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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

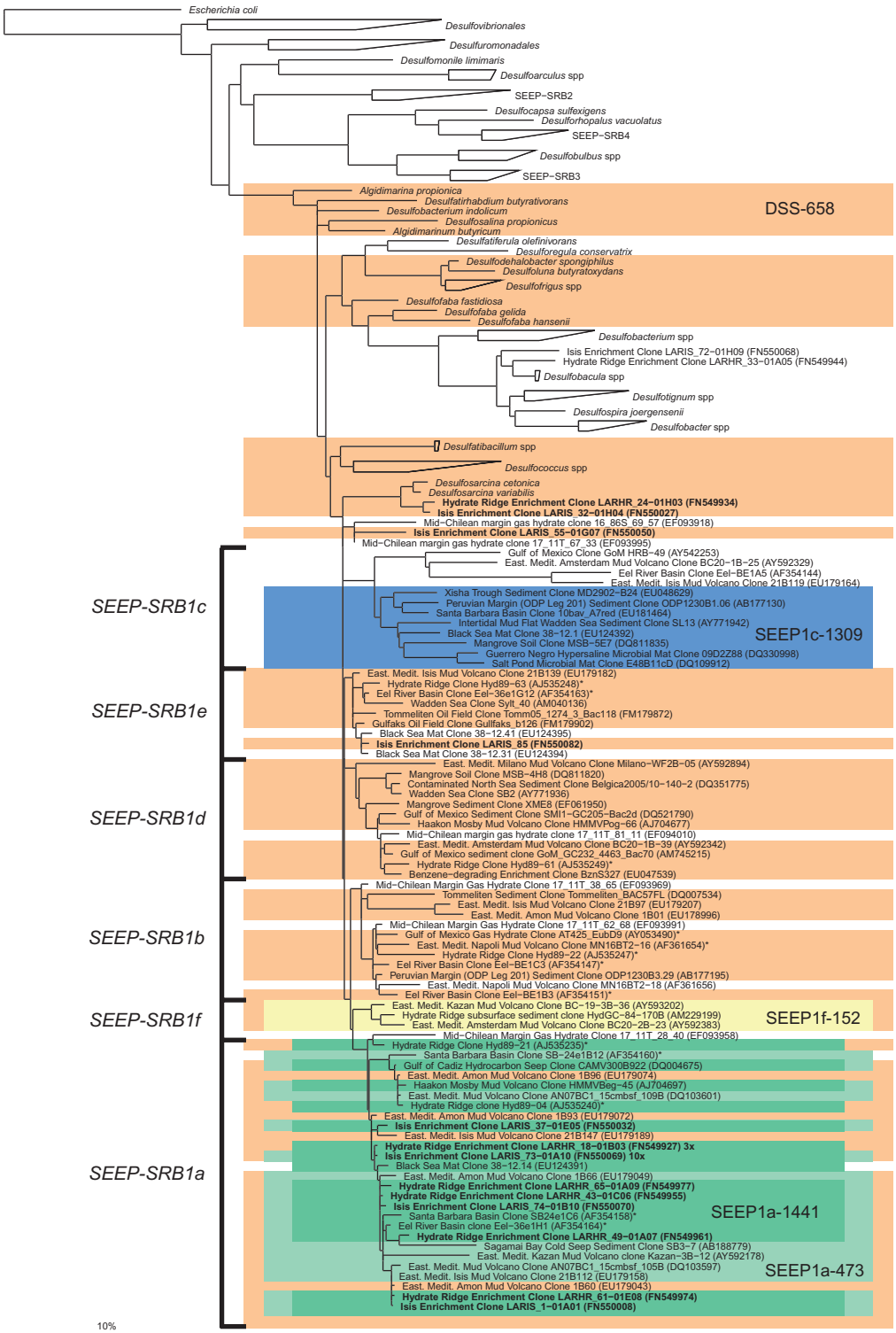

Fig. 1. Tree showing the phylogenetic positions of six SEEP-SRB1 subgroups compared with related reference sequences of the *Deltaproteobacteria*. Sequences within SEEP-SRB1 were selected in order to represent major habitats of the SEEP-SRB1 subgroups. Selected 16S rRNA sequences obtained from Hydrate 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.

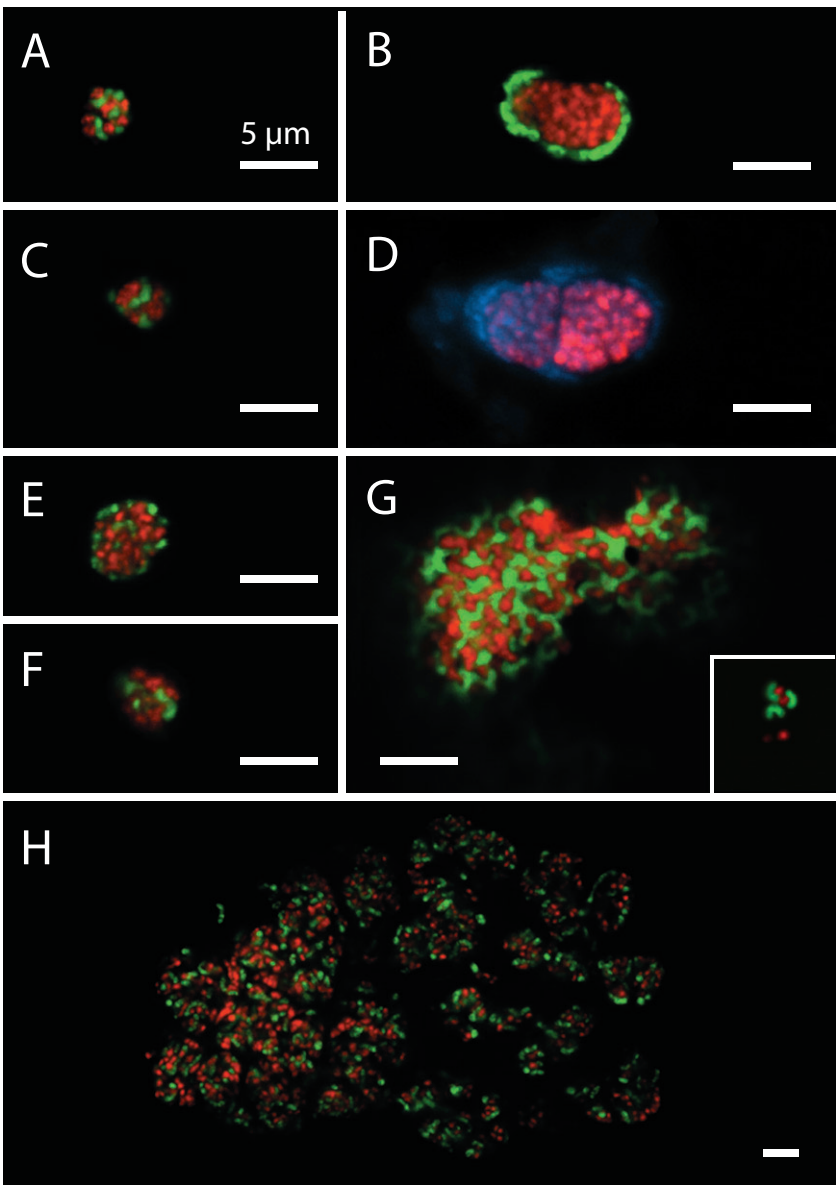

Fig. 2. Cell aggregates of ANME-2 and ANME-3 in AOM enrichments, a Black Sea mat, and sediments from Hydrate Ridge, the Gulf of Mexico, the Gullfaks oil field and the Haakon Mosby Mud Volcano, visualized by CARD-FISH.
A. ANME-2/SEEP-SRB1a aggregate as detected by using probe ANME2-538 (red) and probe 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)

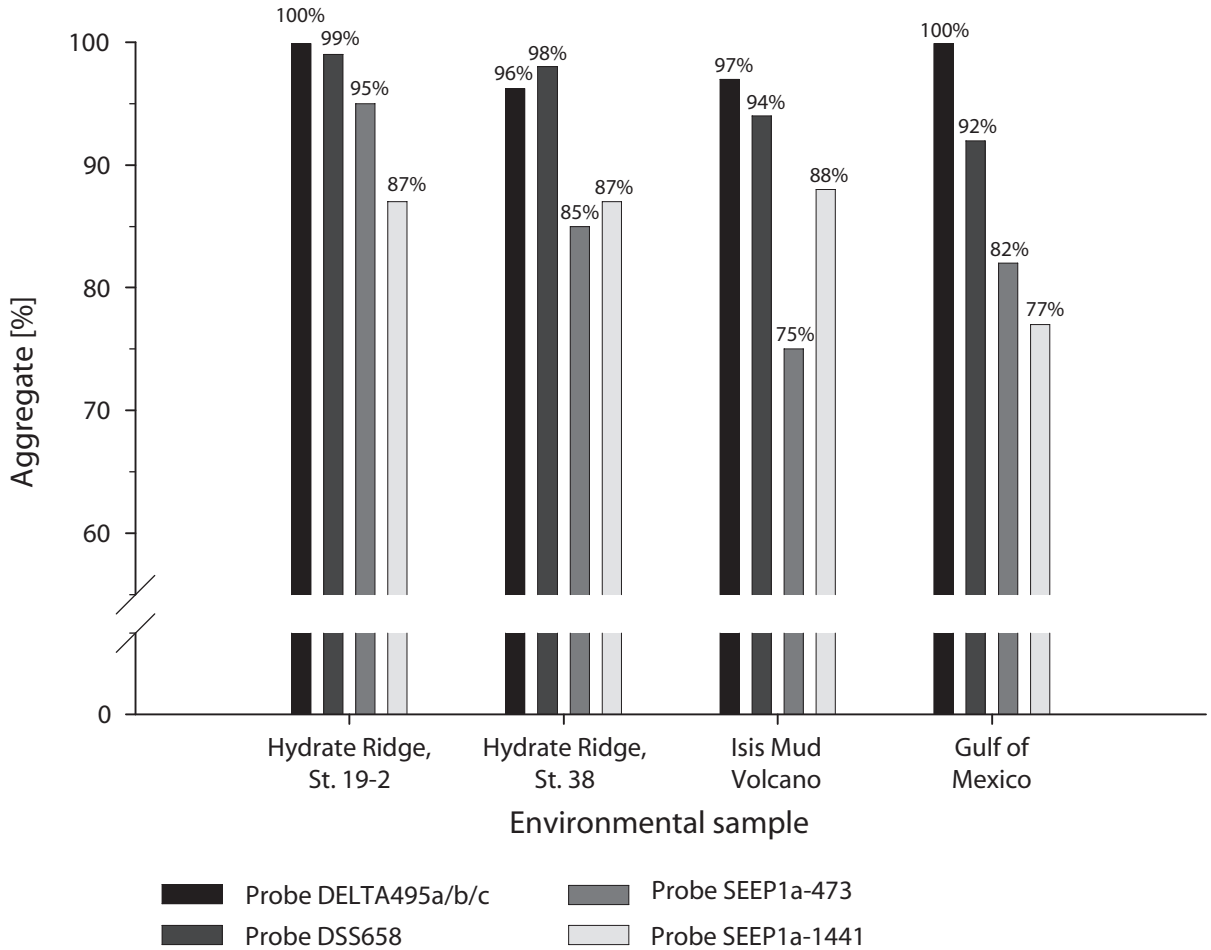

Fig. 3. ANME-2-partners in the environment. The graph shows the percentages of ANME-2/*Deltaproteobacteria* (probes: ANME2-538, DELTA495a/b/c), and ANME-2/DSS (probes: ANME2-538, DSS658) aggregates (Table S4) as well as of ANME-2/SEEP-SRB1a detected with probe SEEP1a-473, or probe SEEP1a-1441 (Table 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.
Table S4. Non-SEEP-SRB1 oligonucleotide probes used in the present study.
Table S5. Average cell and aggregate sizes and aggregate percentages in sediments at different AOM sites.
Table S6. Clones used for Clone-FISH.

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