, 2007) was used for amplification of *aprA* fragments of samples HRSed, HREnrPr, BSBlack, and BSPink. For samples HREnrLT and BSExt, primer set AprA-1-FW/AprA-10-FW-deg was used. The *dsrAB* fragments of all samples were amplified with the primer set DSR1F/DSR4R (Wagner et al., 1998). The PCR was performed in a Mastercycler Gradient (Eppendorf, Germany) in a 50 µl reaction volume. Each PCR reaction contained: 0.5 µM of each primer, 200 µM of each deoxyribonucleoside triphosphate, 15 µg bovine serum albumin, 1× PCR buffer (5Prime, Germany), 1.25 – 2 U *Taq* DNA polymerase, and 5-100 ng of template DNA. *Taq* polymerase used for PCR amplification of samples from BSBlack, BSPink, HRSed, and HREnrPr was produced in-house (Max Planck Institute for Marine Microbiology, Bremen, Germany), while *Taq* polymerase used for samples BSExt and HREnrLT was purchased from 5Prime (Germany). AprA gene fragments were amplified using the 'touchdown' PCR protocol described by Meyer and Kuever (2007). The initial annealing temperature for the touchdown interval (10°C) was 58°C.

PCR with primer set DSR1F/DSR4R was performed with two protocols. For samples HRSed, HREnrPr, BSBlack, and BSPink, the amplification included 30 cycles with each cycle consisting of 60 s at 96°C, 60 s at the annealing

temperature, and 120 s at 72°C. The reaction was completed by a final extension at 72°C for 10 min. For samples HREnrLT and BSExt, PCR was performed with an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 30 s at 94°C, 30 s at the annealing temperature, 90 s at 72°C, and a final extension of 10 min at 72°C. Amplification was performed at annealing temperatures of 57°C for BSBlack, 56°C for BSPink, 54°C for HRSed and HREnrPr, and 52°C for BSExt and HREnrLT.

Amplification products resulting from the PCR of *dsrAB* were purified by agarose (1%) gel electrophoresis. Bands of the expected size were excised from the agarose gel and either purified with the Perfect Gel Clean-up Kit (Eppendorf, Germany; samples HRSed, HREnrPr, BSBlack and BSPink) or melted for 10 min at 50°C (HREnrLT and BSExt) prior to cloning. Amplification products of the *aprA* amplification were either gel purified with subsequent agarose melting as described above (HREnrLT and BSExt) or used directly for cloning (samples HRSed, HREnrPr, BSBlack and BSPink). Amplification products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Germany) according to the manufacturer's recommendations. Full length insert sequences were determined by Sanger sequencing either on an ABI377 sequencer (Applied Biosystems, USA) or at the GATC Biotech AG (Germany). Only for the *dsrAB* clone library from HREnrLT and BSExt single reads were clustered into OTUs on a 90% amino acid identity level using the *mothur* software package (Schloss et al., 2009), and full length insert sequences were determined for one reference clone for each OTU.

Phylogenetic Analysis

All obtained *aprA* and *dsrAB* sequences were aligned and translated in amino acids by using the ARB software package (Ludwig et al., 2004). For phylogenetic analyses, deletions and highly variable regions were removed by using a 30% positional conservatory filter. The filter for the DsrAB alignment left a total of 483 amino acid positions (292 positions for the α subunit, and 191 positions for

the β subunit), while the filter for the AprA alignment left a total of 351 amino acid positions.

Distance matrix (DM; using neighbor joining with global rearrangements and randomized input order of species) and maximum parsimony (MP) trees were calculated using the PHYLogeny Inference Package version 3.65 (Felsenstein, 2005). The robustness of the DM and MP trees was tested by bootstrap analysis with 1,000 resamplings using the Seqboot, ProtDist (Kimura distance), ProtPars, and Consense programs of PHYLIP. Maximum likelihood (ML) trees were calculated by using RaxML 7.04 (Stamatakis et al., 2008) as provided by the CIPRES cluster at the San Diego Supercomputing Center (http://www.phylo.org/). The robustness of the ML trees was tested by bootstrap analysis with 100 resamplings. Prior to ML analysis, the best suited model of protein evolution was determined to be the WAG-model by using ProtTest (Abascal et al., 2005). A consensus tree based on the maximum likelihood tree was drawn in which branches were collapsed that were not observed in all three trees. Groups of OTUs with bootstrap support >75% and inter-OTU identities of >70% were categorized in different phylogenetic clusters.

Rarefaction analysis, Chao1 estimation, similarity analysis

Diversity of the clone libraries was investigated by rarefaction analysis. Rarefaction curves were produced applying the analytical approximation algorithm of Hurlbert (1971) and 95% confidence intervals estimated as described by Heck et al. (1975).Calculations were based on OTU's formed with a 90% amino acids identity cut-off and the software program *aRarefactWin* version1.3 (Holland, 2003). The absolute number of AprA- and DsrAB-OTUs in the samples was estimated by calculating the Chao1 diversity estimate (Chao, 1984) with the software EstimateS version 8.2 (Colwell, 2009). Cluster analysis to compare the sulfate-reducing communities of the AOM habitats were carried out with the software program PAST (PAlaeontological Statistics, ver. 2.0, http://folk.uio.no/ohammer/past/). Calculations were based on the presence or

absence of AprA and DsrAB clusters detected during phylogenetic analysis and the corresponding Jaccard distance between the sites.

Identification of SEEP-SRB1a genes

Catalyzed reporter deposition (CARD)-FISH was performed as described elsewhere (Pernthaler et al., 2002) with the following modifications:. Subsamples of Hydrate Ridge cultures were homogenized with a Dounce type glass homogenizer (tight, gap size 25.4 – 76.2 µm). Samples were afterwards fixed for 1 h in 1% formaldehyde, washed with 1× phosphate buffered saline (130 mM NaCl, 10 mM sodium phosphate; pH 7.4; PBS), and finally stored in 1× PBS-ethanol (1:1) at -20°C. Fixed samples were treated by sonication for 90 s with a MS73 probe (Sonopuls HD70, Bandelin, Germany) at an amplitude of 42 μm <10 W. Aliquots of 250 µL were centrifuged (3 min, 16,000xg) and the supernatant was removed. All subsequent steps were performed in solution. A change of solutions was performed by centrifugation (3 min, 16,000xg) and discarding of the supernatant. Target cells were permeabilized by resuspending samples in 500 µL 0.1 M HCl and incubation for 1 min at room temperature (RT). Endogenous peroxidases were inactivated by resuspending samples in 1 ml methanol containing 0.15% H_2O_2 and an incubation for 30 min at room temperature. Samples were washed with 1 ml PBS and resuspended in hybridization buffer (45% formamide) containing probe SEEP1a-1441 (Schreiber et al., 2010) at a final concentration of 2 ng/µL. Hybridization was performed for 2.5 h at 46°C, followed by a washing step with 2 ml of pre-heated washing buffer. Washing was performed for 15 min at 48°C. Samples were incubated in 1x PBS for 5 min at RT. CARD was performed with 1 ml of amplification buffer with the fluorochrome Alexa Fluor 488 for 20 min at 37°C. Samples were resuspended in 1x PBS and stored at 4°C. Samples were diluted 1:10 to 1:100 and stained with 4',6'-diamidino-2-phenylindole (DAPI) immediately prior to flow cytometry.

Cell sorting was done with a MoFlo flow cytometer (Beckman Coulter GmbH, Krefeld, Germany) equipped with two argon ion lasers. The first laser was tuned

to a wavelength of 488 nm (400 mW) for the excitation of hybridized cells. The second laser was tuned to UV (multilines at 351.1 to 363.8 nm, 100 mW) to detect DAPI-stained cells. Side angle light scatter (SSC) was detected through a 488- ± 10-nm band-pass filter. Green fluorescence from hybridized cells was detected by using a 530- ± 20-nm band-pass filter. DAPI fluorescence was measured with a 450- ± 32-nm band-pass filter. The system threshold was set in SSC and green fluorescence. We used a nozzle with an orifice diameter of 70 µm. Prior to measurements, the instrument was aligned by using Fluoresbrite® Multifluorescent 1.00 µm polystyrene beads (Polysciences, USA). For every measured event all parameters were recorded as pulse height signals and stored in list mode files. Online analysis, sort control, and postanalysis were done with the Summit software, version 4.3 (Beckman Coulter). By bivariant dot plot analyses of SSC, blue and green fluorescence objects with medium scatter signals and of high blue and green fluorescence were selected by logical gating and sorted out. Sorting was performed at 98,280 Hz at an amplitude around 10.5 V and a delay of usually 45 14/16 droplets. Instrument tubings were rinsed prior to sorting with autoclaved 0.1% (wt/vol) NaCl which was also used as the sheath fluid. The sample line was cleaned by using FACSRinse® solution (Becton Dickinson, San Jose, CA, USA) and autoclaved 0.1% NaCl. In order to keep the instrument as sterile as possible, autoclaved sheath fluid was prefiltered with an in-line filter cartridge; pore size 0.2 µm and 0.1 µm (Sartorius Stedim, Goettingen, Germany). The amount of sorted objects ranged from 40,000 to 100,000 cells per tube in replicates.

Aliquots of *c*. 150,000 sorted cells were spotted onto GTTP polycarbonate filters (Millipore, Germany) with a diameter of 3 mm, and a pore size of 0.2 μm. Filter pieces were immersed in 6 µl of PCR water and subjected to three freeze/thaw cycles (-20°C/RT). Multiplex PCR (Multiplex PCR kit, Qiagen) was performed with primers 907RM (Muyzer et al., 1998), GM5 (Muyzer et al., 1998), AprA-1-FW, Apr-5-RV, Dsr1F, and Dsr1334 (Santillano et al., 2010) and a final volume of 50 µl according to the manufacturer's protocol. PCR products were purified and

size selected by agarose gel electrophoresis. Purified PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Germany) according to the manufacturer's recommendations. Insert sequences were determined by Sanger sequencing (GATC Biotech AG).

Sequence accession numbers

The nucleotide sequence data reported in this paper have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers xxx - xxx

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Supporting Information

Supporting Tables

Table S1. Description of sampling sites

Site	Cruise	Site description	Position	Water depth [m]	Date	References
Hydrate Ridge	SO148-1 (RV SONNE)	Crest of southern Hydrate Ridge, coast of Oregon; gas hydrate bearing sediment covered by *Beggiatoa* mat; sediment layer 1-3 cm	44° 34' N, 125° 09' W	777	Jul 2000	Linke and Suess, 2001; Nauhaus et al., 2002
Black Sea, Paleo-Dnepr area (reef 822)	POS317-3 (RV POSEIDON)	Exterior part of a microbial mat from microbial reef 822	44° 47' N 31° 59' E	191	Sept/Oct 2004	unpubl.
Black Sea, lower Crimean shelf	RV Prof. LOGACHEV	Microbial reef structure at cold, anoxic methane seep	44° 46' N 31° 60' E	230	Jul 2001	Michaelis et al., 2002

Table S2. Primers used in the present study

Primer	Specificity	Sequence 5'-3'	Binding site[†]	Reference
AprA-1-FW	*aprA* subunit of most SRP and sulfur-oxidizing bacteria	TGG CAG ATC ATG ATY MAY GG	1236–1256	Meyer and Kuever, 2007
AprA-5-RV	*aprA* subunit of most SRP and sulfur-oxidizing bacteria	GCG CCA ACY GGR CCR TA	1615–1631	Meyer and Kuever, 2007
AprA-10-RV-deg	*aprA* subunit of most SRP and sulfur-oxidizing bacteria	YKR WAG TAG WAR CCR GGR TA	2611–2630	*modified from* Meyer and Kuever, 2007
DSR1F	*dsrAB* subunits of most SRP	ACS CAC TGG AAG CAC G	298-313	Wagner et al., 1998
DSR4R	*dsrAB* subunits of most SRP	GTG TAG CAG TTA CCG CA	2569-2585	Wagner et al., 1998
DSS1334R	*dsrAB* subunits of most SRP	TYT TCC ATC CAC CAR TCC	1281-1298	Santillano et al., 2010

[†] Corresponding nucleotide positions of the *aprBA* or *dsrAB* operon of *Desulfovibrio vulgaris* ssp. *vulgaris* strain Hildenborough (GenBank accession no. NC_002937).

Table S3 Operational taxonomic units and diversity estimates for AprA and DsrAB. Number of OTUs and Chao1 diversity estimates for translated *aprA* and *dsrAB* clone libraries of the studied AOM samples. The grey intensity of each block corresponds to diversity relative to the sample with the highest diversity.

(A) *aprA* gene diversity

BSBlack OTUs: 6 Chao1: 6	HRSed OTUs: 14 Chao1: 22
BSPink OTUs: 1 Chao1: 1	HREnrPr OTUs: 2 Chao1: 2
BSExt OTUs: 7 Chao1: 7	HREnrLT OTUs: 6 Chao1: 7

(B) *dsrAB* gene diversity

BSBlack OTUs: 8 Chao1: 8	HRSed OTUs: 19 Chao1: 36
BSPink OTUs: 5 Chao1: 8	HREnrPr OTUs: 5 Chao1: 8
BSExt OTUs: 2 Chao1: 2	HREnrLT OTUs: 7 Chao1: 8

Table S4. Operational taxonomic units of SRB and sulfur-oxidizing bacteria based on comparative sequence analysis of *aprA* genes retrieved from 6 different AOM habitats

Sample	OTU	clones	% of clones	Cluster affiliation	Group affiliation
BSBlack	BSB-a01	14	21.2	R	uncertain affiliation
(66 clones)	BSB-a02	43	65.2	K	uncertain affiliation
	BSB-a03	3	4.5	C	I (*Desulfobacteraceae*)
	BSB-a04	3	4.5	S	uncertain affiliation
	BSB-a05	1	1.5	O	II (*Syntrophobacteraceae*)
	BSB-a06	2	3.0	L	uncertain affiliation
BSPink	BSP-a01	46	100	K	uncertain affiliation
(46 clones)					
BSExt	BSExt-a01	1	2.1	K	uncertain affiliation
(48 clones)	BSExt-a02	23	47.9	C	I (*Desulfobacteraceae*)
	BSExt-a03	16	33.3	M	uncertain affiliation
	BSExt-a04	1	2.1	A	I (*Desulfobacteraceae*)
	BSExt-a05	2	4.2	K	uncertain affiliation
	BSExt-a06	3	6.3	*Thiocystis*	*Chromatiaceae*
	BSExt-a07	2	4.2	N	uncertain affiliation
HRSed	HRS-a01	5	10.2	*Thiobacillus*	*Hydrogenophilaceae*
(49 clones)	HRS-a02	2	4.1	*Thiococcus*	*Chromatiaceae*
	HRS-a03	2	4.1	K	uncertain affiliation
	HRS-a04	16	32.7	J	I (*Desulfobacteraceae*)
	HRS-a05	1	2.0	F	I (*Desulfobacteraceae*)
	HRS-a06	9	18.4	T	uncertain affiliation
	HRS-a07	1	2.0	P	uncertain affiliation
	HRS-a08	6	12.2	T	uncertain affiliation
	HRS-a09	2	4.1	D	uncertain affiliation
	HRS-a10	1	2.0	L	uncertain affiliation
	HRS-a11	1	2.0	Q	IV (*D.anilini*)
	HRS-a12	1	2.0	S	uncertain affiliation
	HRS-a13	1	2.0	*Thiocystis*	*Chromatiaceae*
	HRS-a14	1	2.0	*Thiobacillus*	*Hydrogenophilaceae*
HREnrPr	HRP-a01	70	98.6	J	I (*Desulfobacteraceae*)
(71 clones)	HRP-a02	1	1.4	A	I (*Desulfobacteraceae*)
HREnrLT	HREnr-a01	3	6.7	I	I (*Desulfobacteraceae*)
(45 clones)	HREnr-a02	3	7.0	H	I (*Desulfobacteraceae*)
	HREnr-a03	2	4.4	G	I (*Desulfobacteraceae*)
	HREnr-a04	1	2.2	E	I (*Desulfobacteraceae*)
	HREnr-a05	13	28.9	B	I (*Desulfobacteraceae*)
	HREnr-a06	23	51.1	J	I (*Desulfobacteraceae*)

Table S5. Operational taxonomic units of SRB based on comparative sequence analysis of *dsrAB* genes retrieved from 6 different AOM habitats

Sample	OTU	Clones	% of clones	Cluster affiliation	Group affiliation
BSBlack	BSB-d01	26	45.6	I-b	I (*Desulfobacteraceae*)
(57 clones)	BSB-d02	15	26.3	IV	IV (*D. anilin*i related)
	BSB-d03	5	8.8	IX	IX (*Desulfoarculaceae*)
	BSB-d04	3	5.3	X	II (*Syntrophobacteraceae*)
	BSB-d05	3	5.3	I-b	I (*Desulfobacteraceae*)
	BSB-d06	2	3.5	IV	IV (*D. anilini* related)
	BSB-d07	2	3.5	IX	IX (*Desulfoarculaceae*)
	BSB-d08	1	1.8	I-a	I (*Desulfobacteraceae*)
BSPink	BSP-d01	34	70.8	I-a	I (*Desulfobacteraceae*)
(48 clones)	BSP-d02	11	22.9	I-g	I (*Desulfobacteraceae*)
	BSP-d03	1	2.1	IV	IV (*D. anilini* related)
	BSP-d04	1	2.1	VI	VI
	BSP-d05	1	2.1	X	II (*Syntrophobacteraceae*)
BSExt	BSExt-d01	44	91.7	I-b	I (*Desulfobacteraceae*)
(48 clones)	BSExt-d02	4	8.3	I-a	I (*Desulfobacteraceae*)
HRSed	HRS-d01	16	30.8	V-a	V (Deep-branching)
(52 clones)	HRS-d02	9	17.3	V-a	V (Deep-branching)
	HRS-d03	4	7.7	I-a	I (*Desulfobacteraceae*)
	HRS-d04	4	7.7	V-b	V (Deep-branching)
	HRS-d05	3	5.8	II	II (*Syntrophobacteraceae*)
	HRS-d06	2	3.8	V-a	V (Deep-branching)
	HRS-d07	2	3.8	V-a	V (Deep-branching)
	HRS-d08	1	1.9	V-a	V (Deep-branching)
	HRS-d09	1	1.9	I-f	I (*Desulfobacteraceae*)
	HRS-d10	1	1.9	I-h	I (*Desulfobacteraceae*)
	HRS-d11	1	1.9	I-e	I (*Desulfobacteraceae*)
	HRS-d12	1	1.9	IV	II (*Syntrophobacteraceae*)
	HRS-d13	1	1.9	IV	II (*Syntrophobacteraceae*)
	HRS-d14	1	1.9	IV	II (*Syntrophobacteraceae*)
	HRS-d15	1	1.9	IX	IX (*Desulfoarculaceae*)
	HRS-d16	1	1.9	X	II (*Syntrophobacteraceae*)
	HRS-d17	1	1.9	VII	VII (*Desulfobulbaceae*)
	HRS-d18	1	1.9	V-c	V (Deep-branching)
	HRS-d19	1	1.9	V-c	V (Deep-branching)
HREnrPr	HRP-d01	21	56.8	I-g	I (*Desulfobacteraceae*)
(37 clones)	HRP-d02	13	35.1	I-b	I (*Desulfobacteraceae*)
	HRP-d03	1	2.7	VII	VII (*Desulfobulbaceae*)
	HRP-d04	1	2.7	I-e	I (*Desulfobacteraceae*)
	HRP-d05	1	2.7	I-i	I (*Desulfobacteraceae*)
HREnrLT	HREnr-d01	13	30.2	I-g	I (*Desulfobacteraceae*)
(43 clones)	HREnr-d02	6	14.0	I-d	I (*Desulfobacteraceae*)
	HREnr-d03	3	7.0	I-e	I (*Desulfobacteraceae*)
	HREnr-d04	12	27.9	I-c	I (*Desulfobacteraceae*)
	HREnr-d05	6	14.0	I-c	I (*Desulfobacteraceae*)
	HREnr-d06	2	4.7	I-d	I (*Desulfobacteraceae*)
	HREnr-d07	1	2.3	I-b	I (*Desulfobacteraceae*)

Table S6 Jaccard distance between studied samples based on the presence of specific *aprA* and *dsrAB* gene clusters

	BSBlack	BSPink	BSExt	HRSed	HREnrPr	HREnrLT
BSBlack	1.00					
BSPink	0.31	1.00				
BSExt	0.27	0.17	1.00			
HRSed	0.26	0.16	0.11	1.00		
HREnrPr	0.06	0.08	0.15	0.11	1.00	
HREnrLT	0.05	0.06	0.06	0.06	0.29	1.00

Table S7. Phylogenetic affiliation of 16S rRNA sequences retrieved from FACS-sorted cells and sorting sheath fluid.

Phylum	Order	Hydrate Ridge enrichment; sorted cells	Hydrate Ridge enrichment; sheath fluid
Alphaproteobacteria	Rhizobiales	0	1
	Sphingomonadales	0	2
Betaproteobacteria	Burkholderiales	1	8
	Xanthomonadales	1	0
Deltaproteobacteria	Uncultured	1	2
Gammaproteobacteria	Pseudomonadales	12	2
	Enterobacterales	0	1
Bacteroidetes	Bacteroidales	4	2
	Sphingobacteriales (Chitinophaga)	1	2
Firmicutes	Clostridiales	1	0
Actinobacteria	Actinomycetales	1	1
Total		*22*	*21*

Supporting Figures

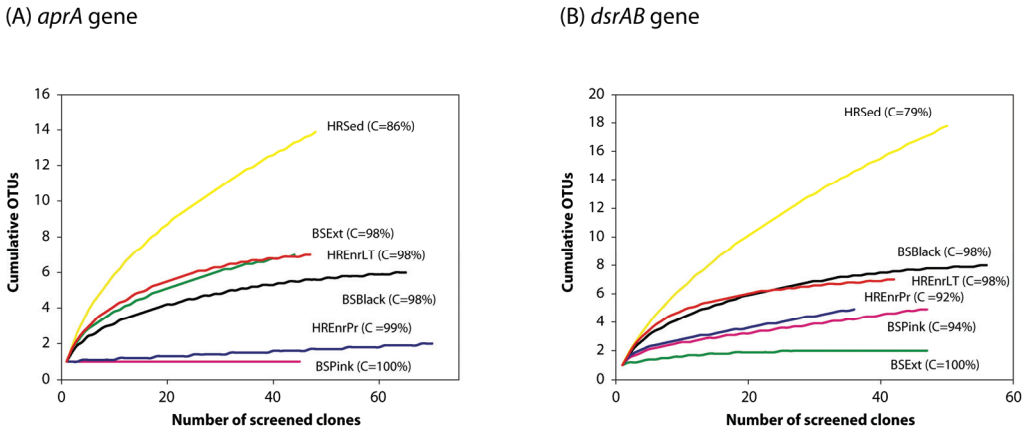

Figure S1: Rarefaction analysis of AprA and DsrAB sequences from different AOM samples. The analysis is based on OTUs based on a 90% amino acid identity threshold. Black part of Black Sea microbial mat, BSBlack, black (–); Pink part of Black Sea microbial mat, BSPink, pink (–); Exterior part of Black Sea microbial mat, BSExt, green (–); Hydrate Ridge sediment, HRSed, yellow (–); Pressure-incubated Hydrate Ridge enrichment, HREnrPr, blue (–); Long-term Hydrate Ridge enrichment, HREnrLT, red (–). The homologous coverage C is shown for each library.

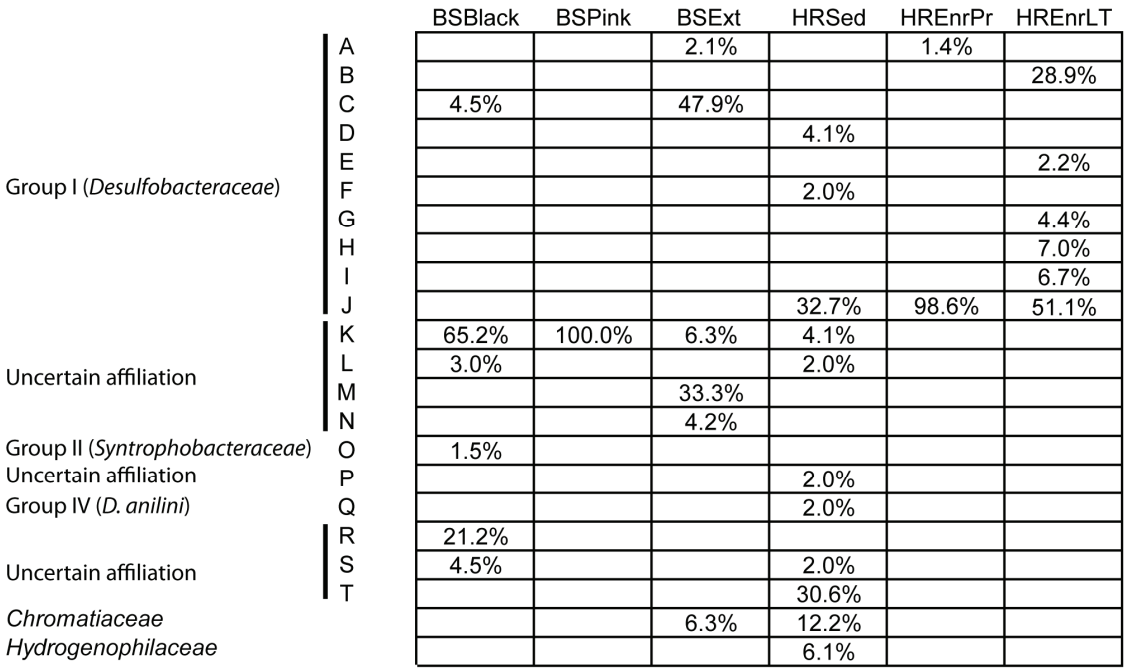

Figure S2 Comparison of the detailed phylogenetic affiliations of AprA sequences

		BSBlack	BSPink	BSExt	HRSed	HREnrPr	HREnrLT
Group I (*Desulfobacteraceae*)	I-a	1.8%	70.8%	8.3%	7.7%		
	I-b	50.9%		91.7%		35.1%	2.3%
	I-c						41.9%
	I-d						18.7%
	I-e				1.9%	2.7%	7.0%
	I-f				1.9%		
	I-g		22.9%			56.8%	30.2%
	I-h				1.9%		
	I-i					2.7%	
Group IV (*D.anilini* group)	IV	29.8%	2.1%		5.8%		
Group VII (*Desulfobulbaceae*)	VII				1.9%	2.7%	
Group II (*Syntrophobacteraceae*)	II				5.8%		
Group X	X	5.3%	2.1%		1.9%		
Group IX (*Desulfoarculaceae*)	IX	12.3%			1.9%		
Group VI	VI		2.1%				
Group V (deep-branching)	V-a				57.5%		
	V-b				7.7%		
	V-c				3.8%		

Figure S3 Comparison of the detailed phylogenetic affiliations of DsrAB sequences

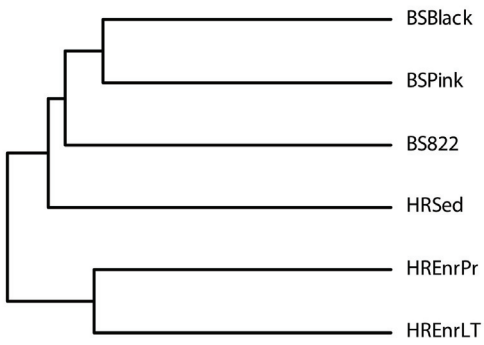

Figure S4 Similarity between the studied sulfate-reducing communities. Cluster analysis based on Jaccard distance for the combined AprA and DsrAB data set as shown in Table S6

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IV. Metagenomic analysis of the dominating sulfate-reducing bacteria in ANME-2 dominated enrichments catalyzing the anaerobic oxidation of methane.

Contributions to this chapter:

Lars Schreiber, Thomas Holler, Michael Kube, Richard Reinhard, Anke Meyerdierks, and Rudolf Amann. Metagenomic analysis of the dominating sulfate-reducing bacteria in ANME-2 dominated enrichments catalyzing the anaerobic oxidation of methane.

L.S.: developed the concept, constructed fosmid library, analyzed metagenomic dataset, conceived and wrote the manuscript; T.H.: provided AOM enrichment culture; M.K.: developed the concept, sequenced fosmid endsequences, extracted DNA for pyrosequencing and performed pyrosequencing, performed sequence assembly; R.R.: developed the concept, sequenced fosmid endsequences, extracted DNA for pyrosequencing and performed pyrosequencing, performed sequence assembly; A.M.: developed the concept, conceived and edited the manuscript; R.A.: developed the concept, conceived and edited the manuscript

Metagenomic analysis of the dominating sulfate-reducing bacteria in ANME-2 dominated enrichments catalyzing the anaerobic oxidation of methane

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ABSTRACT

The anaerobic oxidation of methane with sulfate (AOM) is a widespread process and the main methane sink in marine systems. AOM is catalyzed by consortia of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB). In spite of the importance of AOM, our understanding of the interaction between ANME and SRB as well as the underlying biochemistry of AOM is still limited. Attempts to answer these questions are hampered by the current inability to cultivate the involved microorganisms in pure culture. Here we used a culture-independent metagenomic approach to attempt a genome reconstruction of SEEP-SRB1a, the main sulfate-reducing partner of anaerobic methanotrophs of the ANME-2 clade. We used DNA from an enrichment culture dominated by ANME-2 and SEEP-SRB1a for constructing a large-insert fosmid library and for performing next-generation pyrosequencing. Almost 92% of the assembled contigs featured a length of only 1.5 kbp or shorter indicating an insufficient coverage of the enrichment community. Within the metagenome, we detected 11 contigs carrying 16S rRNA genes of SEEP-SRB1a. This observation indicated a considerable microdiversity of SEEP-SRB1a within the enrichment. The metagenome contained too few long contigs to reconstruct the genome of SEEP-SRB1a. However, we identified the 23S rRNA gene as well as putative *apr* and *dsr* genes of SEEP-SRB1a which might be used as alternative genetic markers in future studies. *Apr* and *dsr* genes of SEEP-SRB1a identified two contigs with a length of >18kbp and thereby provided a first glimpse of the genetic potential of SEEP-SRB1a. Finally, we identified 211 fosmid inserts whose end-sequences map onto the genome of *Desulfococcus oleovorans* Hxd3, the closest fully-sequenced relative of SEEP-SRB1a. Sequencing of these inserts might provide a robust scaffold for the reconstruction the SEEP-SRB1a genome.

INTRODUCTION

Only a fraction of the microorganisms inhabiting our planet is currently culturable (Amann et al., 1995; Curtis et al., 2002). Thus, in order to still explore the physiology and ecological function of all the uncultivated microbial species culture-independent approaches are necessary. One such approach, increasingly used since the advent of next-generation sequencing (Margulies et al., 2005), is metagenomics (Liolios et al., 2010). Metagenomics is characterized by bulk sequencing of whole microbial communities (Handelsman et al., 1998; Riesenfeld et al., 2004). In an ideal case scenario, the resulting sequence data is subsequently used for genome and metabolic reconstruction of individual species of the community. The immense diversity within typical microbial communities, however, has so far limited successful genome reconstructions from metagenomes to low-diversity habitats such as an acid mine drainage biofilm (Tyson et al., 2004), the symbiont community within a marine oligochaete (Woyke et al., 2006), or microbial mats from the Black Sea (Meyerdierks et al., 2010).

The anaerobic oxidation of methane with sulfate (AOM) is the main methane sink in the marine environment (Reeburgh, 2007). The process was shown to be catalyzed by consortia of anaerobic methanotrophic archaea (ANME) and physically-attached sulfate-reducers affiliated with *Deltaproteobacteria* (Boetius et al., 2000). None of the involved microorganisms has been cultured so far and thus the underlying biochemistry of AOM is currently unresolved. Due to the inability to obtain pure cultures, recent attempts focussed on metagenomic approaches to shed some light on the AOM process. Such metagenomic studies provided an initial characterization of the ANME-subclades ANME-1 (Krüger et al., 2003; Meyerdierks et al., 2010) and ANME-2 (Hallam et al., 2004; Meyerdierks et al., 2005). Furthermore, they supported the hypothesis that ANME perform the anaerobic oxidation of methane via a reversal of the methanogenesis pathway (Krüger et al., 2003; Hallam et al., 2004). How methane-oxidation is

coupled to sulfate-reduction as well as the role of sulfate-reducing bacteria in AOM consortia however remained unclear.

In spite of the inability to obtain pure cultures, several AOM enrichment cultures have been reported (Nauhaus et al., 2007; Holler et al., 2009). This study investigated one such enrichment originating from sediment above gas hydrates at Hydrate Ridge (NE Pacific). The enrichment was dominated by archaea of the ANME-2 clade and their sulfate-reducing partner. A previous study identified this partner to belong to the SEEP-SRB1a group within the *Desulfosarcina*/*Desulfococcus* group (Schreiber et al., 2010). In order to learn more about the role of SRB within AOM consortia in general, we aimed at a genome reconstruction of SEEP-SRB1a. We used a dual metagenomic approach combining large-insert fosmid libraries and next-generation pyrosequencing sequencing to achieve this goal. This study provides a first characterization and evaluation of the generated metagenomic data.

RESULTS AND DISCUSSION

Characterization of the enrichment culture

Hydrate Ridge sediment was incubated for more than 84 month which resulted in detritus-free enrichments of loose flocks essentially composed of microbial cells (Nauhaus et al., 2007; Holler et al., 2009). Analysis by fluorescence *in situ* hybridization (FISH) showed that the enrichment is dominated by archaea of the ANME-2 clade and their associated sulfate-reducing partners (Holler et al., 2009). More precisely the enrichment contained 17% ANME-2a, 64% ANME-2c, and 18% ANME-2 associated SRB (Schreiber et al., 2010). Other microorganisms made up less than 1% of the total cell numbers (Holler et al., 2009). The dominant sulfate-reducing-partner of ANME-2 within the enrichment was identified to belong to the SEEP-SRB1a group within the *Desulfosarcina*/*Desulfococcus* group (Schreiber et al., 2010). Based on retrieved 16S rRNA genes, the enrichment was also shown to contain other *Deltaproteobacteria* related to *Desulfosarcina*, *Desulfuromonas*, and *Desulfobulbus.* ANME-2/SEEP-SRB1a consortia in the enrichment are estimated to grow with a generation time of *c*. 6 month.

Metagenome sequencing

In order to attempt a reconstruction of the SEEP-SRB1a genome from the enrichment culture, a parallel metagenomic approach was used which combined next-generation shotgun sequencing and a large insert fosmid library. A fosmid library containing *c*. 24,500 clones was constructed from DNA extracted from the enrichment. Assuming an average insert size of 35 kbp, this library represents about 858 Mbp of sequence information. A total of 13,086 clones were end-sequenced, yielding 24,440 high-quality end-sequences with an average read length of 721 bp. In total, 17.6 Mbp of sequence data was obtained from this sequencing effort (Table 1).

Table 1 Overview of the Hydrate Ridge enrichment metagenome

Fosmid statistics	
Fosmid library size	*c.* 24,500 clones
End-sequences of fosmids	24,440
Average end-sequence read length	721 bp
Total amount of end-sequence data	17.6 Mbp
Desulfosarcinales associated end-sequences[a]	390 (1.6%)
Methanosarcinales associated end-sequences[a]	1070 (4.4%)
Pyrosequencing statistics	
Number of reads	1,377,651
Average read length	413
Total amount of sequence data	570 Mbp
Assembly statistics	
Number of assembled contigs	94,367
Total amount of assembled sequence data	73.4 Mbp
Contig size range	27 – 99,180 bp
Average contig size	779 bp
Number of predicted ORFs	177,208

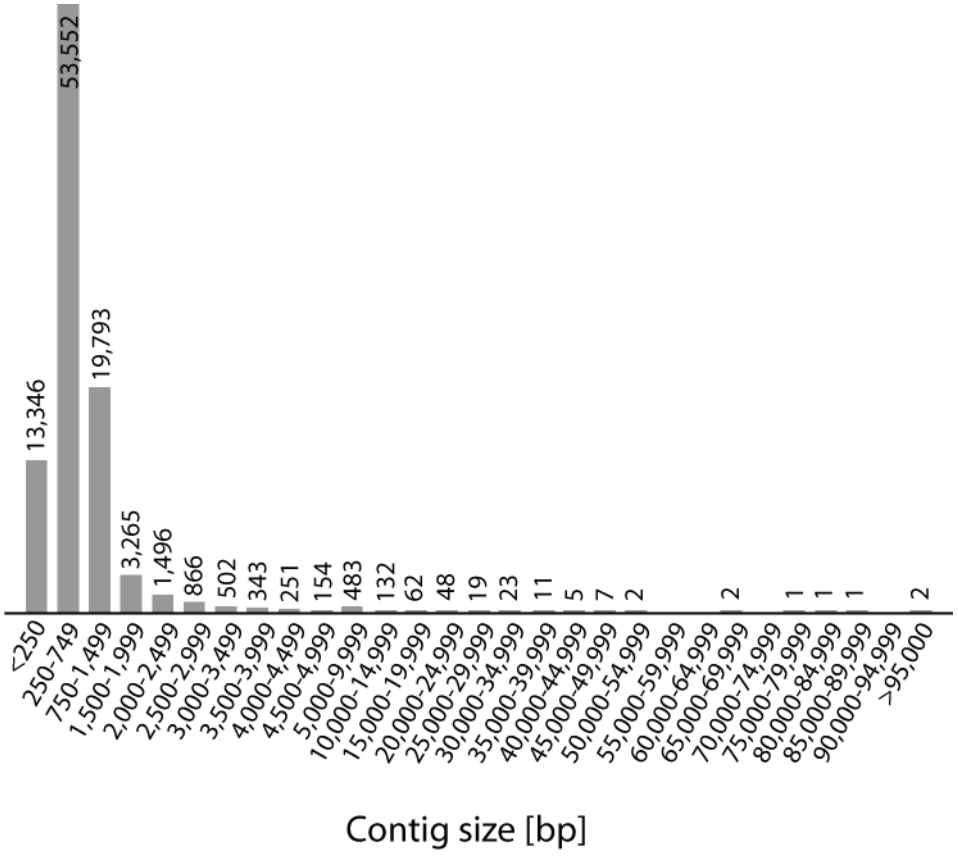


Figure 1 Size distribution of the 94,367 assembled contigs. Shown are the numbers of contigs for the indicated size ranges.

Next-generation shotgun sequencing of the enrichment was performed using pyrosequencing sequencing technology (Margulies et al., 2005). A total of 570 Mbp of DNA sequence was generated from 1,377,651 reads (averaging 413 bp per read; Table 1)

Prior to further analysis, the pyrosequencing dataset was assembled together with the fosmid end-sequences using MIRA V3rc2 (Chevreux et al., 1999. The pyrosequencing reads and end-sequecnes were assembled into 94,260 contigs with a contig size range of 27 to 99,180 bp (Table 1). Of these only 319 contigs (0.3%) had a size of 10 kbp or more (Figure 1).

Diversity within the metagenome

The diversity within the fosmid end-sequences was assessed with the MG RAST server (Meyer et al., 2008) using the SEED reference dataset. In total, 11,733 (48%) of the end-sequences could be phylogenetically classified. Manual evaluation showed that at least 46% of the classified sequences were aligned with phage-associated reference sequences. It is not known if these sequences represent prophage sequences or if the originated from the phage population within the enrichment culture. Phage-associated sequences were excluded from further analysis, leaving a total of 6329 classified sequences. Of the classified end-sequences, 4497 (71%) could be assigned to the bacterial domain, compared to 1326 (21%) assigned to the archaeal domain (Figure 2).

Within the *Bacteria* most of the sequences (3213; 73%) affiliated with the *Proteobacteria*. The proteobacterial species with the most assigned end-sequences, were *Thiomicrospira crunogena* XCL-2 (*Gammaproteobacteria*; 662 hits) and *Desulfococcus oleovorans* Hxd3 (*Deltaproteobacteria*; 270). Most of the archaeal end-sequences (1017; 77%) could be assigned to methanogens from within the *Euryarchaeota*. Most of these end-sequences hit three *Methanosarcinaceae* species closely related to ANME-2a and ANME-2c: *Methanosarcina acetivorans* C2A (375 hits), *Methanococcoides burtonii* DSM 6242 (224), and *Methanosarcina mazei* Go1 (111).

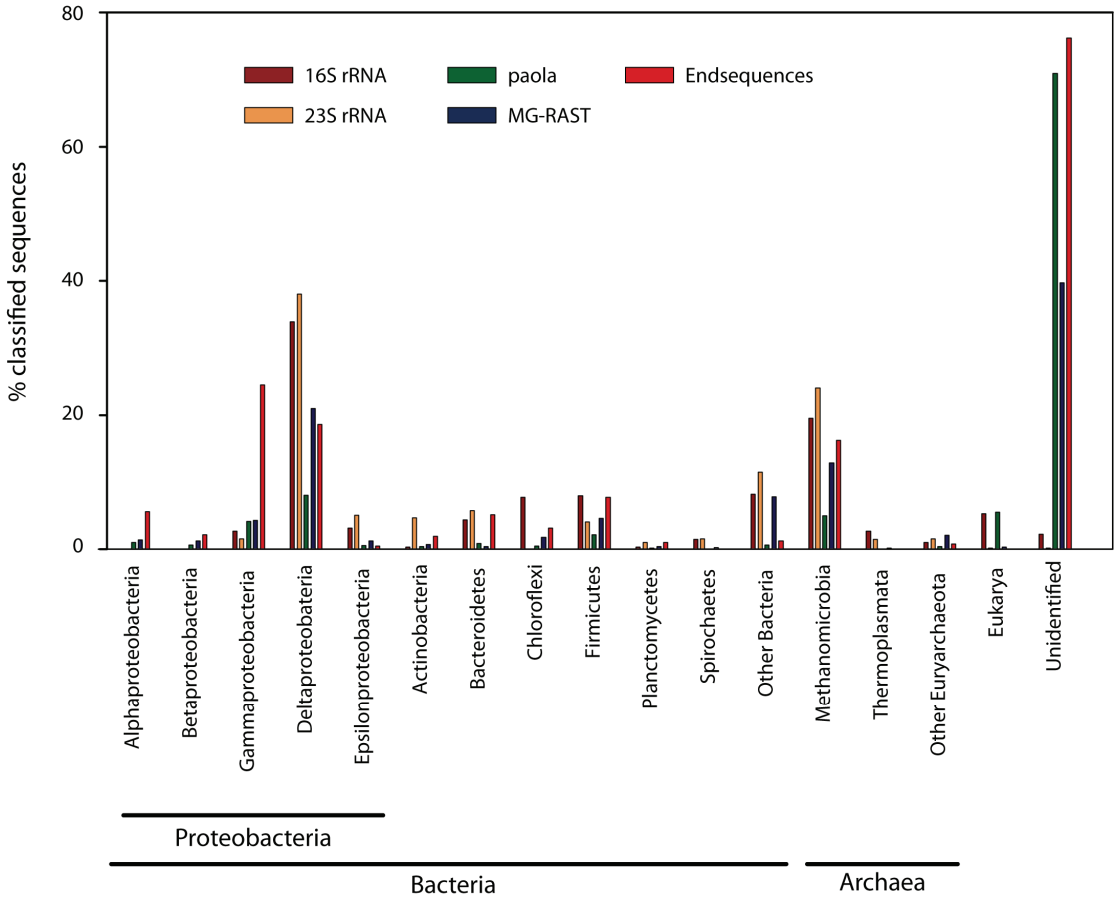


Figure 2 Phylogenetic assessment of the combined pyrosequencing and fosmid end-sequences set. End-sequences were analyzed using the MG-RAST server (Meyer et al., 2008) and a minimum alignment length of 98 bp. The alignment length was chosen to reduce the high number of alignments with phage-associated genes, which dominated the phylogenetic classification at lower values.

To assess the diversity within the pyrosequencing dataset, all reads were screened for 16S and 23S rRNA genes using the SILVA NGS pipeline, an unpublished version of the SILVA pipeline optimized for next-generation sequence data (Pruesse et al., 2007). In total 419 16S and 712 23S rRNA gene fragments were detected. Most of the retrieved rRNA gene sequences affiliated with *Deltaproteobacteria* (34%, 16S rRNA; 38%, 23S rRNA) and *Methanomicrobia*

(20%, 16S rRNA, 24%, 23S rRNA), the two groups harboring SEEP-SRB1a and ANME-2, respectively (Figure 2).

The rRNA-based approach was complemented by two alternative taxonomic classification approaches; one based on BLAST and PFAM hits as implemented in the *paola* software tool (Huang, 2009) and one based on the SEED dataset of the MG-RAST server (Meyer et al., 2008). This analysis was performed on the assembled dataset combining fosmid end-sequences with pyrosequencing data. Similar to the rRNA-based approach, MG-RAST and *paola* reported that most of the classified contigs affiliated with *Deltaproteobacteria* and *Methanomicrobia* (Figure 2). Besides the expected *Deltaproteobacteria* and *Methanomicrobia,* other microbial groups, most notably *Bacteroidetes, Firmicutes*, and *Gammaproteobacteria*, were also consistently detected in the metagenome with the used approaches (Figure 2).

Comparing the diversity of the enrichment with the one determined for the metagenome, two results are surprising: (1) the considerable amount of metagenomic data supposedly not originating from ANME-2 or SEEP-SRB1a, even though these groups together make up more than 99% of the enrichment culture, and (2) the high number of bacterial sequences within the metagenome, despite the fact that the enrichment featured an archaeal fraction of *c*. 81%. One possible explanation for these discrepancies is the fact that microorganisms can differ considerably in their susceptibility to different DNA extraction methods (Zhou et al., 1996). This is especially important considering that consortia of ANME and SRB were shown to be embedded in an extracellular matrix (Knittel and Boetius, 2009) which might protect them during DNA extraction. In addition, *Methanosarcina mazei*, a close relative of ANME-2, was shown to contain a large amount of bacterial genes acquired by horizontal gene transfer (HGT) (Deppenmeier et al., 2002). Considering a similar genome structure of ANME-2, some of the contigs classified as bacterial might in fact originate from ANME-2. Finally, a biased reference data set might have caused an overprediction of contigs of bacterial origin. The SEED reference dataset of the MG-RAST server for

example currently contains 1,120 bacterial genomes compared to only 69 archaeal ones.

Reconstruction of a SEEP-SRB1a genome

In order to reconstruct microbial genomes from metagenomic data sets, previous studies mainly used bioinformatic binning based on intrinsic sequence signatures, such as GC content (Tyson et al., 2004) or other nucleotide frequencies (Teeling et al., 2004; Woyke et al., 2006). One prerequisite of this approach is the identification of the formed 'bins' by specific marker genes or based on previous knowledge about the DNA signatures of the target species. Currently, no information is available about the genome of SEEP-SRB1a. Thus, in order to identify SEEP-SRB1a contigs which could serve as anchors for sequence binning, we screened the data set for SEEP-SRB1a 16S rRNA genes. With this approach we identified 11 SEEP-SRB1a contigs with a size range of 582 to 8862 bp. Of the contigs possessing sequence information at the binding site, 5 of 7 featured a perfect match to the SEEP-SRB1a-specific oligonucleotide probe SEEP1a-473, while 5 of 5 featured a perfect match with the second SEEP-SRB1a-specific oligonucleotide probe SEEP1a-1441. On two of the contigs we identified the 23S rRNA gene of SEEP-SRB1a, which could serve as an alternative genetic marker for the group. The average GC-content of the SEEP-SRB1a contigs ranged from 40-54%. Due to the short length of the contigs it is, however, questionable to what extent they can be used to reliably infer the GC content of the whole genome (Tyson et al., 2004).

We investigated the diversity of *apr* and *dsr* genes within the metagenome to identify additional SEEP-SRB1a contigs. Both genes are key genes of the sulfate-reduction pathway and are frequently used as phylogenetic markers (Wagner et al., 1998; Klein et al., 2001; Meyer and Kuever, 2007). We detected 37 and 30 contigs carrying *apr* and *dsr* genes in the metagenome, respectively. Phylogenetic analysis showed a dominance of sequences from within the *Desulfobacteraceae* (Figure 3). The single biggest group of *apr* sequences affiliated with

Desulfobacteraceae cluster J (17 sequences; Figure 3); the *apr* cluster putatively originating from SEEP-SRB1a (Chapter III). For the *dsr* gene, most of the sequences (19 sequences) affiliated with *Desulfobacteraceae* cluster I-b (Chapter III).

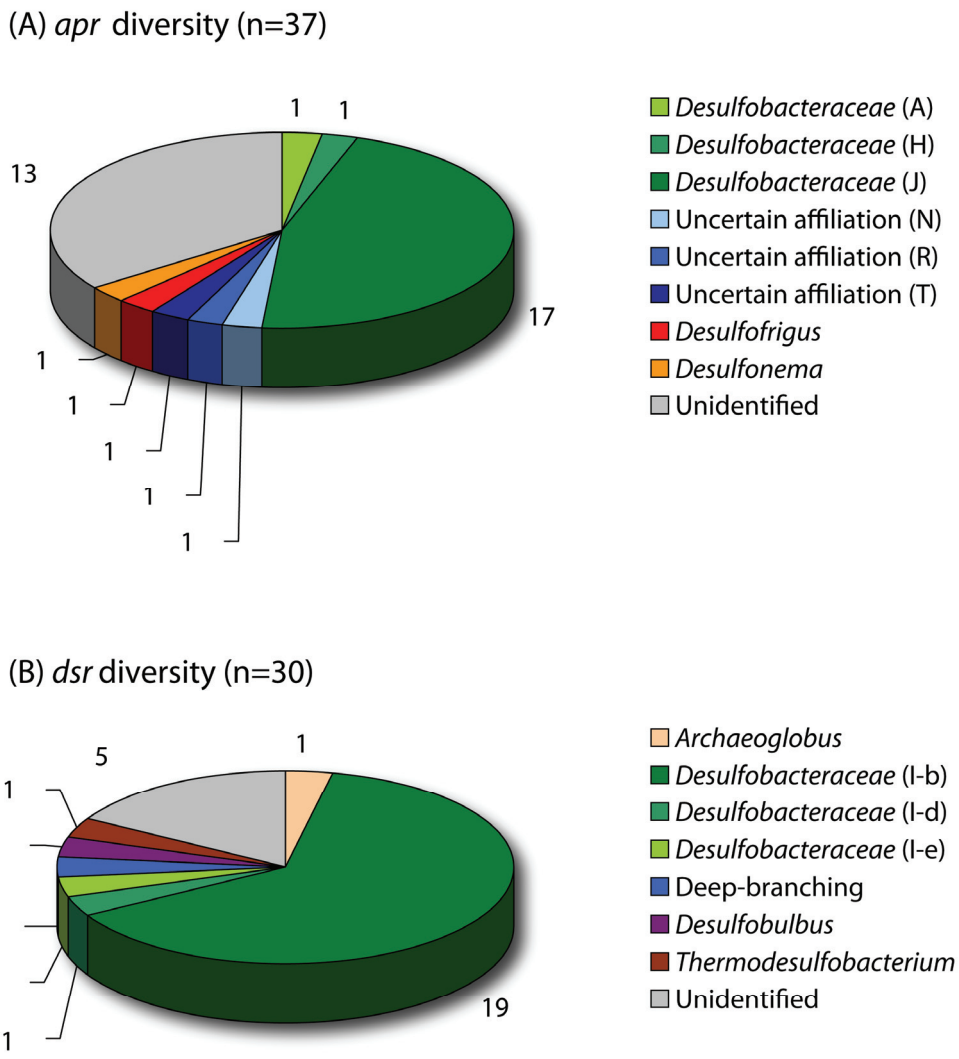


Figure 3. Diversity of *apr* and *dsr* detected on contigs of the assembled metagenome. Contigs carrying *apr* or *dsr* genes were detected automatically with the *paola* software tool. Phylogenetic assignment was performed by adding the corresponding amino acid sequences to AprA and DsrAB reference trees using maximum parsimony criteria in the ARB software package (Ludwig et al., 2004).

The high abundance of these *dsr* sequences suggests that cluster I-b represents the *dsr* gene of SEEP-SRB1a. Based on publicly available genomes of SRB, *apr* and *dsr* are usually single-copy genes. Thus, our data suggests that 17-19 SEEP-SRB1a strains are represented in the metagenome. However, it cannot be excluded that these numbers overestimate the number of SEEP-SRB1a strains and that some of the differences between the SEEP-SRB1a *apr* and *dsr* sequences are in fact sequencing artefacts (Kunin et al., 2010).

One *apr* gene from cluster J and one *dsr* gene from cluster I-b identified two contigs with lengths of 18.3 kbp and 22.6 kbp, respectively, to putatively originate from SEEP-SRB1a (Figure 4). Both contigs should have a sufficient length to infer the nucleotide signatures of SEEP-SRB1a and might therefore prove highly valuable for identifying SEEP-SRB1a sequence bins. Based on these contigs the SEEP-SRB1a genome has a GC content in the range of 38% to 40%. These values are much lower than those of close relatives of SEEP-SRB1a which have GC contents between 51% and 59% (Table S1). The estimated GC content of SEEP-SRB1a is also much lower than the estimated GC contents of ANME-2a (46%) and ANME-2c (53%), indicating that the separation of ANME-2 and SEEP-SRB1a based on GC content alone might prove successful (Tyson et al., 2004).

Manual annotation of the putative SEEP-SRB1a contigs offered a first glimpse of the genetic potential of SEEP-SRB1a. Similar to the gene order in other SRB (Mußmann et al., 2005), the *sat* gene, a gene coding for the third key enzyme of the sulfate-reduction pathway, was identified in close proximity and upstream of the *aprBA* operon. Interestingly, the contig segment starting from the *aprB* gene up to the *sat* gene (Figure 4), features the same gene order as detected in *Desulfococcus oleovorans* Hxd3, the closest fully-sequenced relative of SEEP-SRB1a. The majority of orfs on the *apr* contig code for enzymes most likely involved in energy production and conversion during sulfate-reduction, such as heterodisulfide reductase subunit A (HdrA), methyl-viologen-reducing hydrogenase subunit D (Strittmatter et al., 2009), as well as enzymes responsible for cytochrome *c* synthesis (Figue 4A). *Dsr* genes detected on the second SEEP-

SRB1a contig, feature the *dsrABD* gene order also detected in other SRB (Mußmann et al., 2005). Besides that, this contig contains *tatA* and *tatC* genes, the two key genes for the twin-arginine translocation system (Wu et al., 2000).

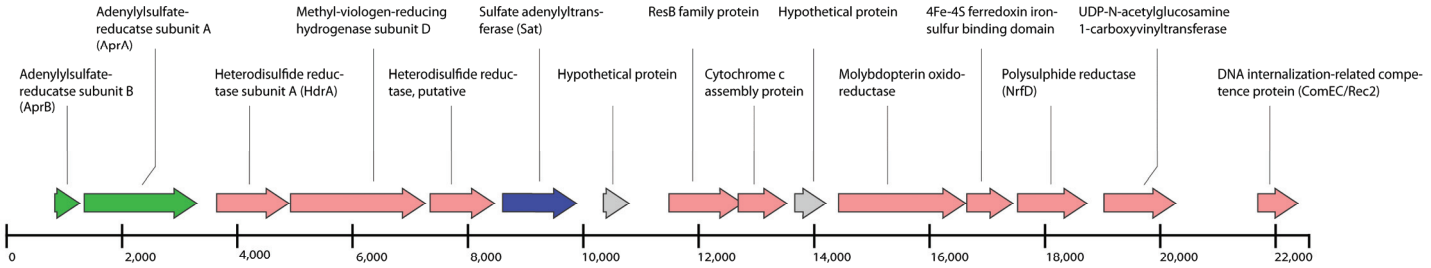
The twin-arginine translocation system is responsible for translocating fully folded proteins across the cytoplasmic membrane (Wu et al., 2000) and is also detected in other SRB, such as *Desulfotalea psychrophila* LSv54 (acc. no. NC_006138), *D. oleovorans* Hxd3 (NC_009943), and several *Desulfovibrio* species (NC_011883, NC_008751, NC_012796, NC_012881). Directly upstream of the *tatAC* genes, a gene encoding for a MltA domain protein was detected. Proteins featuring this domain are involved in cleaving the peptidoglycan cell wall to facilitate cell growth or to create a cell wall opening without a loss of integrity (van Straaten et al., 2007). Other genes on the *dsr* contig include genes encoding for enzymes involved in the biosynthesis of molybdenum-containing enzymes, a cobrinic acid a,c-diamide synthase, and a uracil phosphoribosyl-transferase (Figure 4). In conclusion, the majority of genes on the two contigs are also frequently found in other SRB, such as *D. oleovorans*, and therefore did not allow any conclusion about how SEEP-SRB1a is involved in the AOM process.

For sequence binning based on nucleotide signatures, contigs should have a length that is not significantly compromised by local heterogeneities. When binning sequences based on GC content, a minimum contig length of 10 kbp is suggested to obtain a reliable average unbiased by local fluctuations (Tyson et al., 2004). Applying this observation to the here investigated metagenome only 319 contigs, representing a total of about 6.5 Mbp, would be suitable for bioinformatical binning. The low number of long contigs indicates an insufficient coverage of the enrichment community. One explanation for this could be microdiversity within the enrichment. When performing conventional shotgun sequencing of microbial genomes, all sequence fragments are usually derived from a single clone. In contrast, when applying a shotgun approach to reconstruct microbial genomes from more complex samples, variation within each species population might complicate the assembly process. If this intraspecies variation is limited to local

polymorphisms or homologous recombination, it should still be possible to define a composite genome for a given species population. On the other hand, if the degree of variation between individual species members becomes too large, e.g. caused by large recombinations, or a high number of polymorphisms, the assembly of a composite genome might become impossible.

Retrieved 16S rRNA, *apr* and *dsr* genes sequences from the metagenomic data set showed that there is a considerable amount of microdiversity within the SEEP-SRB1a group. This result was confirmed by 16S rRNA clone (Schreiber et al., 2010) which detected ribotypes similar to those of the pyrosequencing data (Figure 5). The different SEEP-SRB1a ribotypes were most likely introduced during the initial inoculation of the enrichment. Considering an estimated generation time of 6 months (this study), the persistence of different SEEP-SRB1a ribotypes within the enrichment even after an incubation time of 84 months is not surprising. In substrate competition experiments with bacteria with slightly different growth kinetics, more than 70 generations were necessary for one species to completely outcompete the other (Hansen and Hubbell, 1980). Assuming a similar trend and different growth kinetics for the SEEP-SRB1a ribotypes, an incubation time of about 35 years might be necessary in order to obtain a single winning SEEP-SRB1a ribotype. Moreover, it is also possible that the different SEEP-SRB1a ribotypes do not exhibit different growth kinetics under the enrichment conditions. In this case the microdiversity may be maintained indefinitely.

(A) contig F1GTUB102IZ82Q carrying *aprBA* operon



(B) contig F1GTUB101EGDT3 carrying *dsrAB* operon

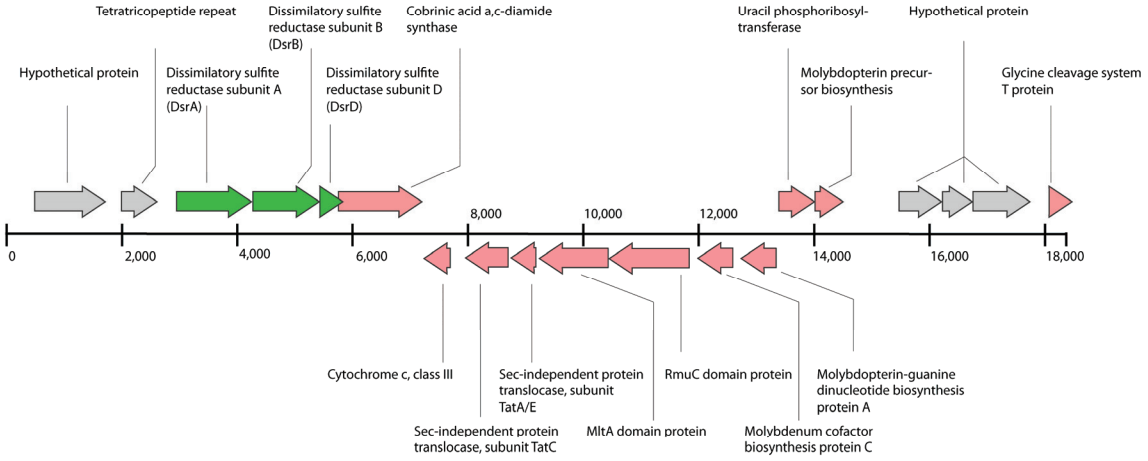


Figure 4. Contigs carrying the putative *aprBA* and *dsrAB* genes of SEEP-SRB1a.

(A) Shown is contig F1GTUB102IZ82Q with a length of 22,580 bp that carries the putative *aprBA* operon (green) of SEEP-SRB1a. Among other genes, the contig also carries a second key gene of the sulfate-reduction pathway, the *sat* gene (blue).

(B) Shown is contig F1GTUB101EGTD3 with a length of 18,340 bp that carries the putative *dsrAB* operon (green) of SEEP-SRB1a.

Genes with a predicted function are shown in pink, while genes coding for hypothetical proteins are shown in grey.

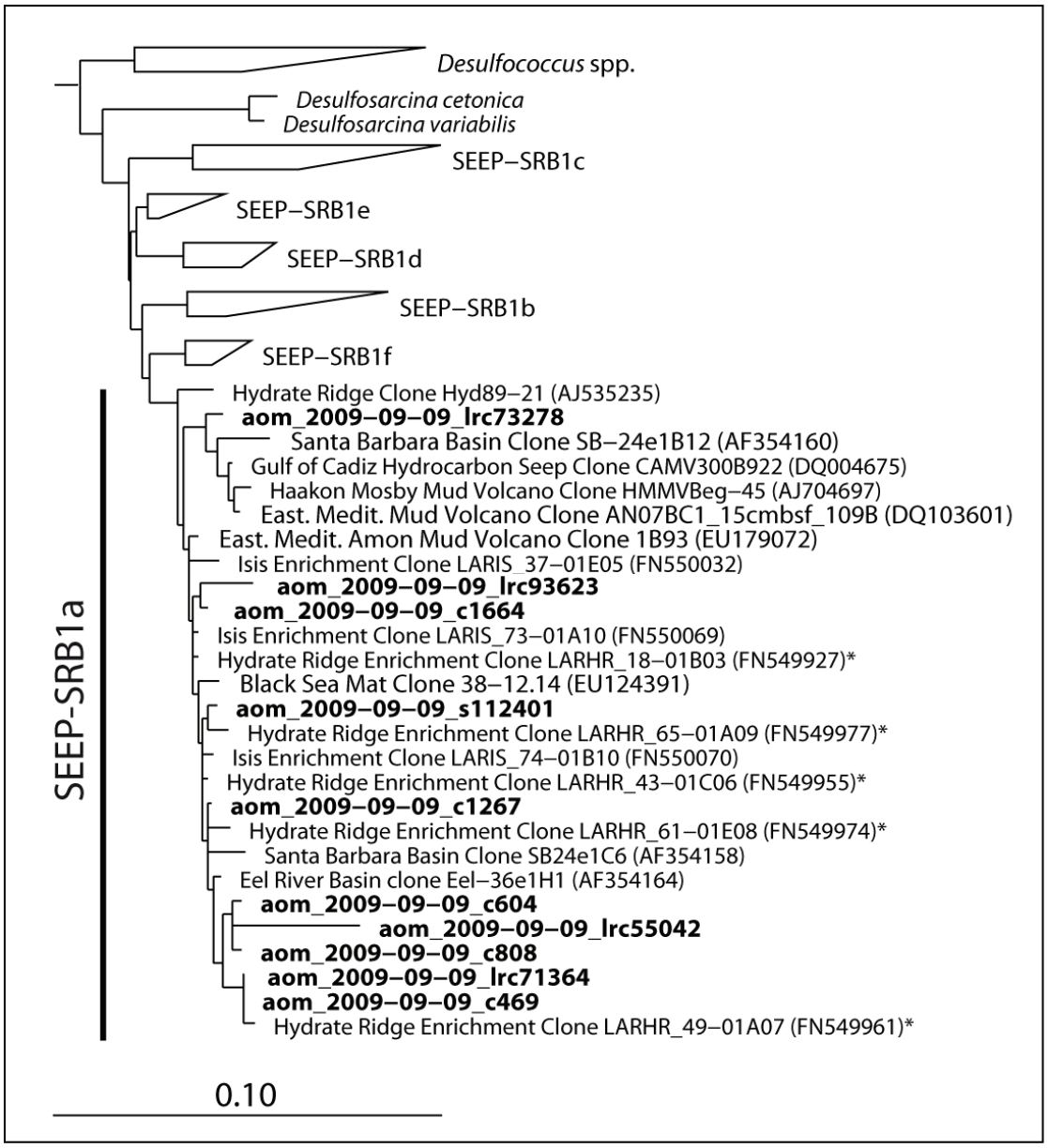


Figure 5. Microdiversity of SEEP-SRB1a in the Hydrate Ridge enrichment culture. Shown are 16S rRNA gene sequences retrieved from the pyrosequencing data set (bold face), a bacterial 16S rRNA gene clone library from the enrichment (marked with an asterisk), and a selected set of related sequences.

An alternative approach for genome reconstruction is the mapping of contigs onto reference genomes of closely-related species (e.g. Pop et al., 2004). Using this approach we could map 9075 contigs onto the genome of *D. oleovorans* Hxd3.

These contigs cover 1425 of 3315 genomic features of *D. oleovorans* and represent 1.17 Mbp of sequence information (Table S3). The low coverage of the *D. oleovorans* genome again suggests that the current metagenome provides only an insufficient coverage of the SEEP-SRB1a genome. Alternatively, however, it is also possible that the SEEP-SRB1a differs considerably from the one of *D. oleovorans* and that the features not covered are simply missing in SEEP-SRB1a. One important problem of the mapping approach is the presence of additional deltaproteobacterial SRB, such as *Desulfosarcina*, *Desulfuromonas*, and *Desulfobulbus*, in the enrichment. Contigs originating from these bacteria might potentially also map onto the genome of *D. oleovorans,* so that a successful mapping not necessarily indicates an origin of SEEP-SRB1a. Despite this problem, the mapping approach might provide an elegant way to further the genome reconstruction of SEEP-SRB1a. As stated above 270 fosmid end-sequences also mapped on the *D. oleovorans.* These end-sequences show a homogeneous coverage of the *D. oleovorans* genome (234 of 3324 genomic features covered; Figure S1, Table S2) and represent 211 fosmid inserts. Assuming an average fosmid insert size of 35 kbp, these clones represent about 7.5 Mbp of sequence data. Even though this translates only to a 1.5x genome coverage (i.e. statistically only *c*. 80% of the SEEP-SRB1a genome would be covered), the fosmids might provide a robust scaffold which can be used as a seed for re-assembling the pyrosequencing data.

CONCLUSION

This study provides the first characterization of a metagenome generated from an ANME-2/SEEP-SRB1a dominated enrichment culture. We identified the SEEP-SRB1a 23S rRNA gene as well as *apr* and *dsr* genes putatively originating from SEEP-SRB1a. In addition we also obtained a first glimpse of the genetic potential of SEEP-SRB1a. However, the metagenome contained too few contigs with sufficient length to reconstruct the genome of SEEP-SRB1a. Possible reasons for this include an insufficient coverage of the genome and microdiversity of SEEP-SRB1a within the enrichment. Therefore, in order to obtain a SEEP-SRB1a genome the generation of more sequence data is necessary. An alternative to generating more pyrosequencing data is the more directed approach of sequencing the full inserts of fosmid clones whose end-sequences map onto close-relatives of SEEP-SRB1a.

EXPERIMENTAL PROCEDURES

Hydrate Ridge enrichment

The AOM enrichment used in this study originated from Hydrate Ridge (NE Pacific, 044° 34.2' N, 125° 08.7' W, taken during RV Sonne cruise SO-148/1 in August 2000) and is described in more detail elsewhere (Nauhaus et al., 2007; Holler et al., 2009).

Fosmid library construction and analysis

DNA was extracted from 31.5 ml of the enrichment culture (*c.* 4.4×10^{11} cells in total) according to the SDS-based DNA extraction protocol by Zhou et al. (1996). The protocol encompassed three cycles of chemical lysis in a high-salt extraction buffer (1.5 M NaCl) by heating of the suspension in the presence of sodium dodecyl sulfate (SDS) and hexadecyltrimethylammonium bromide (CTAB), and a proteinase K step. Prior to the first cycle a lysozyme step was performed. Additionally, prior to the third cycle three freeze/thaw steps (-70°C/42°C) were added. The extracted DNA was purified with a preparative low melting point agarose gel (1%, *Biozym Plaque Agarose*; Biozym, Germany).

A fosmid library was constructed using the CopyControl™ Fosmid Library Production Kit (Epicentre, USA) according to the manufacturer's protocol. In short, purified DNA was end-repaired and then separated by pulse-field gel electrophoresis (PFGE). DNA fragments with a size of ≈30-50 kbp were recovered and ligated into the pCC1FOS vector. After *in vitro* packaging into lambda phages and transfection into *Escherichia coli* strain EPI300-T1^{R}, the bacterial cells were plated onto 1x LB plates containing 12.5 µg/ml chloramphenicol and incubated for 16-18at 37ºC. At the MPI for Molecular Genetics (Berlin, Germany), ≈24,500 fosmid clones were picked and arrayed into 384-well plates using automated colony picking robots. Picked fosmid clones were replicated and then stored at -80ºC. Fosmid insert end-sequences were

determined at the MPI for Molecular Genetics (Germany). Fosmid end-sequences were analyzed using the MG-RAST server (Meyer et al., 2008). For the analysis, a minimum alignment length of 98 bp was chosen to reduce the high number of alignments with phage-associated genes.

Pyrosequencing

Genomic DNA was extracted from the enrichment by using the Genomic-tip 500/G kit in combination with the genomic DNA buffer set (Qiagen, Germany) according to the manufacturer's manual for extracting DNA from gram-negative bacteria. A total of 3 µg of DNA was used for direct sequencing using the GS DNA Library Preparation Kit, following the instructions of the GS FLX Shotgun DNA Library Preparation Manual (Roche Diagnostics, USA). The SP3 processing pipeline (MPI for Molecular Genetics, Germany) was used for vector and quality clipping. Pyrosequecing data were assembled by using MIRA V3rc2 (Chevreux et al., 1999).

Data assembly and analysis

Pyrosequecing raw reads were screened for 16S and 23S rRNA genes using the SILVA NGS pipeline, an unpublished version of the SILVA pipeline optimized for next-generation sequence data (Pruesse et al., 2007). Detected rRNA genes were phylogenetically analyzed by using the ARB software tool (Ludwig et al., 2004) and the release 102 of the ARB/SILVA reference databases LSURef and SSURef (Pruesse et al., 2007).

Pyrosequecing data and fosmid end-sequences were assembled by using MIRA V3rc2 (Chevreux et al., 1999). The contigs were analyzed using the MG-RAST server Meyer et al., 2008 by mapping contigs onto reference genomes of the SEED database. The MG-RAST approach was complemented by a second approach to assign contigs to taxonomic groups: All contigs underwent ORF prediction by Metagene (Noguchi et al., 2006). Taxonomic assignment of contigs was done based on BLASTP and PFAM models by using the *paola* software tool

(Huang, 2009). *Paola* combines results of the taxonomic prediction tools DarkHorse (Podell and Gaasterland, 2007), CARMA (Krause et al., 2008) as well as the newly developed software tool Kirsten (Kinship Reestablishment; unpublished) to predict a taxonomic affiliation for a given contig.

Contigs carrying a 16S rRNA gene of SEEP-SRB1a were detected by using BLASTn with a known 16S rRNA gene sequence of SEEP-SRB1a (accession no. FN549936) as a query. Phylogenetic identity of the detected 16S rRNA genes was confirmed by using the ARB software tool (Ludwig et al., 2004) and publicly available deltaproteobacterial 16S rRNA reference sequences. *Apr* and *dsr* sequences within the metagenome were detected by using the *paola* software tool (Huang, 2009). Phylogenetic affiliations of the *apr* and *dsr* sequences were determined by using the ARB software tool (Ludwig et al., 2004) and, *apr* and *dsr* reference sequences, respectively. Manual annotation of contigs was performed within the JCoast software environment (Richter et al., 2008). The function of individual ORFs was predicted based on hits of BLASTp and PFAM.

Sequence accession numbers

The nucleotide sequence data reported in this paper have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers xxx- xxx

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Supporting Tables

Table S1 Genome characteristics of species closely-related to SEEP-SRB1a, and of ANME and its relatives. [a]currently only genome draft

Species	Family	GC%	Genome size / genes / rRNA operons	References
Sulfate-reducing bacteria				
Desulfosarcina variabilis Montpellier (DSM 2060)	*Desulfobacterales*	51	9.4 Mbp[a] 10,444[a] 2[a]	Craig Venter Institute
Desulfosarcina ovata	*Desulfobacterales*	51	n/d	Kuever et al., 2005
Desulfosarcina cetonicum	*Desulfobacterales*	59	n/d	Kuever et al., 2005
Desulfococcus multivorans	*Desulfobacterales*	57	n/d	Widdel, 1980
Desulfococcus oleovorans Hxd3	*Desulfobacterales*	56	3.9 Mbp 3,323 1	Joint Genome Institute
Desulfococcus biacutus	*Desulfobacterales*	56.5	n/d	Platen et al., 1990
Desulfococcus sp. DSM 8541 (*Desulfobacterium anilini*)	*Desulfobacterales*	59	n/d	Schnell et al., 1989
Anaerobic methane-oxidizers (ANME) and related methanogens				
ANME-1	*Methanosarcinales*	43	3.3 – 3.6 Mbp 3,578 1	Meyerdierks et al., 2010
ANME-2a	*Methanosarcinales*	46	n/d	Meyerdierks et al., 2010
ANME-2c	*Methanosarcinales*	53	n/d	Meyerdierks et al., 2010
ANME-3	*Methanosarcinales* / *Methanomicrobiales*	45	n/d	Meyerdierks et al., 2010
Methanococcoides burtonii	*Methanosarcinales*	43	2.6 Mbp 2,273 3	Joint Genome Institute
Methanosarcina acetivorans	*Methanosarcinales*	43	5.8 Mbp 4,540 3	Galagan et al., 2002
Methanosarcina barkeri Fusaro	*Methanosarcinales*	39	4.8 3,698 3	Maeder et al., 2006
Methanosarcina mazei	*Methanosarcinales*	41.5	4.1 Mbp 3,371 3	Deppenmeier et al., 2002

Table S2 End-sequences mapped onto reference genomes of species closely related with ANME-2 and SEEP-SRB1a.

Reference genome	ES mapped	Corresponding fosmids	Feature coverage of mapped genome
ANME-2 related			
Methanococcoides burtonii DSM 6242	224	202	183 / 2440
Methanosarcina acetivorans C2A	375	311	118 / 4639
Methanosarcina barkeri	60	60	56 / 642
Methanosarcina barkeri str. *fusaro*	34	33	29 / 3722
Methanosarcina mazei Go1	111	105	102 / 3456
SEEP-SRB1a related			
Desulfococcus oleovorans Hxd3	270	211	234 / 3315

Table S3 Contigs mapped onto reference genomes of species closely related with ANME-2 and SEEP-SRB1a.

Reference genome	Contigs mapped	Feature coverage of mapped genome	Total length of mapped fragments
ANME-2 related			
Methanococcoides burtonii DSM 6242	3682	956 / 2440	465.7 kbp
Methanosarcina acetivorans C2A	2322	878 / 4639	274.7 kbp
Methanosarcina barkeri	1107	526 / 2422	125.5 kbp
Methanosarcina barkeri str. fusaro	593	272 / 3722	6.7 kbp
Methanosarcina mazei Go1	1930	781 / 3456	230.7 kbp
(Total)	5952		1103.3 kbp
SEEP-SRB1a related			
Desulfococcus oleovorans Hxd3	9075	1425 / 3315	1169 kbp

Supporting Figures

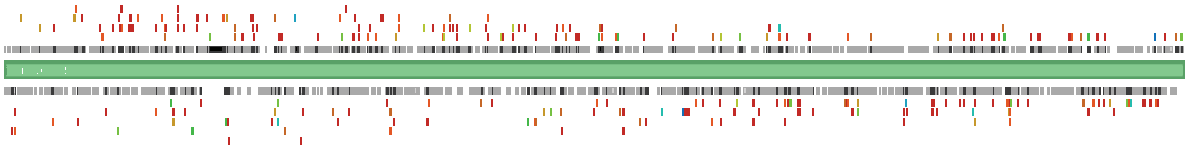

Figure S1. Mapping of fosmid end-sequences onto the genome of *Desulfococcus oleovorans* Hxd3.
The end-sequences represent 211 fosmid inserts and cover 234 of 3315 genomic features of *D. oleovorans*. Source, MG-RAST server.

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V. General Conclusion and Outlook

V. General Conclusion and Outlook

1. Sulfate-reducing bacteria associated with ANME-2

The anaerobic oxidation of methane (AOM) is the main methane sink in marine sediments (Reeburgh, 2007) and is performed by consortia of anaerobic methane-oxidizing archaea (ANME) and sulfate-reducing bacteria (SRB) (Boetius et al., 2000). Prior to this thesis, the identity of SRB associated with anaerobic methanotrophs of the ANME-2 clade was limited to its placement within the *Desulfosarcina/Desulfococcus* (DSS) group (Boetius et al., 2000). As for the position within the DSS group, it was hypothesized that the ANME-2 partner most likely belongs to the SEEP-SRB1 group (Knittel et al., 2003), a group frequently detected in ANME-2 dominated habitats. However, proof for this hypothesis was lacking. During the first part of my thesis, I tested this hypothesis by using the full-cycle rRNA approach. I proposed six subgroups, SEEP-SRB1a to SEEP-SRB1f and designed specific oligonucleotide probes for each subgroup. Using these probes on environmental samples from six different AOM sites, I discovered bacteria from the SEEP-SRB1a subgroup as the sulfate-reducing partner in up to 95% of total ANME-2 consortia. In contrast to that, single SEEP-SRB1a cells were very rare (less than 1% of total numbers of single cells) in all but one of the investigated samples which suggested a highly adapted if not even obligate syntrophic lifestyle of the SEEP-SRB1a group in ANME-2 consortia.

SEEP-SRB1a bacteria were observed as coccoid cells but also as rod/vibrio-shaped morphotypes (Chapter II). This morphological variability likely reflected the genomic variations within the SEEP-SRB1a group (92% 16S rRNA sequence similarity) which might be at the level of genera. Different ANME-clades, species within a particular clade, or environmental parameters seem to select for different strains within the SEEP-SRB1a group. Future FISH studies involving probes of a higher resolution, e.g. by targeting ITS sequences, might be useful to test this hypothesis. In this respect it is also interesting to note that different SEEP-SRB1a strains seem to be able to coexist in AOM habitats. In addition to detecting

different SEEP-SRB1a morphotypes in environmental samples, a similar observation was also made for a Hydrate Ridge enrichment culture in which I detected multiple SEEP-SRB1a strains even after an incubation time of 84 month.

In addition to its association with ANME-2, I also detected SEEP-SRB1a as an alternative partner of ANME-3. This was unexpected, as this ANME clade was previously only reported to be associated with bacteria related to the *Desulfobulbus* genus (Niemann et al., 2006; Lösekann et al., 2007). Unfortunately, due the lack of suitable samples, I could not test if SEEP-SRB1a is also a partner of archaea from the ANME-1 clade. Previous studies have shown that ANME-1 also features a partner from the DSS group. However, Niemann and colleagues (2008) proposed significant differences between lipid biomarkers of ANME-1 and ANME-2 associated DSS-partners. It would be interesting to investigate if the differences reported by the Niemann et al. study (2008) reflect different phylogenetic identities or are caused by environmental factors.

The association between ANME-3 with *Desulfobulbus*-related bacteria (Lösekann et al., 2007) and with bacteria from the SEEP-SRB1a group (this study) showed that there are diverse partnerships between ANME and SRB. In fact, similar observations were made for ANME-2 which was reported to be also associated with *Desulfobulbus*-related SRB, as well as with *Alphaproteobacteria* and *Betaproteobacteria* (Pernthaler et al., 2008). Even though I also observed ANME-2 aggregates with non-SEEP-SRB1a partners, my investigations could not confirm an association between ANME-2 and one of the afore-mentioned groups. This suggests that the association between ANME-2 and *Alphaproteobacteria*, *Betaproteobacteria*, or *Desulfobulbus*-related bacteria might be restricted to certain AOM habitats. Nonetheless, the association of ANME with diverse partners raises the interesting question what factors determine if a bacterium is suited as a partner of ANME? Additionally, it is even today not yet absolutely clear whether the relationship in AOM consortia is a syntrophic one as proposed by Hoehler et al. (1994) or if the bacterial partners are rather commensals feeding on ANME metabolites (Widdel et al., 2007). Answering these questions will be an

important step to elucidate the interaction between ANME and its partners in general and thereby resolve the still puzzling biochemistry of AOM.

One approach to answer these questions would be to assess the genomic potential of the involved microorganisms. Previous studies focusing on the archaeal part of ANME/SRB consortia yielded as yet only a fragmentary understanding of AOM (Hallam et al., 2003; Krüger et al., 2003; Hallam et al., 2004; Meyerdierks et al., 2005; Pernthaler et al., 2008; Meyerdierks et al., 2010). Therefore, for my thesis, it was decided to instead focus on the genomic potential of the involved SRB or more precisely of the SEEP-SRB1a group.

Prior to this thesis genomic data of ANME-associated SRB was limited to a single metagenomic study of AOM consortia which provided only little information on the associated SRB (Pernthaler et al., 2008). Thus, the second part of my thesis aimed at reconstructing the genome of bacteria from the SEEP-SRB1a group. Since no pure culture of SEEP-SRB1a bacteria existed, I used a metagenomic approach to attempt this genome reconstruction. The source DNA for this study originated from an AOM enrichment culture in which ANME-2 and its DSS partner formed together more than 99% of total cell numbers (Holler et al., 2009). The metagenomic approach I used combined the construction of a large insert fosmid library with next-generation pyrosequencing. Almost 590 Mbp of sequence data was thus generated. However, only about 0.3% of the assembled sequences featured a length of ≥10 kbp and would therefore have been suited for a genome reconstruction by bioinformatic binning (Tyson et al., 2004). Phylogenetic analysis of the generated data as well as 16S rRNA gene clone libraries (Chapter II) suggested microdiversity within the ANME-2 and SEEP-SRB1a groups in the enrichment culture as the cause for the high number of short contigs (Chapter IV). Consequently, additional sequencing will be needed in order to reconstruct the genome of SEEP-SRB1a from the used enrichment culture. An alternative to generating more pyrosequencing data is the more targeted approach of sequencing the 211 fosmid clones whose end-sequences were successfully mapped onto *Desulfococcus oleovorans* Hxd3, the closest fully-sequenced relative

of SEEP-SRB1a. Alternatively, the use of single-cell techniques might also provide a way to reconstruct the genome of SEEP-SRB1a. Even though SEEP-SRB1a is usually only found aggregated with ANME 2 (Chapter II), I discovered that single SEEP-SRB1a cells can be generated from the enrichment culture by a protocol combining aggregate homogenization and sonication. Isolation of single SEEP-SRB1a cells followed by whole genome amplification would circumvent the problem of microdiversity and would even permit a comparison of different SEEP-SRB1a strains.

Even though only fragments of the genome of SEEP-SRB1a could be reconstructed, I detected 11 contigs carrying SEEP-SRB1a 16S rRNA genes within the metagenomic data set. Unfortunately all of these contigs had a length ≤10 kbp and were therefore not suited to serve as "anchor" contigs in a bioinformatic binning approach. However, on two of the contigs, I identified the 23S rRNA gene of SEEP-SRB1a which could serve as an alternative genetic marker for the group in future studies. When assessing the diversity of Apr and Dsr genes (two key genes of the sulfate-reduction pathway) within the data set, two dominant sequence clusters were detected. Their dominance and their position within the *Desulfobacteraceae* suggested that both originated from the SEEP-SRB1a group. The retrieval of the same *apr* gene from SEEP-SRB1a cells isolated by fluorescence-activated cell sorting (FACS) confirmed the SEEP-SRB1a origin of the dominant Apr cluster (Chapter III). Interestingly, a study by Basen (2010) focusing on ANME-2 dominated Black Sea mat sections, identified the Apr of the SEEP-SRB1a group as the dominant Apr enzyme in these mats.

The identification of SEEP-SRB1a Apr and Dsr genes enabled to take a first look at the genomic potential of these bacteria as I detected two contigs with lengths of 18.3 kbp and 22.6 kbp carrying the putative Apr and Dsr genes of SEEP-SRB1a, respectively. The majority of predicted genes on these contigs coded for enzymes involved in energy production and conversion. Besides that genes for the twin-arginine translocation system were also detected. Unfortunately, most of the annotated genes are also frequently found in other SRB (e.g. *Desulfococcus*

oleovorans, *Desulfotalea psychrophila*, and several *Desulfovibrio* species) so that it was not possible to infer the role of SEEP-SRB1a in the AOM process or how to obtain SEEP-SRB1a in pure culture based on this data.

2. Sulfate-reducing bacteria in AOM habitats

Previous studies have shown that AOM habitats also contain a diversity of SRB not associated with ANME (Knittel et al., 2003; Treude, 2003). I contributed to these studies and investigated the diversity of SRB in microbial mats from the Black Sea, Hydrate Ridge sediment, and two Hydrate Ridge enrichment cultures. To study these habitats, I constructed *aprA* and *dsrAB* clone libraries from a Black Sea mat sample and a Hydrate Ridge enrichment culture. These data were then combined with *apr* and *dsr* data previously generated by Friedrich Reinhard (2005) which originated from non-enriched Hydrate Ridge sediment, a second Hydrate Ridge enrichment culture, and two additional mat samples from the Black Sea.

Most of the retrieved AprA and DsrAB sequences affiliated with clusters that possessed no cultured representative and could, if at all, only be assigned down to the family level. Because of that and previously described events of horizontal gene transfer (Klein et al., 2001; Friedrich, 2002; Meyer and Kuever, 2007), the approach of using *aprA* and *dsrAB* as molecular markers is currently limited in its ability to predict the presence of phylogenetic lineages of SRB. The lack of cultured representatives also made an ecological interpretation of the data difficult since these unidentified sequences most likely represent SRB with currently unknown physiological properties. Thus, in order to gain a better understanding of the ecology of SRB, future studies should focus on linking AprA and DsrAB clusters with phylogenetic identities as well as on culturing currently uncharacterized SRB.

When comparing the results from different AOM habitats, I observed that the respective SRB communities were highly heterogenous and only featured similarity scores of ≤31% based on the presence of *aprA* and *dsrAB* sequence clusters (Chapter III). Clone libraries from Black Sea mats were dominated by

sequences from within the *Desulfobacteraceae*, the cluster also harboring the sulfate-reducing partners associated with ANME-1 and ANME-2. Nonetheless, sequences within the *Desulfobacteraceae* showed a high diversity and affiliated with several different clusters. The diversity of SRB within the mat sections was comparable to the diversity within the two Hydrate Ridge enrichment cultures and considerably lower compared to non-enriched Hydrate Ridge sediment. This showed that Black Sea mats represent naturally enriched microbial communities.

Clone libraries from Hydrate Ridge enrichment cultures were also dominated by *Desulfobacteraceae* (98-100% of the analyzed clones); however even in these enrichments a considerable diversity within this family was detected. Besides the putative SEEP-SRB1a *apr* and *dsr* sequence clusters (Chapter III&IV), both enrichments also contained a considerable number of other *Desulfobacteraceae* sequences. It is currently not clear if these other sequences simply represent SEEP-SRB1a microdiversity within the enrichments or if they originated from other *Desulfobacteraceae* groups.

Another integral part of this part of my thesis was to identify the *aprA* and *dsrAB* genes of SEEP-SRB1a. Even though my previous metagenome study already suggested the identity of SEEP-SRB1a's AprA and DsrAB genes, a final proof of this association was missing. Thus, I isolated SEEP-SRB1a cells from a Hydrate Ridge enrichment culture by using FACS. The isolated cells were then used in a multiplex polymerase chain reaction (PCR) to identify the AprA and DsrA genes of these cells. Even though the amplification of the DsrA and the 16S rRNA genes failed, I was able to successfully amplify and sequence the AprA gene from the isolated SEEP-SRB1a cells. All of the obtained AprA sequences affiliated with the putative SEEP-SRB1a cluster from the metagenome study and thereby confirmed the phylogenetic identity of this cluster. The success of this method to identify functional genes of an uncultured bacterium might be very helpful for future studies. In that respect, the sulfate-reduction genes of many other AOM-associated SRB, such as bacteria from the SEEP-SRB2, 3 and 4 groups (Knittel et

al., 2003), are still unidentified and would therefore provide interesting targets for future studies.

3. Single-cell techniques

One of the main topics of this thesis was assessing the genomic potential of SRB associated with ANME-2. Since these bacteria were at the time of this thesis not available in pure culture, several culture-independent genomic approaches were tried to reach this goal. Besides the eventually used metagenomic approach, I also tested so-called single-cell approaches which were previously successfully used to characterize uncultured microorganisms (Raghunathan et al., 2005; Marcy et al., 2007; Gloess et al., 2008; Rodrigue et al., 2009; Woyke et al., 2009; Woyke et al., 2010).

My first attempts in this direction involved the use of laser-capture microdissection (LCM). The goal of these experiments was to isolate ANME-2/DSS aggregates, amplify their DNA by whole genome amplification (WGA), and finally identify the bacterial DSS-partner by PCR. Even though, LCM was successfully applied to isolate aggregates and subsequent WGA yielded high amounts of DNA (unpublished), I was not able to obtain amplicons of 16S rRNA genes of either SRB or ANME-2 cells. Instead only 16S rRNA genes of contaminant bacteria such as *Propionibacterium acnes* or *Stenotrophomonas maltophila* were detected (unpublished).

Similar results were obtained when using FACS as a second single-cell technique to analyze SEEP-SRB1a cells to identify their sulfate-reduction genes (Chapter III). Even though, this approach eventually succeeded in obtaining the *aprA* gene of SEEP-SRB1a, I again only retrieved 16S rRNA genes of contaminating bacteria (Chapter III). One possible explanation for these results is the used approach to identify the target cells in both methods. My approach included formaldehyde fixation of the sample followed by an identification by CARD-FISH. Unfortunately, formaldehyde fixation (Chang and Loew, 1994; Bucklin and Allen, 2004) as well as radicals generated during CARD-FISH

(Pernthaler et al., 2002; Demple and Harrison, 2003) most likely damaged the target DNA to a degree that contaminating DNA was more readily amplified. If this was the case, a recently reported fixation-free FISH protocol which does without the CARD amplification step (Yilmaz et al., 2010) might provide a solution to minimize damage of the target DNA in future studies. Alternatively, an *a posteriori* identification of the target cells (as described by Marcy et al., 2007 and Woyke et al., 2009) also provides a possible solution. In this case, random single unfixed and unlabelled cells are isolated from environmental samples and are only afterwards identified by molecular genetic techniques.

When using single-cell techniques, the problem of contamination and bad quality of target DNA is enhanced when WGA is necessary to obtain sufficient DNA for sequencing. The WGA protocol currently used most often employs multiple displacement amplification (MDA) to amplify DNA. MDA is capable of yielding micrograms of DNA from single bacterial cells (Raghunathan et al., 2005) and its amplification bias is sequence-nonspecific (Zhang et al., 2006). Despite these advantages, my attempts using MDA to amplify DNA of isolated cells, confirmed earlier studies which reported a high susceptibility of MDA for contaminations (Marcy et al., 2007). Even when employing elaborate measures to avoid contaminations, negative controls frequently also yielded high amounts of DNA (Raghunathan et al., 2005). Secondly, when using isolated cells as MDA template, I could only retrieve DNA from contaminating bacteria after MDA. During this thesis, I identified several reasons for these observations. First of all, it is nearly impossible to totally avoid contamination. Bacteria are all around us and are even found in clean rooms with the highest classification level (Moissl et al., 2007). Even when specifically isolating single cells, contaminating DNA attached to these cells or present in reagents is difficult to avoid (Marcy et al., 2007). In addition, while target cells will often have gone through several potentially DNA damaging steps (fixation, identification), contaminating DNA introduced late in the pipeline will have a better quality and is likely less fragmented. Considering MDA's inefficiency to amplify fragmented DNA <2 kb in size (Qiagen), this

contaminating DNA will not only be present in the MDA product, but also be severely overrepresented. The best way to currently reduce this problem is the above mentioned *a posteriori* identification approach of the target cells. Even this approach must then however be complemented by bioinformatic approaches to distinguish between target and contaminating DNA.

4. Outlook

During the preparation of this thesis, the world of microbial ecology was revolutionized by the advent of next-generation sequencing and single-cell technologies which both significantly improved our ability to characterize uncultured microorganisms. Next-generation sequencing platforms drastically reduced the costs of sequencing and made metagenome projects feasible even for small labs. With the increased use of metagenomics, however, also new challenges were identified. The first challenge, in that respect, is the immense diversity of microorganisms in environmental samples (Curtis et al., 2002). Probability theory states that a genome coverage of *c*. 4.5 is needed to achieve a probability of 99% that a genomic library contains all genes of the original genome. Applying that observation to a metagenomic study, an assumed environmental sample with 2,000 different species, a homogenous species distribution, and an average genome size of 4 Mbp, would require a metagenomic dataset with a minimum size of 36 Gbp Even though this number does not seem insurmountable in the light of next-generation sequencing technology, it is still almost six times more sequence data than generated during the global ocean sampling expedition (Rusch et al., 2007). Even if such a dataset was generated, it is still an open question how to analyze such datasets as the sheer amount of data would also require an immense amount of computing and man power.

When interested in specific microorganisms from the environment, newly developed single-cell techniques provide a way to circumvent the problems of metagenomic studies when dealing with microbially diverse samples. However, even with the current ability to amplify and sequence DNA from single bacterial

cells, our ability to predict the ecological function or the physiology of the corresponding microorganisms is severely limited. The main reason for this is our approach for annotating microbial genomes which is entirely based on finding similarities with genes of known function. This approach can therefore not succeed in discovering novel biochemical pathways or phenotypic features. Unfortunately, these are potentially the characteristics that define the function of the target microorganism in its environmental setting. Another drawback is that, if there is no pure culture of the sequenced microbe available, there is no way to confirm the correctness of a predicted gene function. Also, even if the majority of genes are annotated correctly, only an idea of the genetic potential of the target is obtained. In order to link this potential to actual function, however, a combination of genomics and gene expression-based approaches, such as transcriptomics or proteomics, is necessary. Gene-expression analysis of uncultured microorganisms can be performed by mRNA-FISH (Pernthaler and Amann, 2004), metatranscriptomics (Vila-Costa et al., 2010) or metaproteomics (Ram et al., 2005; Benndorf et al., 2007; Wilmes et al., 2008). Combining these approaches with metagenomics and single-cell genomics, will be an important step to improve our understanding of microbial ecology. In respect to microorganisms involved in AOM, a combination of genomics and gene-expression based methods will hopefully yield clues on how to obtain pure cultures of these microorganisms. Such pure cultures remain the holy grail of AOM as only they will allow detailed physiological analyses of the involved microorganisms and resolving the biochemistry of the AOM process.

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