Metabolismus und Biomineralisation in anaerob Methan-oxidierenden Lebensgemeinschaften

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Abbreviations

% (v/v)	% (volume/volume)
% (w/v)	% (weight/volume)
<i>A.</i>	<i>Archaeoglobus</i>
AMP	adenosine monophosphate
ANME	anaerobic methane oxidizer
AOM	anaerobic oxidation of methane
AprA	APS reductase α -subunit
AprB	APS reductase β -subunit
APS	adenosine-5'-phosphosulfate/ammonium peroxodisulfate
ATP	adenosine triphosphat
BSA	bovine serum albumin
CDP-Star	disodium 2-chloro-5-(4-methoxyspiro{ 1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1 ^{3,7}]decan}-4-yl)-1-phenyl phosphate
<i>D.</i>	<i>Desulfovibrio</i>
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleic triphosphate
DSS	<i>Desulfosarcina/Desulfococcus</i>
<i>E.</i>	<i>Escherichia</i>
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy/microscope
EPS	extracellular polymeric substances
FE-SEM	field emission scanning electron microscopy/microscope
FISH	fluorescence <i>in situ</i> hybridization
ICM	intracytoplasmatic membranes
IgG	immunoglobulin G
IPTG	isopropyl- β -D-thiogalactopyranoside
LB	Luria Bertani
LEW	lysis/equilibration/wash

<i>M.</i>	<i>Methanosarcina/Methanospirillum/Methylobacterium</i>
MCR	methyl coenzyme M reductase
MDH	Methanol dehydrogenase
NMS	nitrate mineral salts
NTA	nitrilo-tri-acetic acid
OD _x	optical density, measured wavelength $\lambda = x$ nm
OTU	operational taxonomic unit
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)
pMMO	particulate methane monooxygenase
PP _i	pyrophosphate
PVDF	polyvinylidene difluoride
PWS	phosphotungstic acid
RT	room temperature
SDS	sodium dodecyl sulfate
sMMO	soluble methane monooxygenase
SMTZ	sulfate-methane transition zone
SRB	sulfate reducing bacteria
TAE	Tris-acetate-EDTA
<i>Taq</i>	<i>Thermus aquaticus</i>
TB	Transformation buffer
TBS	Tris-buffered saline
TED	Tris-(carboxymethyl)-ethylenediamine
TEM	transmission electron microscopy/transmission electron microscope
TEMED	N,N,N',N'-tetramethylethylenediamine
T _m	melting temperature
Tris	tris-(hydroxymethyl)-aminomethane
ZAP	Zellaufschluss-Puffer (cell lysis buffer)

In addition, leaks from near-surface petroleum deposits and release of methane from clathrate hydrates represent methane sources (Reeburgh, 2007).

In contrast to terrestrial methane sources, oceans emit just a small part (2 %) of methane to the atmosphere due to consumption of methane before emission (Ehhalt, 1978; Lambert and Schmidt, 1993; Bange et al., 1994). The annual rate of methane production in the oceans is calculated to 85–300 Tg methane of which ≥ 90 % is consumed (Hinrichs and Boetius, 2002; Reeburgh, 2007).

Four major methane sources of about equal relevance contribute to the atmospheric inventory, namely: natural wetlands and tundra, animals (especially ruminants), anthropogenic land use (especially rice production), and fossil methane (natural gas and coal mining; Fung et al., 1991). In marine habitats coastal runoff, organic-rich anoxic sediments and seeps, vents and mud volcanoes emitting methane rich gas or fluids are major methane sources. However, the effective microbial consumption lowers the concentration of methane of most oceanic habitats to nanomolar level, undersaturated with respect to the atmosphere (Reeburgh, 2007). Nevertheless it remains uncertain, if rapid release of methane from clathrate hydrates in the future caused by warming can be microbially consumed (Dickens, 2003).

1.1.2. Methane cycling in earth history

Anaerobic oxidation of methane (AOM) results in carbonate formation (see section 1.3.1.). This is the main reason why deposits of ancient seeps are still preserved. To identify limestones formed by methane seepage several aspects have to be considered: (a) the geological setting, (b) the occurrence of characteristic faunal assemblages (Campbell and Bottjer, 1995), (c) specific carbonate fabrics and phases (e.g. inverted stromatoloid cavities), (d) low $\delta^{13}\text{C}$ values of early diagenetic carbonate phases and (e) characteristic ^{13}C -depleted biomarkers (Peckmann and Thiel, 2004). Most ancient methane seepage deposits are described for Cenozoic strata, few are known from Mesozoic strata (Gaillard et al., 1992; Campbell and Bottjer, 1993; Kelly et al., 1995; Kauffman et al., 1996; Kelly et al., 2000; Campbell et al., 2002). The oldest methane dependent deposits with biomarker evidence were tracked back to the Jurassic

(Peckmann et al., 1999b; Birgel et al., 2006). Nevertheless Palaeozoic deposits of methane seeps were demonstrated, too (Peckmann et al., 1999a; Peckmann et al., 2001a). One study is implicating AOM also in the formation of carbonate systems from the Archaean (approximately 2.7 billion years old), based on low $^{13}\text{C}_{\text{org}}$ isotopes (Hinrichs, 2002).

Isotopic data of kerogens and organic carbon reveal an important role of methanotrophy 2.7 billion years ago. It is assumed that with the increase of oxygen concentration in ocean water, methanotrophic bacteria were able to spread (Hayes, 1994). In spite of raising aerobic methanotrophy, the main process of Archaean carbonate formation was suggested to be AOM. This hypothesis is supported by two observations: (a) also in the present oxic oceans, carbonate accumulations are based on AOM metabolism, (b) at the beginning of Proterozoic a significant change in the sulfur cycle came up, putatively based upon AOM activity (Hinrichs, 2002; Hinrichs and Boetius, 2002).

A hypothesis for temporal evolution of biogeochemical cycles is depicted in Fig. 1.1. (Hinrichs and Boetius, 2002). On the left in Fig. 1.1. the sulfate-methane transition zone (SMTZ) is shown, as it is found in almost all anoxic aquatic systems (Reeburgh, 2007). The SMTZ represents the ecological niche for AOM associated organisms. Methane is reduced with sulfate as electron acceptor. Thus, both methane oxidation rate and sulfate reduction rate show a peak in the SMTZ. During the Archaean, fermentation may have been the only relevant process of degradation of organic matter. Methane and carbon dioxide were final products, quite stable due to only few available biologically utilizable electron acceptors. With oxygenation of the earth by oxygenic photosynthesis, also the sulfate concentration increased and enabled methane oxidation with sulfate as electron acceptor. For this time period it is assumed that sulfate was predominately reduced by methane oxidation. Even today, in certain sediments the sulfate is completely metabolized by AOM (Aharon and Fu, 2000; Boetius et al., 2000). Besides sulfate, also other oxidized compounds (e.g. iron or nitrate) promoted anaerobic respiration while fermentation processes decreased. Hence, also methane production per mole C_{org} was reduced. Due to reduced methanogenesis and consumption of accumulated methane, AOM activity declined. In conclusion AOM in marine sediments as well as in earth's history shows the highest activity, where high methane and sulfate concentrations are co-occurring (Hinrichs and Boetius, 2002).

Figure 1.1.: Methanotrophy over the time (Hinrichs and Boetius, 2002).

Left: geochemical profile and sulfate reduction/methane oxidation rate. The high rate of sulfate reduction and methane oxidation is due to AOM activity in the sulfate-methane transition zone (SMTZ) where methane gets in contact to sulfate. Right: hypothetical temporal evolution of carbon cycle in earth's history. First microbial processes were based on fermentation leading to an accumulation of methane. With increasing concentrations of oxidized compounds AOM increased, too. The high AOM activity decreased with methane production, since fermentation processes were mainly displaced by microbial respiration (Figure from Hinrichs and Boetius, 2002; with kind permission of Springer Science + Business Media).

1.2. Microbial processes involved in methane turnover

Estimations vary, but approximately 85 % of the annual methane production and approximately 60 % of its consumption is based on microbial processes (McDonald et al., 2008; Knittel and Boetius, 2009). Ongoing studies mainly aim at metabolic processes, participating microorganisms and fluxes of substrates and products.

Four microbial key processes are presently known (cf. Reeburgh, 2007): (a) the formation of methane by methanogenic archaea (Thauer et al., 2008), (b) the aerobic

consumption of methane by methanotrophic bacteria (Hanson and Hanson, 1996), (c) the anaerobic consumption of methane by syntrophic consortia of anaerobic methane oxidizers (ANME) and sulfate reducing bacteria (SRB; Knittel and Boetius, 2009), (d) anaerobic consumption by bacteria of the NC10 phylum (Ettwig et al., 2009; Ettwig et al., 2010). Especially the recently discovered AOM turned out to be very important in carbon cycling. Over 90 % of the microbially produced methane in the oceans is consumed by this process (Hinrichs and Boetius, 2002).

Figure 1.2.: Methane turnover in global cycle (Thauer et al., 2008).

Continuous arrows indicate a reaction and dashed arrows indicate diffusion and/or convection. 1 Gt methane is produced by methanogenesis sourced from organic compounds. In addition 1 Gt is released from methane hydrates. From this amount 1 Gt is oxidized by AOM and 0.6 Gt by aerobic bacteria. Remaining 0.4 Gt and 0.2 Gt from other sources reach the troposphere (Lelieveld et al., 1998; Lee and Holder, 2001; Krüger et al., 2005; Thauer et al., 2008). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Thauer et al., 2008), copyright 2008.

The metabolic key pathways of the methane cycle are depicted in Fig. 1.2. Dissociation of methane hydrates and methanogenesis produce approximately 2 Gt of methane per year. Approximately 0.6 Gt are oxidized to CO₂ by aerobic bacteria, approximately 1 Gt is oxidized by anaerobic archaea and approximately 0.4 Gt are released to the atmosphere. Further 0.2 GT are emitted by other sources, such as industrial gas leakages or biomass fires (Lelieveld et al., 1998; Lee and Holder, 2001; Krüger et al., 2005; Thauer et al., 2008). These data stress that the importance of AOM is not limited to marine environments, but represents a crucial part of global methane degradation.

1.2.1. Methanogenesis

The formation of methane is restricted to methanogenic archaea, a phylogenetical diverse group of strictly anaerobic Euryarchaeota (Garcia et al., 2000). They are classified in the five orders Methanobacteriales, Methanococcales, Methanomicrobiales, Methanopyrales and Methanosarcinales. Carbon dioxide and hydrogen, formate, methanol, methylamines and acetate represent the compounds for methane formation (Thauer, 1998). The compounds are metabolized by only three methanogenic pathways, namely: reduction of carbon dioxide, methyl-group reduction and the acetoclastic reaction. Only members of the Methanosarcinales ferment acetate to carbon dioxide and methane with C₁ compounds (methanol, methylthiols or methylamines) as sole energy sources (Thauer, 1998; Liu and Whitman, 2008). As depicted in Fig. 1.2. the methanogenic archaea hold an important role in global methane cycle by producing annually approximately 1 Gt methane. Methanogenesis represents the final step of anaerobic biomass degradation (Eq. 3; Thauer, 1998):

Biomass is degraded in anaerobic environments by syntrophic associations of microorganisms. After degradation of biopolymers to monomers, the organic compounds are fermented where concentrations of electron acceptors like sulfate, nitrate, oxidized manganese or iron are low. In the presence of these oxidized compounds, methanogenesis is out-competed by anaerobic respiration due to thermodynamic reasons (Schönheit et al., 1982; Thauer et al., 2008). That is why in the

upper layer of marine sediments, where sulfate concentration is high, methanogenesis is restricted to substrates such as methylamines, which are not metabolized by sulfate reducing bacteria. The prevalent substrate cellulose, for example, is degraded by microorganisms to the monomer glucose. Then, glucose is fermented to acetate, carbon dioxide and hydrogen or to acetate, formate and hydrogen (Eq. 4 and Eq. 5; Thauer, 1998):

In a second step the products are converted by methanogenic archaea to methane (Eq. 6,7,8):

C₁ compounds get disproportionated to carbon dioxide and methane or get reduced to methane with H₂ (cf. Eq. 9 and 10, exemplary for methanol; Thauer, 1998):

The methanogenic archaea represent the only group of microorganisms capable of this metabolic pathway, but have no alternative ways for energy conservation. Thus, they are strictly limited to their ecological niche. In addition, methanogenic archaea are very sensitive to small amounts of oxygen. Nevertheless, they are able to survive in aerated

natural habitats. In that case, activities of other organisms using oxygen for respiration can protect methanogens by providing anoxic microenvironments (Brusa et al., 1987; Belay et al., 1988; Whitman et al., 2006).

1.2.2. Methanotrophic bacteria

The aerobic microbial oxidation of methane has been already discovered by the beginning of the 20th century. The first methanotrophic bacterium was isolated by Söhngen (1906). Thus, today there is already extensive knowledge, in comparison to other methane oxidation pathways, about ecology, physiology and genetic as well as enzymatic background of aerobic methanotrophy (Hanson and Hanson, 1996). Methanotrophs represent a subset of a physiological group of bacteria known as methylotrophs. They distinguish from methylotrophic bacteria by their ability to utilize methane as a sole carbon and energy source. Methylotrophic bacteria utilize one-carbon compounds, higher reduced than formic acid, as substrates. These compounds are for example methanol, methylated amines, halomethanes, and methylated compounds containing sulfur (Anthony, 1982, 1986; Hanson et al., 1990; Anthony, 1991; Lidstrom, 2006).

As shown in Fig. 1.2., methanotrophic bacteria have an important role in the global methane cycle by oxidizing annually approximately 0.6 Gt methane. Methanotrophic bacteria are present in nearly all habitats where methane and oxygen is available, such as in swamps, rivers, rice paddies, soils from meadows, deciduous woods, freshwater and marine water as well as in technical processes, e.g. in sewage sludge (Whittenbury et al., 1970; Hanson, 1980; Heyer et al., 1984; Strand and Lidstrom, 1984; Corpe, 1985; Sieburth et al., 1987; Holmes et al., 1995; Dedysh et al., 1998; McDonald et al., 2008; Conrad, 2009). Moreover, methanotrophs exist as symbionts in mussels, clams and Pogonophora (Distel and Cavanaugh, 1994; DeChaine and Cavanaugh, 2006; Duperron et al., 2009). In particular, methanotrophic bacteria were detected in oxic environments near methane seeps and hydrothermal vents where they provide the basis of a food chain independent of photosynthesis (Childress et al., 1986; Cavanaugh et al., 1987; Fisher, 1990; Cavanaugh et al., 1992; Cavanaugh, 1993; Fisher et al., 1993; Distel and Cavanaugh, 1994; Larock et al., 1994; Dubilier et al., 1998; DeChaine and Cavanaugh, 2006; Conrad, 2009). For example samples of marine mud volcanoes, like Haakon

Mosby (Barents Sea) and Kazan mud volcano (eastern Mediterranean sea), contained methanotrophs (Niemann et al., 2006; Heijs et al., 2007; Lösekann et al., 2007). Also in the water column above methane seeps of the Black Sea type I methanotrophs were detected (Durisch-Kaiser et al., 2005; Schubert et al., 2006).

Type I methanotrophs belong to the subdivision of γ -Proteobacteria including the genera *Methylobacter*, *Methyломicrobium*, *Methyломonas*, *Methylocaldum*, *Methylosphaera*, *Methylothermus*, *Methylosarcina*, *Methylohalobius*, *Methylosoma*, and *Methylococcus*. *Methylococcus* and *Methylocaldum* are also referred as type X methanotrophs, which are distinguished by certain physiological, biochemical and phylogenetic characteristics (Bowman, 2006; McDonald et al., 2008). The group of type I methanotrophs metabolize methane by the ribulose monophosphate (RuMP) pathway and exhibit intracytoplasmic membranes (ICM) as stacks of membrane disks (Fig. 1.3.a; Hanson and Hanson, 1996; Trotsenko and Murrell, 2008).

Figure 1.3.: Intracytoplasmic membranes of methanotrophs (Madigan and Martinko, 2006).

(a) type I methanotrophic bacterium, *Methylococcus capsulatus*, with ICM as stacks of membrane disks (cell diameter: 1 μm). (b) type II methanotrophic bacterium, *Methylosinus*, with ICM parallel to the cytoplasmic membrane (cell diameter: 0.6 μm ; Figure from Madigan and Martinko, 2006; with kind permission of Pearson Education Deutschland GmbH).

Type II methanotrophs *Methylocystis*, *Methylosinus*, *Methylocella*, and *Methylocapsa* are in the subdivision α -Proteobacteria (McDonald et al., 2008). They utilize methane by the serine pathway and exhibit ICM parallel to the cytoplasmic membrane (Fig. 1.3.b; Hanson and Hanson, 1996; Trotsenko and Murrell, 2008). *Methylocella* is also known for vesicular membranes (Dedysh et al., 1998).

Recently some new aerobic methylotrophs were described. *Crenothrix polyspora* and *Clonothrix fusca* are the first described filamentous methane oxidizers, both related to type I methanotrophs (Stoecker et al., 2006; Vigliotta et al., 2007). Moreover, methanotrophs belonging to the phylum Verrucomicrobia (Dunfield, 2007; Pol et al., 2007; Islam et al., 2008) were recently described and placed in the new genus *Methylacidiphilum* (Op den Camp et al., 2009).

By stable isotope probing two groups of methanotrophs were detected at marine hydrocarbon seeps, *Methylophaga* (γ -Proteobacteria) and Methylophilaceae-genera (β -Proteobacteria; Redmond et al., 2010). These new findings indicate that presumably more bacterial phylotypes are capable of methane oxidation than previously thought.

The first enzyme of the methanotrophic pathway (cf. Fig. 3.1.), methane monooxygenase (MMO), oxidizes methane to methanol. Two variants of the enzyme are known, the membrane-bound particulate MMO (pMMO) and the soluble MMO (sMMO). The pMMO is the canonical enzyme, abundant in almost all methanotrophs. The sMMO is only abundant in a few strains. The sMMO has a broader substrate specificity (for diverse aliphatic and aromatic hydrocarbons) and is more stable than the pMMO exhibiting a smaller range for substrates. In methanotrophs with both MMO, the sMMO is only expressed under copper limitation (Hanson and Hanson, 1996; Lidstrom, 2006). Just one genus, *Methylocella*, was described, which contains solely the sMMO (Conrad, 2009).

The methanol dehydrogenase (MDH) catalyzes the second step of aerobic methane oxidation and represents a key enzyme for methanotrophic as well as methylotrophic bacteria. It converts methanol to formaldehyde. For carbon assimilation, formaldehyde is used for ribulose monophosphate pathway or the serine cycle, for type I and type II methanotrophs, respectively. Some methylotrophs assimilate carbon dioxide in the Calvin-Benson-Bassham cycle (Hanson and Hanson, 1996; Lidstrom, 2006).

Since methane represents highly reduced carbon, the energy yield of the methanotrophic pathway is high (see Eq. 11; Widdel, 2002):

1.2.3. Anaerobic oxidation of methane (AOM)

The AOM is a widely abundant process of importance in the global methane cycle. Approximately ≥ 90 % of the annually produced methane in the oceans is oxidized in anoxic, marine sediments (cf. section 1.1.1.; Hinrichs and Boetius, 2002). Thus, only 2 % of the global methane budget originates from oceanic systems (cf. section 1.1.1.). The anaerobic oxidation of methane is usually associated with the precipitation of carbonates and sulfides, which was observed in marine mud volcanoes as well as in cold seeps (Aloisi et al., 2000; Peckmann et al., 2001b; Aloisi et al., 2002). The process of AOM was already postulated in 1974. Methane and sulfate concentration profiles in anoxic sediments and water columns gave indications to this process (Martens and Berner, 1974; Barnes and Goldberg, 1976; Reeburgh, 1976). These findings were confirmed by tracer measurements using ^{14}C -CH₄, ^3H -CH₄ or ^{35}S -SO₄²⁻ for turnover rates of methane and sulfate. The results were also substantiated by natural $^{13}\text{C}/^{12}\text{C}$ ratio of methane and CO₂. In the water column and in oxic sediments these compounds showed a low depletion of ^{13}C isotopes, whereas in deposits of methane seeps a high depletion of ^{13}C isotopes was observed. This shift in the isotopic ratio was due to the preference of compounds containing lighter isotopes in enzyme-catalyzed reactions (Reeburgh, 1980; Iversen and Blackburn, 1981; Oremland and Desmarais, 1983). Therefore, the anaerobic oxidation of methane coupled to reduction of sulfate is usually related to marine, anaerobic sites since in oceanic systems high sulfate concentrations are available (D'Hondt et al., 2002).

It was postulated that archaea can reverse the methanogenic pathway for methane oxidation, while electrons are transferred to SRB (Hoehler et al., 1994). This hypothesis is still corresponding to the opinion of actual research (Knittel and Boetius, 2009). The energy yield of AOM is extreme low (Eq. 12) and methane oxidation is only favorable in syntrophy with sulfate reduction (Eq. 13 and Eq. 14; Thauer and Shima, 2008). The

measured stoichiometric ratios of 1:1 (methane:sulfate) are in accordance with Eq. 12 (Nauhaus et al., 2002; Seifert et al., 2006; Wegener et al., 2008).

The first evidence for anaerobic methanotrophy was given by Hinrichs et al. (1999) based on the isolation of archaeal lipids depleted in ^{13}C . Considering the hypothesis of a reversed methanogenic pathway, ANME archaea require homologous enzymes of methanogenesis. The methyl-coenzyme M reductase (MCR) catalyzes in methanogenic archaea the terminal step of methane formation. In reversal, MCR is needed in a reversed methanogenic pathway for the initial step of AOM. The MCR of methanogens is a 300 kDa enzyme with three subunits ($\alpha_2\beta_2\gamma_2$) and two tightly bound molecules of the nickel porphyrinoid cofactor F430 with a molecular mass of 905 Da (Thauer, 1998). Novel DNA sequences (*mcrA* gene) of MCR could be detected in ANME archaea (Hallam et al., 2003). Krüger et al. (2003) were able to identify the MCR on protein level in cold seeps of the Black Sea. A 951 Da nickel porphyrinoid cofactor, similar to F430, is bound to the enzyme. This Ni-protein I was present in concentrations of maximum 7 % of the bulk protein mass and was assigned to ANME-1 group. The new nickel cofactor was identified as 172-methylthio-F430 (Mayr et al., 2008). MCR from ANME-2 cells use apparently the 905 Da nickel cofactor known from methanogens (Krüger et al., 2003). The MCR expression of ANME-1 and ANME-2 cells was shown by Heller et al. (2008) on cellular level. A further direct proof for anaerobic methanotrophy was based on isotopic evidence in combination with FISH analysis demonstrating the uptake of methane by ANME-2 cells (Orphan et al., 2001a). In addition, almost all methanogenic enzymes were found by genome analysis. Genes of dissimilatory sulfate reduction could not be detected in ANME archaea. Thus, syntrophy with SRB appears to be obligatory (Hallam et al., 2004; Meyerdierks et al., 2005; Pernthaler et al., 2008; Meyerdierks et al., 2010).

Intermediates for a necessary transfer of reducing equivalents between the syntrophic partners are still unknown. Model based calculations suggested formate as a possible intermediate (Sørensen et al., 2001), but in *in vitro* feeding studies, hydrogen, formate, acetate and methanol could be excluded (Nauhaus et al., 2002; Widdel et al., 2006). Also methyl sulfides were suggested (Moran et al., 2008), but this finding could not be proved by other studies (Knittel and Boetius, 2009). A further hypothesis proposed a transfer of reducing equivalents via electron shuttles. Tests with compounds like phenazines and humic acids gave no positive results (Nauhaus et al., 2005). Transfer of reducing equivalents by nanowires (Gorby et al., 2006) was hitherto not reported. Recent analysis of the ANME-1 genome also suggests *c*-type cytochromes as possible carrier (Meyerdierks et al., 2010). *In vitro* analysis of ANME/SRB consortia with stable isotopes suggested autotrophy of the syntrophic SRB partner cell. Only archaeal lipids indicated incorporation of ^{13}C -labeled methane, for SRB only incorporation of ^{13}C -labeled carbon dioxide was observed (Wegener et al., 2008). Thus, it is unlikely that the SRB take up organic substrates derived from the ANME-1 metabolism. Assimilation of CO_2 is known for SRB to occur via the reductive citric-acid cycle (Schauder et al., 1987) or the acetyl-CoA pathway (Jansen et al., 1984; Jansen et al., 1985; Schauder et al., 1989).

Studies of physiology and biochemistry of AOM organisms are challenging since no pure cultures are available in the laboratory. Analyses have to be performed with enrichment cultures exhibiting a very slow growth due to the small energy yield of AOM (Eq. 12). The doubling time of the cells is approximately between 2 and 7 month (Nauhaus et al., 2007; Krüger et al., 2008a).

For the detection of AOM communities the approach of lipid biomarker analysis is well established. Isoprenoidal dialkyl glycerol diethers (archaeol, hydroxyarchaeol), tetramethylhexadecane (crocetane) and pentamethylcosenes are frequently used for identification. Depletion of ^{13}C isotopes in biomarkers indicate consumption of methane by AOM metabolism (usually -40 to -70 ‰ compared to the stable carbon isotope ratio of the source methane; Blumenberg et al., 2004; Blumenberg et al., 2005; Reeburgh, 2007; Niemann and Elvert, 2008). Isotopic analysis of biomarkers can also be applied for identification of ancient AOM systems (Thiel et al., 1999; Peckmann and Thiel, 2004). For identification of sulfate reducing partner of ANME cells, characteristic ^{13}C -depleted fatty acids such as $\text{C}_{16:1\omega5}$, $\text{cy-C}_{17:0\omega5,6}$, and $\text{C}_{17:1\omega6}$ are used (Niemann and

Elvert, 2008). Notably, sometimes hopanoids from methanotrophic bacteria are found at AOM sites (Elvert et al., 2000; Pancost et al., 2000; Thiel et al., 2001; Thiel et al., 2003; Birgel and Peckmann, 2008). It is under discussion, if also certain groups of marine SRB or other anaerobic bacteria produce these hopanoids (Fischer et al., 2005; Härtner et al., 2005; Blumenberg et al., 2006; Birgel and Peckmann, 2008).

Different groups catalyzing AOM have been identified yet. Most studies are based on 16s rRNA and *mcrA* genes, supported by lipid biomarker fingerprints with stable isotope analysis (Knittel and Boetius, 2009).

Figure 1.4.: Phylogenetic tree of ANME cluster (Knittel and Boetius, 2009).

Phylogenetic tree of ANME 16S rRNA gene sequences and other sequences within the archaeal domain. Data in colored boxes give information about the distribution and abundance of sequences. Bar, 10 % estimated sequence divergence. Reproduced with permission of ANNUAL REVIEWS, INC, from Anaerobic Oxidation of Methane: Progress with an Unknown Process, K. Knittel and A. Boetius, 63, 311-334, copyright 2009; permission conveyed through Copyright Clearance Center, Inc.

The three ANME clusters are: ANME-1, ANME-2 with the subclasses -2a, -2b, and -2c, and ANME-3. The euryarchaeal groups are not monophyletic. ANME-1 are distantly related to Methanomicrobiales (Fig. 1.4.; Hinrichs et al., 1999), while ANME-2 and ANME-3 are distantly related to Methanosarcinales (Fig. 1.4.; Orphan et al., 2001b; Niemann et al., 2006). A fourth group has been described as ANME-2d or GoM Arc I; this group is not monophyletic with the other ANME-2 subgroups (Mills et al., 2005; Lloyd et al., 2006; Martinez et al., 2006). AOM metabolism for this novel group has not yet been proven (Knittel and Boetius, 2009). Fig. 1.4. illustrates that ANME-1 and ANME-2 are the most diverse groups detected in a multitude of habitats. ANME-3 and GoM Arc I, representing new described groups, are less abundant.

The ANME groups show differences in cell morphology, aggregate structure and related syntrophic partners.

ANME-1 cells exhibit a cylinder-shaped morphology with an external sheath and were observed as single cells or in filaments (Fig. 1.5.a,b and Fig. 1.6.; Eller et al., 2005; Reitner et al., 2005a). In microbial mats they were found in loose association with SRB of *Desulfococcus/Desulfosarcina* (DSS) group (Reitner et al., 2005a; Reitner et al., 2005b).

ANME-2 cells are coccoid and frequently detected in consortia with SRB. ANME-2a form mixed-type aggregates with SRB showing a randomly distribution of both cell types (Fig. 1.5.c-e), while ANME-2c/SRB aggregates reveal a shell-like structure (Fig. 1.5.f,g). ANME-2 are usually associated with SRB of the DSS group (Boetius et al., 2000), in particular with subgroup SEEP-SRB1a (Schreiber et al., 2010). But also partner cells of α -Proteobacteria, β -Proteobacteria or *Desulfobulbus*-related SRB were described (Pernthaler et al., 2008). Single ANME-2 cells were reported as well (Fig. 1.5h; Orphan et al., 2002; Durisch-Kaiser et al., 2005; Eller et al., 2005; Schubert et al., 2006; Treude et al., 2007).

The phylogenetic group of ANME-3 was observed as shell-type aggregates with SRB (Fig. 1.5.i) or as single cells. Syntrophic partners are related to the *Desulfobulbus* group (Niemann et al., 2006; Lösekann et al., 2007). In some cases SRB of the DSS subgroup SEEP-SRB1a were reported (Schreiber et al., 2010). The described syntrophic associations suggest that ANME cells are not restricted to distinct bacterial partners.

Figure 1.5.: Different ANME cells and aggregates (Knittel and Boetius, 2009).

Epifluorescence micrographs of different ANME single cells and aggregates visualized by fluorescence *in situ* hybridization (FISH) or CARD-FISH (Catalyzed Reporter Deposition-FISH). (a) single ANME-1 cells from a Black Sea microbial mat. (b) mat-type consortia formed by ANME-1 (red) and DSS cells (green). (c–e) mixed-type consortia of ANME-2a (red) and DSS (green) cells observed in different seep sediments. (f,g) shell-type consortia of ANME-2c (red) and DSS cells (green) and (h) single ANME-2c cells observed in different seep sediments. (i) ANME-3/*Desulfobulbus* consortia. Unless otherwise indicated, scale bar is 5 µm. Reproduced with permission of ANNUAL REVIEWS, INC, from Anaerobic Oxidation of Methane: Progress with an Unknown Process, K. Knittel and A. Boetius, 63, 311-334, copyright 2009; permission conveyed through Copyright Clearance Center, Inc.

Figure 1.6.: ANME-1 morphology (Reitner et al., 2005a).

ANME-1 archaea in the Black Sea microbial mat. (a) transmission electron microscopic (TEM) micrograph of cylinder-shaped ANME-1 archaea. (b) Image of field emission scanning electron microscope (FE-SEM) depicts cylinder-shaped ANME-1 sheaths.

Very recently AOM with iron and manganese as electron acceptors were described. Regarding thermodynamic aspects this reaction allows approximately ten times as much as energy as sulfate dependent AOM (with respect to higher energy yield per mol but lower reaction rate). In principle it is likely that this AOM variant depends on reverse methanogenesis, but it remains unknown if the electron acceptors have an direct or indirect influence on the process (Beal et al., 2009).

1.2.4. Nitrate dependent AOM

During the last few years a novel mechanism was discovered for the anaerobic oxidation of methane. An enrichment culture from a water drain in the Netherlands was grown in a methane atmosphere with nitrate and nitrite as electron acceptors. Phylogenetic analysis revealed that 10 % archaeal species, related to the ANME cluster, and 90 % bacterial species were present, suggesting that this AOM variant also depends on ANME related archaea (Raghoebarsing et al., 2006). However, further studies demonstrated that archaea disappeared in the cultures while AOM activity remained. 80 % of the bacterial species were represented by the phylum NC10 (Ettwig et al., 2008). The genome of one organism from enrichment cultures (*Candidatus*

Methyloirabilis oxyfera) representing a member of the NC10 phylum, was assembled and subject of further studies (Ettwig et al., 2010). The energy yield for nitrate and nitrite dependent AOM (Eq. 15 and Eq. 16) is higher than for sulfate dependent AOM (Eq. 12; Thauer and Shima, 2008):

From genome as well as transcriptome and proteome analyses it was concluded that in nitrate/nitrite dependent AOM nitric oxide is decomposed to nitrogen and oxygen by a hitherto unknown mechanism. The oxygen is then used for oxidation of methane by the known aerobic methanotrophic pathway (cf. section 1.2.2.; Ettwig et al., 2010).

1.3. AOM habitats of this work

1.3.1. Cold seeps of the Black Sea

The AOM consortia of the Black Sea cold seeps exhibit a characteristic feature by forming microbial mats, which can reach several centimeters in diameter. In the Black Sea the water column shows a strict stratification caused by a low salt concentration of surface water and a higher salinity of the deep water. Due to this stratification about 80 % of the deep water of the Black Sea is anoxic (Peckmann et al., 2001b). The anaerobic methanotrophic mats, used in this study, are abundant in approximately 230 m depth in the permanent anoxic bottom water along the shallow Crimean shelf (Luth et al., 1999; Michaelis et al., 2002). Further AOM sites of the Black Sea were observed at different locations of the Crimean shelf and at the Sorokin Trough mud volcanoes at 2000 m depth (Stadnitskaia et al., 2005; Krüger et al., 2008b).

Figure 1.7.: Morphology of carbonate towers (Reitner et al., 2005b).

Carbonate towers of Black Sea cold seeps. (a) drawing of a large, active tower. (b) group of growing, carbonate towers in an area of intense gas seepage. (c) subrecent tower devoid of microbial mats with bottom plate of carbonate-cemented sediment.

The anoxic water column and the methane seepage out of the sediment allow extensive AOM activity leading to alkalinization of the surrounding water (cf. Eq. 15; Reitner et al., 2005b):

This reaction causes precipitation of carbonates (Aloisi et al., 2000; Peckmann et al., 2001b; Aloisi et al., 2002; Peckmann and Thiel, 2004) forming cold seep carbonate towers. For the AOM associated carbonates in the Black Sea several morphologies are known (Ivanov et al., 1991; Luth et al., 1999; Peckmann et al., 2001b; Lein et al., 2002; Michaelis et al., 2002; Reitner et al., 2005a; Reitner et al., 2005b; Pape et al., 2008). These precipitates are formed (a) in the sediment as carbonate-cemented plates and large tabular constructions (b) below the chemocline as tall build-ups and tower-like

constructions that may reach several meters in height growing free into the anoxic water column (Peckmann et al., 2001b; Lein et al., 2002; Michaelis et al., 2002).

The carbonate towers studied in this work exhibit no distinct central channel. A diffuse system of cavities within the towers is percolated by methane. Openings release gas in a continuous flow or in intervals (Fig. 1.7.; Reitner et al., 2005b).

It is assumed that the towers grow from microbial mats at the sediment surface slowly into the anoxic water column (Treude et al., 2005). The microbial mats grow at the inner and outer surfaces of the carbonate towers and show a remarkable degree of organization (cf. also Wrede et al., 2008). Macroscopically, three layers were discriminated. On the surface, exposed to the sea water, the black layer consists mainly of ANME-2/SRB aggregates. SRB of this mat type exhibit often intracytoplasmic magnetosome-like chains of greigite precipitations (Reitner et al., 2005a). The underlying pink layer is characterized by a matrix of ANME-1 cells with embedded SRB colonies. A green layer lines the internal open spaces of the carbonate towers and shows a more heterogeneous distribution of cell types (Reitner et al., 2005b). At micrometer scale the sphere-like aggregates composed of ANME/SRB and unknown organisms, are embedded in a matrix of EPS {Reitner, 2005a #10; Wrede, 2008 #138}.

Figure 1.8.: Structure of microbial mats (Reitner et al., 2005b).

The figure depicts a broken tower revealing the microbial mats. In the enlarged figure (right), the three microbial layers are visible. On the surface, exposed to the sea water, the black layer is present. The underlying layers show pink or orange colors. The cavities are lined by a green layer. (Photo: GHOSTDABS - BMBF & Jago team: K. Hissmann & J. Schauer)

1.3.2. Terrestrial mud volcanoes in the Northern Apennines

Terrestrial mud volcanoes are peculiar geological structures within zones of tectonical compression that are mostly associated with local plate tectonic activity. They are found in the forelands of Alpidic orogenic structures, namely in the Apennines, the Carpathians or the Caucasus (Dimitrov, 2002). Along the Apennines, these fluid venting structures could be traced back to the Oligocene (e.g. Taviani, 2001). Recent mud volcanoes at the Emilia Apennines, but also elsewhere in Italy, are mostly cold springs, expelling hydrocarbon-enriched, saline waters (Martinelli and Judd, 2004). The transported sedimentary material leads to the formation of mud cones, which can reach several meters in height.

Figure 1.9.: Geological setting of the Nirano mud volcano field.

(a) panoramic view of the Nirano mud volcano field towards NE. (b) active mud cone analyzed in this work. (c) geological cross section in the direction of the view axis of (a) (adapted from Bonini, 2008).

At the Nirano mud volcano site, fluids enriched with clay fractions are transported to the surface forming mud cones of maximum 5 m height (cf. Fig. 1.9.). Analysis of microfossils retrieved from mud volcano fluids revealed that the fluids pass the Pliocene-Pleistocene Argille Azzurre formation (marine silty clays) and the underlying Epi-Ligurian (Eocene-Miocene) and Ligurian (Cretaceous) units (Bonini, 2007 and references therein). Thus, fluids pass through sedimentary material of different permeability (cf. Fig. 1.9.). Along lithological boundaries, overpressure or expansion will lead to the formation of fluid reservoirs, namely, along the Upper Marnoso Arenacas unit boundary (below the rather impermeable Ligurian unit) and along the Epi-Ligurian unit (below the impermeable claystones of the Argille Azzurre formation). Along the two km-paths between the deep fluid reservoir and the surface, a temperature difference of 20 °C per km has to be expected (della Vedova et al., 2001).

Moreover, the upwelling pressurized fluid will be enriched with minerals from different geological units. It is likely that the fluids come also into contact with the lagoonal and marine deposits of the late Messinian, but possibly not with early Messinian evaporitic (gypsum-rich) deposits. However, it may be expected that due to a hiatus readily at the Nirano mud volcano site, some of the fluids also pass directly the Epi-Ligurian/Argille-Azzurre boundary (Fig. 1.9.c). This feature may also be reflected by geochemical data, collected from different mud cones over time, showing a certain variability of the mineral concentrations in the fluid, especially sulfate (Martinelli and Judd, 2004; Duchi et al., 2005; this work). Thus, it must be expected that microbial communities from these mud volcanoes have to be adapted to different temperatures and available electron acceptors along the path of the fluid to the surface.

1.4. Aims of this work

Up to now, most facts of AOM, i.e. phylogenetic or genomic analyses, are based on environmental DNA (cf. sections 1.2.3. and 1.3.1.). FISH analyses were used to localize the phylotypes inside environmental samples, but direct information about metabolic activities of the cells are still missing, even after combining FISH analysis with isotopic data (Orphan et al., 2001a). Proteomic data are rare and most studies do not discriminate between cells of distinct species (cf. sections 1.2.3. and 1.3.1.).

In this work antibody based marker systems were generated. The antibodies are directed against key enzymes of anaerobic methane consumption. Though MCR, the key enzyme of (reverse) methanogenesis, could be already detected in ANME cells (Heller et al., 2008), sulfate reduction could not be directly assigned to a specific cell type. Therefore, a conserved key enzyme of the dissimilatory sulfate reduction pathway, the dissimilatory adenosine-5'-phosphosulfate (APS) reductase was selected to detect and localize active SRB on protein level.

Another focus of this work is the metabolic pathway of aerobic, including nitrate/nitrite dependent methanotrophs (Ettwig et al., 2010). Regarding this metabolic pathway there is a big gap in knowledge for anoxic Black Sea cold seeps. To analyze active methano- or methylotrophic bacteria, a marker system based on the methanol dehydrogenase (MDH) was developed.

Regarding current knowledge of AOM habitats, there are only few data concerning terrestrial sites (Alain et al., 2006). For this reason, the Nirano mud volcano field near Modena, Emilia Romagna area, Northern Italy, was also one focus of this work. It is reasonable to assume that also in terrestrial mud volcanoes methane is oxidized either anaerobically with various electron acceptors (Beal et al., 2009; Knittel and Boetius, 2009) or aerobically, if oxygen (or nitrite, cf. Ettwig et al., 2010) is present. This study aimed at the detection of the relevant organisms.

2. Materials and Methods

Materials: Chemicals, utilities, equipments, commercial systems as well as proteins, antibodies, enzymes, oligonucleotides and respective manufacturers are listed in the appendix (section 7.).

2.1. Organisms and plasmids

2.1.1. Archaeal and bacterial strains

Cloning and protein overexpression experiments were performed with *Escherichia coli* strains. *Methanosarcina mazei* Gö1, *Methanospirillum hungatei* strains, *Desulfovibrio vulgaris* Hildenborough, *Methylobacterium extorquens* AM1 and isolates of aerobic methanotrophs were used for structural and comparative analyses. Details are given in Tab. 2.1.

Table 2.1.: Applied archaeal and bacterial strains.

Strain	Relevant genotype/strain no.	Reference/construction
<i>D. vulgaris</i> Hildenborough	DSM 644	(Postgate and Campbell, 1966)
<i>E. coli</i> DH5 α	<i>recA1 endA1 gyrA96 thi</i> <i>hsdR17r_K⁻m_K⁺relA1 supE44</i> $\Phi 80\Delta lacZ\Delta M15 \Delta (lacZYA-$ <i>argF)</i> U169	(Sambrook et al., 1989)
<i>E. coli</i> TOP10	F– <i>mcrA</i> $\Delta(mrr-hsdRMS-$ <i>mcrBC)</i> $\Phi 80lacZ\Delta M15$ $\Delta lacX74$ <i>recA1 araD139</i> $\Delta(ara$ <i>leu)</i> 7697 <i>galU galK rpsL</i> (StrR) <i>endA1 nupG</i>	Commercial product of Invitrogen, Carlsbad, USA
<i>M. extorquens</i> AM1	DSM 1338	(Vuilleumier et al., 2009)
<i>M. hungatei</i>	DSM 864 ^T	(Ferry et al., 1974)

Strain	Relevant genotype/strain no.	Reference/construction
<i>M. hungatei</i>	DSM 1101	(Patel et al., 1976)
<i>M. mazei</i> Göl	DSM 3647	(Maestrojuan et al., 1992)

2.1.2. Samples of Black Sea cold seeps (Reitner et al., 2005b)

Microbial mat samples were taken from the methane seep area on the lower Crimean shelf, Black Sea, explored during a German–Russian–Ukrainian joint expedition (GHOSTDABS) in 2001. The submersible Jago was used from aboard the Russian R/V ‘Professor Logachev’. The samples originated from the GHOSTDABS-field (44° 46.510’ N, 31° 59.570’ E; Fig. 2.1.) at approximately 230 m water depth. Material for transmission electron microscopy (TEM) and immunofluorescence analyses was chemically fixed in a 4.0 % (w/v) formaldehyde solution and kept at 4 °C in 100 mM PBS (phosphate buffered saline, pH 7.0). For DNA extractions and protein analyses, unfixed samples were stored at -20 °C.

Figure 2.1.: GHOSTDABS-field, Black Sea.

Satellite image of Black Sea. Marker shows location of the GHOSTDABS-field (44° 46.510’ N, 31° 59.570’ E) in approximately 230 m depth.

2.1.3. Samples of terrestrial mud volcanoes of the Emilia Apennines, Italy

Samples were collected from two mud volcano cones, showing high and permanent activity of gas bubbling (44° 30.759' N, 10° 49.290' E; 44° 30.848' N, 10° 49.527' E; Fig. 2.2.). Sites were designated as volcanoes NR4 and NR9.

Figure 2.2.: GHOSTDABS-field, Black Sea.

Map of northern Italy. Marker shows location of the Salse di Nirano.

The samples were taken with a sterilized fluid sampler. The sampler consisted of a short metal pipe connected by a 3 m flexible tube with a flask, which is evacuated with a hand-operated vacuum pump. The sampling depth was about 1.5 m. Pipe, tube and flask were sterilized and kept under sterile conditions until sampling.

For chemical analysis the fluids were kept in completely filled, tightly sealed glass bottles and transported to the laboratory under continuous refrigeration at 7 °C. For DNA isolation, fluids were immediately frozen in dry ice. Samples from mud volcano fluids for fluorescence *in situ* analysis (FISH) were fixed for 24-48 h at 4 °C with 4 % (w/v) formaldehyde in phosphate buffered saline (PBS; 10 mM sodium phosphate, 130 mM NaCl), washed twice with PBS at 4 °C and stored in PBS/EtOH (1:1) at -20 °C. Serum bottles for cultivation experiments were inoculated on site and kept refrigerated at 7 °C until appropriate incubation conditions (see 2.2. and 2.3.3.) were possible. AOM cultures were inoculated with anaerobic fluids from 1.5 m depth.

Cultures for aerobic methanotrophs were inoculated with samples either taken from the aerobic fluid outlet or from a depth of 1.5 m. Temperature, redox potential and the pH-values were measured directly in the fluids on-site.

2.1.4. Plasmids

The Tab. 2.2. shows the plasmids applied in this work.

Table 2.2.: Used plasmids.

Plasmid	Resistance	Reference
pWH844	ampicillin	(Schirmer et al., 1997)
pCR2.1-TOPO	ampicillin, kanamycin	commercial product of Invitrogen, Carlsbad, USA
This work:		
pCW4	ampicillin	1.8 kb <i>mxoF</i> gene from <i>M. extorquens</i> by <i>Bam</i> HI und <i>Hind</i> III in pWH844
pCW5	ampicillin	0.5 kb <i>aprB</i> gene from Black Sea cold seeps by <i>Bam</i> HI und <i>Hind</i> III in pWH844 (subcloned from pCR2.1-TOPO)

2.2. Growth media

Buffers, solutions and media were prepared with deionized water and autoclaved for 20 min at 121 °C and 2 bar. Thermolabile substances were dissolved and sterilized by filtration. Solutions are related to water, other solvents are indicated. Agar plates were prepared by adding 15 g/l agar. In case of minimal medium Bacto-Agar was used.

AOM Media (Widdel et al., 2006; modified)**Full marine medium**

NaCl	26.0	g
MgCl ₂ · 6 H ₂ O	5.0	g
CaCl ₂ · 2 H ₂ O	1.4	g
NH ₄ Cl	300	mg
KH ₂ PO ₄	100	mg
KCl	0.5	g
Na ₂ SO ₄	4.0	g
Resazurin	1	mg
Trace element mixture	1.0	ml
NaHCO ₃ solution	30.0	ml
Na ₂ S solution	0.5	ml
Vitamin mixture	1.0	ml
Thiamine solution	1.0	ml
Vitamin B ₁₂ solution	1.0	ml
H ₂ O	ad 1000	ml

Saltwater medium

NaCl	20.0	g
MgCl ₂ · 6 H ₂ O	3.0	g
CaCl ₂ · 2 H ₂ O	150	mg
NH ₄ Cl	300	mg
KH ₂ PO ₄	200	mg
KCl	0.5	g
Na ₂ SO ₄	3.0	g
Resazurin	1	mg

Trace element mixture	1.0	ml
NaHCO ₃ solution	30.0	ml
Na ₂ S solution	0.5	ml
Thiamine solution	1.0	ml
Vitamin B ₁₂ solution	1.0	ml
Vitamin mixture	1.0	ml
H ₂ O	ad 1000	ml
Nirano fluid medium		
NH ₄ Cl	300	mg
KH ₂ PO ₄	200	mg
Na ₂ SO ₄	3.0	g
Resazurin	1	mg
NaHCO ₃ solution	30.0	ml
Na ₂ S solution	0.5	ml
Filtered and autoclaved fluids of the habitat	ad 1000	ml

The Nirano fluid medium is a modification of the media adapted to the mud volcano fluid composition. For this, salts, trace elements and vitamins were substituted by using sterilized and filtered original fluids of the habitat.

Trace element mixture

HCl 25 % (w/v)	13	ml
Boric acid	10	mg
MnCl ₂ · 4 H ₂ O	5	mg
FeSO ₄ · 7 H ₂ O	4	g
CoCl ₂ · 6 H ₂ O	190	mg
NiCl ₂ · 6 H ₂ O	24	mg
CuCl ₂ · 2 H ₂ O	2	mg
ZnSO ₄ · 7 H ₂ O	200	mg
Na ₂ MoO ₄ · 2 H ₂ O	36	mg
H ₂ O	ad 1000	ml

NaHCO₃ solution

NaHCO ₃	84	g
H ₂ O	ad 1000	ml

The solution was saturated with CO₂ (by shaking in a stoppered bottle under a head space of CO₂) and autoclaved in vessels tightly sealed with butyl rubber stoppers.

Na₂S solution

Na ₂ S · 9 H ₂ O	24	g
H ₂ O	ad 50	ml

Only clear crystals of sodium sulfide were used. Opaque or milky surface layers were removed by brief rinsing with distilled water on a plastic sieve. The sodium sulfide was dissolved by stirring under an N₂ atmosphere. The vessels were tightly sealed with butyl rubber stoppers and autoclaved under a head space of N₂ ($\geq 1/4$ of total volume).

Vitamin mixture

Sodium phosphate (10 mM; pH 7.1)	100	ml
4-Aminobenzoic acid	4	mg
D(+)-Biotin	1	mg
Nicotinic acid	10	mg
D(+)-Pantothenic acid, calcium salt	5	mg
Pyridoxine dihydrochloride	15	mg

Sterilized by filtration and stored dark at 4 °C.

Thiamine solution

Sodium phosphate (10 mM; pH 3.4)	100	ml
Thiamine chloride dihydrochloride	10	mg

Sterilized by filtration and stored dark at 4 °C.

Vitamin B₁₂ solution

H ₂ O	100	ml
Cyanocobalamin	5	mg

Sterilized by filtration and stored dark at 4 °C.

Preparation of the AOM media:

The media were autoclaved and then cooled to room temperature under gassing with oxygen-free nitrogen. After this, the supplements were added (except Na₂S solution) and the pH-value was adjusted with sulfuric acid or 1 M Na₂CO₃ to pH 7.8. The medium was filtered (25 ml aliquots) through a 0.2 µm filter in 100 ml serum bottles tightly sealed, sterilized and under N₂ atmosphere. Finally, Na₂S solution was added.

Desulfovibrio* medium (Postgate's Medium C, modified; Postgate, 1984)*Solution A**

K ₂ HPO ₄	0.5	g
NH ₄ Cl	1.0	g
Na ₂ SO ₄	1.0	g
CaCl ₂ · 2 H ₂ O	0.1	g
MgSO ₄ · 7 H ₂ O	2.0	g
DL-Na-Lactate	2.0	g
Yeast extract	1.0	g
Resazurin	1.0	g
H ₂ O	ad 980	ml

Solution B

FeSO ₄ · 7 H ₂ O	0.5	g
H ₂ O	ad 10	ml

Solution C

Na-Thioglycolate	0.1	g
Ascorbic acid	0.1	g
H ₂ O	ad 10	ml

For preparation of *Desulfovibrio* medium, solution A was boiled for several minutes and then cooled to room temperature under gassing with oxygen-free nitrogen. Subsequently, solution B and C were added and the pH-value was adjusted to 7.8. Under continuous nitrogen gassing the medium was dispensed in Hungate tubes or serum bottles. Permanent stirring guaranteed an even distribution of the precipitates. The vessels were tightly sealed with butyl rubber stoppers and autoclaved.

LB medium

Yeast extract	5.0	g
Tryptone	10.0	g
NaCl	10.0	g
H ₂ O	ad 1000	ml

***Methanosarcina mazei* medium (Ehlers et al., 2002)**

KH ₂ PO ₄	456	mg
K ₂ HPO ₄	227	mg
NH ₄ Cl	500	mg
MgSO ₄ · 7 H ₂ O	500	mg
FeSO ₄ · 7 H ₂ O	2	mg
CaCl ₂ · H ₂ O	250	mg
NaCl	2.25	g
Na-Acetate · 3 H ₂ O	1.5	g
Trace element solution SL6	300	μl
Vitamin solution	1	ml
Yeast extract	2	g
Casitone	2	g
Resazurin	1	mg
H ₂ O	ad 1000	ml

Sterilized by filtration and stored dark at 4 °C.

Trace element solution SL6 (Pfennig and Lippert, 1966)

ZnSO ₄ · 7 H ₂ O	100	mg
MnCl ₂ · 4 H ₂ O	30	mg
Boric acid	300	mg
CoCl ₂ · 6 H ₂ O	200	mg
CuCl ₂ · 2 H ₂ O	10	mg
Na ₂ MoO ₄ · 2 H ₂ O	30	mg
H ₂ O	ad 1000	ml

Vitamin solution (Wolin et al., 1963; modified)

Biotin	2	mg
Folic acid	2	mg
Pyridoxine hydrochloride	10	mg
Thiamine hydrochloride	15	mg
Riboflavin	5	mg
Nicotinic acid	5	mg
Calcium D-pantothenate	5	mg
Vitamin B ₁₂	0.01	mg
p-aminobenzoic acid	5	mg
Thioctic acid (alpha lipoic acid)	1	mg
H ₂ O	ad 1000	ml

Sterilized by filtration and stored dark at 4 °C.

The medium was gassed for 30 min with 80 % N₂ and 20 % CO₂, followed by the addition of:

NaHCO ₃	850	mg
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The pH-value was adjusted to 6.9. The medium was dispensed in Hungate tubes (5 ml) under continuous gassing with 80 % N₂ and 20 % CO₂. The Hungate tubes were then tightly sealed with butyl rubber stoppers and gassed for 2 min with 80 % N₂ and 20 % CO₂. After autoclaving, following solutions (separately autoclaved under N₂ atmosphere) were added in each Hungate tube:

Methanol	30.4	μl
Cysteine-HCl · H ₂ O (stock solution 30 g/50 ml)	3.0	μl
Na ₂ S · 9 H ₂ O (stock solution 24 g/50 ml)	2.5	μl

Mineral medium for methylotrophs**Solution A**

Na ₂ HPO ₄	4.5	g
KH ₂ PO ₄	2.25	g
H ₂ O	ad 50	ml

Solution B

MgSO ₄ · 7 H ₂ O	100	mg
NH ₄ Cl	500	mg
Trace element solution SL7	500	μl
H ₂ O	ad 50	ml

Solution C

Fe(III)NH ₄ -Citrate or Fe(III)-Citrate	7.8	mg
CaCl ₂ · 2 H ₂ O	15.6	mg
H ₂ O	ad 16.6	ml
Autoclaved.		

Cycloheximide stock solution 20 mg/ml

Cycloheximide	200	mg
H ₂ O	ad 10	ml
Sterilized by filtration.		

Trace element solution SL7 (Pfennig and Trüper, 1981; modified)

HCl 25 % (w/v)	1.3	ml
Boric acid	6	mg
CoCl ₂ · 6 H ₂ O	190	mg
CuCl ₂ · 2 H ₂ O	2	mg
MnCl ₂ · 4 H ₂ O	100	mg
Na ₂ MoO ₄ · 2 H ₂ O	36	mg
NiCl ₂ · 6 H ₂ O	24	mg
ZnCl ₂	70	mg
Boric acid	300	mg
H ₂ O	ad 1000	ml
Adjusted to pH 6.5.		

Medium preparation

Solution A	50	ml
Solution B	50	ml
Bacto-Agar	7.5	g
H ₂ O	ad 490.5	ml
Autoclaved.		

Added, after cooling to 50 °C:

Solution C	5	ml
Methanol	2	ml
Cycloheximide stock solution	2.5	ml

The plates were stored after a short time period for gelling in tightly sealed bags to prevent evaporation of methanol.

Nitrate Mineral Salts Medium (NMS medium; Bowman, 2006):

MgSO ₄ · 7 H ₂ O	1	g
KNO ₃	1	g
Na ₂ HPO ₄ · 12 H ₂ O	0.717	g
KH ₂ PO ₄	0.272	g
CaCl ₂ · 6 H ₂ O	200	mg
Ferric Ammonium EDTA	5	mg
Trace element solution	1	ml
H ₂ O	ad 1000	ml

Adjusted to pH 7.8 with KOH.

Trace element solution

Disodium EDTA	0.5	g
FeSO ₄ · 7 H ₂ O	200	mg
Boric acid	30	mg
CoCl ₂ · 6 H ₂ O	20	mg
CuSO ₄ · 5 H ₂ O	30	mg
ZnSO ₄ · 7 H ₂ O	10	mg
MnCl ₂ · 4 H ₂ O	3	mg
Na ₂ MoO ₄ · 2 H ₂ O	3	mg
NiCl ₂ · 6 H ₂ O	2	mg
H ₂ O	ad 1000	ml

Adjusted to pH 6.8 with KOH (neutralize).

25 ml medium was dispensed in 100 ml serum bottles. Serum bottles were tightly sealed with gray butyl rubber stoppers and autoclaved. Methane was added to an overpressure of 0.5 bar.

SOB medium

Tryptone	5.0	g
Yeast extract	1.25	g
NaCl	146	mg
KCl	47	mg
MgCl ₂	508	mg
MgSO ₄	516	mg
H ₂ O	ad 250	ml

Antibiotics and X-Gal

Antibiotics were prepared as 1000-fold concentrated stock solutions. Ampicillin was dissolved in deionized water. The solution was sterilized by filtration and stored at -20 °C. Autoclaved medium was cooled down to approximately 50 °C and antibiotics were added to their final concentration of 100 µg/ml.

For screenings with X-Gal, a stock solution of 40 mg/ml X-Gal dissolved in DMF was prepared. The final concentration in the medium was 40 µg/ml.

Media with light sensitive additives were incubated in the dark.

2.3. Methods

2.3.1. General methods

Some general methods used in this work that are described in the literature are listed in Tab. 2.3.

Table 2.3.: General methods.

Method	Reference
Absorption measurement	(Sambrook et al., 1989)
Ethidium bromide staining	(Sambrook et al., 1989)
Gel electrophoresis of DNA	(Sambrook et al., 1989)
Ligation of DNA fragments	(Sambrook et al., 1989)
Determination of protein amounts	(Bradford, 1976)
Gel electrophoresis of proteins (denaturing)	(Laemmli, 1970)
Sequencing according to the chain termination method	(Sanger et al., 1977)

2.3.2. Geochemical analysis

Particles were removed from sampled fluids by centrifugation at 4 °C. The analyses of Cl^- , SO_4^{2-} , NO_3^{2-} , NO_2^{2-} were performed according to DIN EN ISO 103041-1, NH_4^+ according to DIN EN ISO 11732, S_2^- according to DIN 38405-7, SO_3^{2-} according to DIN EN ISO 103041-3, HCO_3^{2-} according to DEV D8 and Fe (total), Fe^{2+} , K^+ , Mg^{2+} , Na^+ , Ni^{2+} , P (total) according to DIN EN ISO 11885 by the SGS INSTITUT FRESENIUS GmbH (Göttingen, Germany).

2.3.3. Cultivation of archaea and bacteria

Cultivation of *D. vulgaris* Hildenborough

Manipulations were carried out under an anoxic N₂-atmosphere using the Hungate technique and its modifications (Sowers and Noll, 1995). *D. vulgaris* Hildenborough was grown in *Desulfovibrio* medium in Hungate tubes or serum bottles with nitrogen in the Headspace (1/3 of the total volume). For inoculation sterile disposable syringes were used. Cultures were incubated at 30 °C. Growth was controlled by microscopy.

Cultivation of *E. coli*

Unless otherwise stated, *E. coli* was grown in LB medium at 37 °C under agitation at 200 rpm in test tubes and culture flasks with baffles. Fresh colonies from plates or overnight liquid cultures were used for inoculation. Growth was measured at a wavelength of $\lambda = 600$ nm.

Cultivation of *M. extorquens* AM1 and methylotrophic isolates

M. extorquens AM1 and methylotrophic isolates were preferentially grown on plates (Mineral medium for methylotrophs) in tightly sealed bags at 30 °C. Methylotrophic bacteria were isolated from leaves of dicot plants according to Green (2006).

Cultivation and enrichment of methanotrophs

100 ml serum bottles with 25 ml NMS medium were inoculated with 3.5 ml original fluid directly on-site. For the controls, nitrogen instead of methane was injected into the headspace. For inoculation, sterile disposable syringes were used. The cultures were grown in a rotary shaker (200 rpm) at 28 °C. Growth was controlled by microscopy.

Cultivation of *M. mazei* Gö1

Manipulations were carried out under an anoxic N₂-CO₂-atmosphere using the Hungate technique and its modifications (Sowers and Noll, 1995). *M. mazei* Gö1 was grown in *Methanosarcina mazei* medium in serum bottles with 80 % N₂ and 20 % CO₂ in the Headspace (3/4 of the total volume). For inoculation sterile disposable syringes were used. Cultures were incubated at 30 °C. Growth was controlled by microscopy.

AOM cultures

Manipulations were carried out under an anoxic N₂-atmosphere using the Hungate technique and its modifications (Sowers and Noll, 1995). Prepared 100 ml serum bottles with 25 ml AOM medium were inoculated with 3.5 ml original fluid directly on-site. The cultures were incubated under a slight overpressure of pure methane at 20 °C and shaken for approximately 0.5 min once per 24 h. For the controls, nitrogen instead of methane was injected into the headspace. For inoculation disposable syringes were used. Activity was checked by determining sulfide concentrations measured with the Nanocolor[®] Sulfid Kit, which is based on colorimetric detection of methylene blue formation. The commercial system was used according to the manufacturer's instructions with miniaturized reaction batches.

2.3.4. Genetic methods

Preparation of chromosomal DNA from *D. vulgaris* Hildenborough

A *D. vulgaris* Hildenborough culture (500 ml) was vacuum filtrated with a Büchner funnel using 3 layers of filter paper to remove iron precipitates. The cells were harvested by centrifugation 5,000 rpm for 10 min at 4 °C. The pellet was resuspended in 4 ml medium and 200 µl lysozyme (10 mg/ml), then 200 µl EDTA (0.25 M) were added. The solution was mixed by repeated inverting and incubated at 37 °C for 20-30 min. Subsequently, 200 µl proteinase K (2.5 mg/ml) and 200 µl SDS (12.5 % [w/v]) were added and the solution was inverted until a cleared lysate was obtained. After an incubation time of 30-40 min at 37 °C, 0.5 ml NaCl (5 M) were mixed into the solution and 4 ml chloroform-octyl alcohol (5 vol. + 1 vol.) were added. The reaction mixture

was shaken until a fine emulsion was obtained. The solution was mixed by 5 further inverting steps. By centrifugation (45 min, 5,000 rpm, 4 °C) the two phases were separated. The upper, aqueous phase containing the DNA was removed with a cut pipet tip. This step was repeated until no proteins were left at the phase boundary. In a next step the DNA was precipitated with 2 volumes isopropanol at -40 °C for 30 min. The mixture was centrifuged (30 min, 13,000 rpm, 4 °C) and the pellet was air dried. Finally the pellet was washed in 70 % ethanol and resuspended in 100 µl water.

Preparation of DNA from cold seep microbial mats

A volume of approximately 200 µl microbial mats was resuspended by pipeting with a cut pipet tip and by vortexing in 1 ml sterile saline. A cushion of 2 ml 60 % (w/v) Nycodenz was layered below the slurry with a syringe and the sample was centrifuged (3 h, 5,000 rpm, swing-out rotor, without brake). Cells were removed and were mechanically disrupted with a Mikro-Dismembrator (3 min, 1800 rpm). The DNA was extracted from the disrupted cells with the High Pure PCR Template Preparation Kit. The DNA was eluted from the column with water.

Preparation of plasmid DNA from *E. coli*

Plasmid DNA was prepared from *E. coli* carrying the desired plasmid. An overnight culture (4 ml) with cells carrying the desired plasmid was harvested (2 min; 13,000 rpm). The plasmid DNA was isolated using the pGOLD Plasmid Miniprep Kit I according to the manufacturer's instructions. The DNA was eluted from the column with water.

Agarose gel electrophoresis

For analytical and preparative separation of DNA fragments, agarose gels containing 1 to 2 % (w/v) agarose (according to the expected fragment size) were prepared in TAE buffer. The DNA samples were mixed with 5 x DNA loading dye to facilitate loading and to indicate the migration of the samples in the gel. A voltage of 80–120 V was applied until the bromophenol blue marker reached the last third of the gel. DNA fragments migrate towards the anode with a velocity proportional to the negative

logarithm of their length. After electrophoresis, gels were incubated in ethidium bromide solution for 5 min and briefly rinsed with water. The DNA was detected and documented via its fluorescence under UV light ($\lambda = 254$ nm). For estimation of the size of the DNA fragments, the Quick-Load[®] 1 kb DNA Ladder (Fig. 2.3.) was used. For the isolation of DNA fragments from preparative gels, the bands were visualized at a wavelength of $\lambda = 365$ nm. The bands were cut out and extracted with the peqGOLD Gel Extraction Kit according to the manufacturer's instructions.

Solutions for agarose gel electrophoresis:

1 % Agarose gel

Agarose	1	g
TAE buffer	ad 100	ml

5 x DNA loading dye

Glycerol (100 %)	5	ml
TAE buffer (50 x)	200	μ l
Bromophenol blue	10	mg
Xylene cyanol	10	mg
H ₂ O	4.5	ml

50 x TAE buffer

Tris	242	g
acetic acid (100 %)	57.1	ml
EDTA (0.5 M, pH 8.0)	100	ml
H ₂ O	ad 1000	ml

Figure 2.3.: Quick-Load® 1 kb DNA Ladder.

1 kb DNA Ladder on a 0.8% TAE agarose gel. Mass values are for 0.5 µg/lane.

Sequencing of DNA

Sequencing was done based on the chain termination method (Sanger et al., 1977) with fluorescence labeled dideoxynucleotides. The sequencing reactions were conducted by the Göttingen Genomics Laboratory (Göttingen, Germany).

Restriction and ligation of DNA

The digestion of DNA with restriction endonucleases was performed with buffers recommended by the manufacturer. Reaction buffers, concentration of enzymes and DNA as well as incubation temperatures were chosen according to the manufacturer's instructions. To avoid re-circularization of a digested plasmid, the 5' phosphate groups of the linearized vector were removed prior to the ligation reaction. The dephosphorylation of the 5' end was performed with the calf intestinal alkaline phosphatase (CIAP). The products were purified with the peqGOLD MicroSpin Cycle-Pure Kit following the manufacturer's instructions.

DNA fragments were ligated with the T4 DNA ligase with buffers supplied by the manufacturer. The ligation reaction contained 20-200 ng of vector DNA and an excess of the DNA fragment (insert to vector molar ratio of 5:1 to 20:1). The reaction was performed with 5 U T4 DNA ligase in a final volume of 20 µl for 1-2 h at RT or overnight at 16 °C.

TOPO TA cloning

For cloning of PCR products of environmental DNA with unknown sequence, e.g. the *aprBA* gene fragments from Black Sea cold seep microbial mats, the vector pCR2.1-TOPO was used. The linearized TOPO TA vector has single, overhanging 3'-deoxythymidine residues. Thus, DNA fragments with a desoxyadenosine overhang can be ligated with the vector. The TOPO TA vector includes a covalently bound topoisomerase I from the *Vaccinia* virus. The topoisomerase I binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5'-CCCTT in one strand (Shuman, 1991). Subsequently, the phosphotyrosyl bond between the DNA and enzyme can be attacked by the 5'-hydroxyl of the original cleaved strand, reversing the reaction and releasing the topoisomerase (Shuman, 1994). The cloning reaction was carried out as recommended by the manufacturer.

Polymerase chain reaction (PCR)

DNA was amplified by PCR with specific oligonucleotide primers. For each DNA fragment of interest, primers were designed. In case of partly unknown target sequences degenerate primers were applied. For cloning of DNA fragments into plasmid vectors, recognition sequences for specific restriction endonucleases were added via primers at both ends of the amplified DNA fragments. All oligonucleotides used in this work are listed in the appendix. The PCR reactions were performed in a total volume of 50 µl. For amplifying genes from environmental templates and control PCR the *Taq* DNA polymerase was used. For other templates the Phusion[®] High-Fidelity DNA Polymerase was applied according to the manufacturer's instructions.

The primers CWAprB1-fw and CWAprB3-fw were designed with Blockmaker (Henikoff et al., 1995) and CODEHOP (Rose et al., 1998) based on the *aprBA*-sequences of *Desulfovibrio desulfuricans* G 20, *Desulfotalea psychrophila* LSv54, *D. vulgaris* Hildenborough and *Archaeoglobus fulgidus*.

Reaction mixture for the *Taq* DNA Polymerase (50 µl):

- 5 µl forward primer (5 µM)
- 5 µl reverse primer (5 µM)
- 2 µl template DNA (approximately 100 ng)
- 5 µl 10 x ThermoPol Reaction Buffer
- 0.5 µl *Taq* Polymerase (5 U/µl)
- 2 µl dNTPs mixture (10 mM)
- ad 50 µl PCR water

The prepared reaction mixture was applied for the listed PCR programs (see below) in a thermocycler. The annealing temperatures were adapted to the melting temperatures (T_m) of the used primers. The elongation times were adjusted to the DNA fragment length.

Reaction conditions for standard PCR

Cycles	Step	Temperature	Reaction time
1	initial denaturation	94 °C	10 min
30-35	denaturation	94 °C	1 min
	annealing	$T_m - 5$ °C	1.5 min
	elongation	72 °C	1 min per 1 kb
1	final elongation	72 °C	10 min
1	break	12 °C	∞

Touchdown and nested PCR conditions for *aprBA* fragment:

First step with degenerate primers CWAprB1-fw and AprA-5-RV;

Second step with degenerate primers CWAprB3-fw and APS-RV:

Cycles	Step	Temperature	Reaction time
1	initial denaturation	94 °C	3 min
20	{	denaturation	94 °C
		annealing	55 °C
		each cycle	
		-0.5 °C	
20	{	elongation	72 °C
		denaturation	94 °C
		annealing	45 °C
		elongation	72 °C
1	final elongation	72 °C	10 min
1	break	12 °C	∞

Primer 8f and 1492r for 16S rDNA amplifications were used as described elsewhere (Alain et al., 2006).

For DGGE analysis, products were used for a nested PCR adding a GC-clamp (341f-GC/907aR and 907cR; Schäfer et al., 2001; Stadnitskaia et al., 2005):

Cycles	Step	Temperature	Reaction time
1	initial denaturation	94 °C	5 min
20	{	denaturation	94 °C
		annealing	65 °C
		each cycle	
		-0.5 °C	
15	{	elongation	72 °C
		denaturation	94 °C
		annealing	45 °C
		elongation	72 °C
1	final elongation	72 °C	7 min
1	break	12 °C	∞

For amplification of archaeal rDNA, the primer pair Arch21F/Arch958R were applied (Delong, 1992):

Cycles	Step	Temperature	Reaction time
1	initial denaturation	95 °C	2 min
25		denaturation	95 °C
		annealing	56 °C
		to	1 min
		66 °C	
	elongation	72 °C	80 s
1	final elongation	72 °C	10 min
1	break	12 °C	∞

Purification of PCR products

PCR products were purified with the peqGOLD MicroSpin Cycle-Pure Kit following the manufacturer's instructions.

Denaturant gradient gel electrophoresis (DGGE) analysis

Denaturant gradient gel electrophoresis was performed by using the INGENYphorU system according to the manufacturer's instructions. 25 µl purified PCR of the products were mixed with 10 µl loading buffer (25 % [w/v] Ficoll 400, 0.25 % bromophenol blue, 0.25 % xylene cyanol, in H₂O). PCR purification was done with peqGOLD MicroSpin Cycle-Pure Kit. Polyacrylamide gels (6 % [w/v]) were applied in 1× TAE, with a denaturing gradient from 20 % to 70 % (100 % denaturant corresponds to 7 M urea and 40 % formamide). Electrophoresis was performed at a constant voltage of 100 V and a temperature of 60 °C for 16 h. The gels were stained for 20-40 min in 1× SybrGold solution (1:10,000) in 1× TAE and documented by a digital imaging system. DGGE bands were excised for sequence analysis using a sterile scalpel and incubated in 200 µl of PCR water for 1 h at RT, followed by 30 µl for 24 h at 4 °C. The solution was taken as a template for the reamplification using the same PCR conditions as described above. The purified amplification products were sequenced.

Construction of 16S rDNA clone library and phylogenetic tree

The DNA was extracted from 5 g fluids as already described elsewhere (Henne et al., 1999). Obtained PCR products (primers Arch21F/Arch958R, see above) were analyzed for appropriate size and then purified by gel extraction peqGOLD Gel Extraction Kit. PCR products were cloned into pCR2.1-TOPO as recommended by the manufacturer. The PCR product-containing vectors were used to transform *E. coli* TOP10. Recombinant plasmids were isolated from randomly selected *E. coli* clones and sequenced. The 16S rRNA gene sequences from clone libraries were edited by using the gap4 program (Staden Package; Bonfield et al., 1995). All sequences were checked for chimeric artifacts by using Mallard (Ashelford et al., 2006), the CHIMERA_CHECK program (Maidak et al., 2001) of the Ribosomal Database Project (RDP) II database (Cole et al., 2003) and Bellerophon (Huber et al., 2004). 133 clones were used for further analyses. Taxonomic classification was performed by using the BLAST tool of the National Center for Biotechnology Information (NCBI) database (Altschul et al., 1990). Operational taxonomic units (OTUs) were determined at sequence similarity levels of 97 % and 80 % by using the furthest-neighbor method of DOTUR (Schloss and Handelsman, 2005). To determine the number of observed unique OTUs as a function of the distance between sequences and the number of sequences sampled, rarefaction analysis was performed. In addition to the Shannon-Weaver diversity index, the Chao1 richness estimator, and the abundance-based coverage estimator (ACE) were calculated by using DOTUR. Only one sequence per OTU (> 99 % identity cutoff) was used for construction of phylogenetic trees. Sequences of the nearest neighbors were retrieved from the NCBI database. The 16S rRNA gene sequences from clone libraries and the most similar neighbors were imported into the most recent SSU Ref SILVA database (www.arb-silva/download/) of the ARB program package (Ludwig et al., 2004). Multiple sequence alignments were checked manually and improved by using the ARB editor tool. Phylogenetic trees were created by using the maximum-parsimony algorithm implemented in ARB. The robustness of obtained tree topologies was evaluated by bootstrap analysis with 100 resamplings.

Preparation of competent *E. coli* cells (Inoue et al., 1990)

250 ml SOB medium (see 2.2.) were inoculated with one colony *E. coli* DH5 α in a 1 l culture flask without baffles and grown at RT under agitation (200 rpm) for at least 36 h ($OD_{600\text{ nm}} = 0.6$). Then, the cells were incubated for 10 min on ice, before centrifugation (10 min, 2,500 g, 4 °C). The pellet was resuspended in 80 ml TB and held on ice for 10 min, followed by a centrifugation step (10 min, 2,500 g, 4 °C). The pelleted cells were resuspended in 20 ml TB and under gentle shaking DMSO was added (final concentration 7 %). The cell suspension was kept for 10 min on ice and then frozen in 200 μ l aliquots in liquid nitrogen. The cells were stored at -70 °C.

Transformation buffer (TB)

PIPES	0.76	g
CaCl ₂ · H ₂ O	0.55	g
KCl	4.66	mg
MnCl ₂ · H ₂ O	2.71	mg
H ₂ O	ad 250	ml

The pH-value was adjusted to pH 6.7 and the buffer was sterilized by filtration.

Transformation of *E. coli*

Competent cells were thawed on ice, and 10-100 ng DNA was added to 200 μ l cells. The suspension was mixed and incubated on ice for 30 minutes. Then, a heat shock was performed at 42 °C for 90 seconds. Afterwards, the samples were incubated for 5 minutes on ice. After addition of 600 μ l LB medium, the samples were incubated for 1 h at 37 °C under agitation (200 rpm). 100 μ l and the concentrated rest were plated on LB selection plates (with ampicillin or kanamycin, respectively).

2.3.5. Preparation and analysis of proteins

Discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of proteins was analyzed for by SDS-PAGE as described by Laemmli (1970). Protein samples were denatured by boiling in SDS loading dye at 95 °C for 10 min. The polyacrylamide concentrations of the gels were chosen according to the expected protein sizes varying from 12 to 16 % (v/v). The gels consisted of a separating gel and a stacking gel. The whole gel measures 8.6 x 7.7 cm (W x L) with a thickness of 1 mm. The wells of the prepared stacking gel were loaded with the samples.

Electrophoresis was performed at 120 V until the bromophenol blue had reached the lower end of the gel. During electrophoresis, proteins were first focused in the stacking gel and subsequently separated according to their molecular mass in the running gel. A prestained protein molecular weight marker was used as size standard (Fig. 2.4.). Gels were stained with staining solution containing Coomassie Brilliant Blue R-250 and destained until the protein bands were clearly visible.

Preparative gels were run at low temperatures of approximately 8 °C. The separated target proteins were excised with a sterile razor blade.

Solutions for SDS-PAGE:

5 x SDS loading dye

Glycerol	3	ml
1 M Tris-HCl pH 7.0	1.4	ml
H ₂ O	2	ml
20 % (w/v) SDS	2	ml
2-Mercaptoethanol	1.6	ml
Bromophenol blue	0.01	g

Electrophoresis buffer

Tris	30	g
Glycine	140	g
SDS	10	g
H ₂ O	ad 1000	ml

Stacking gel

30 % Acrylamide-Bisacrylamide (37.5:1)	1.3	ml
1.5 M Tris-HCl pH 6.8	0.87	ml
H ₂ O	6.83	ml
20 % (w/v) SDS	50	μl
10 % (w/v) APS	100	μl
TEMED	20	μl

Separating gel (12 %)

30 % Acrylamide-Bisacrylamide (37.5:1)	4	ml
1.5 M Tris-HCl pH 8.8	2.6	ml
H ₂ O	3.2	ml
20 % (w/v) SDS	50	μl
10 % (w/v) APS	100	μl
TEMED	10	μl

Separating gel (16 %)

30 % Acrylamide-Bisacrylamide (37.5:1)	5.12	ml
1.5 M Tris-HCl pH 8.8	2.6	ml
H ₂ O	2.08	ml
20 % (w/v) SDS	50	μl
10 % (w/v) APS	100	μl
TEMED	10	μl

Staining solution

Coomassie Brilliant Blue R250	5	g
Acetic acid	100	ml
Methanol	450	ml
H ₂ O	ad 1000	ml

Destaining solution

Acetic acid	100	ml
H ₂ O	ad 1000	ml

8-16% Tris-glycine SDS-PAGE

Figure 2.4.: Prestained protein molecular weight marker.**Overexpression of proteins in *E. coli***

Prior to large scale overproduction, a small scale pretest was performed. For this purpose, 50 ml LB medium with ampicillin were inoculated with an overnight culture of an *E. coli* strain carrying the relevant overexpression vector to $OD_{600} = 0.1$. The culture was incubated at 37 °C under agitation (200 rpm). The expression was started with a final concentration of 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 2 h. As control a parallel culture was grown, which was not induced. To test the expression, small aliquots (sample [μ l] = 100/ OD_{600}) were taken directly before and 2 h after the induction. The samples were boiled in SDS loading dye and analyzed by SDS-PAGE. In case of a positive pretest two 1 l cultures of LB medium with ampicillin were inoculated with an overnight culture to an $OD_{600} = 0.1$ in a culture flask with baffles. The cultures

were induced as described above. After 2 h the cells were harvested (15 min, 8,000 rpm, 4 °C). After removing the supernatant the cells were stored at -20 °C.

Prior to cell disruption the pooled pellets were resuspended in 24 ml LEW buffer with 8 M urea and 10 mM imidazole.

Solutions for overexpression:

IPTG stock solution (1 M)

IPTG	2.38	g
H ₂ O	ad 10	ml

Sterilized by filtration and stored at -20 °C.

LEW buffer

NaH ₂ PO ₄ · 2 H ₂ O	7.8	g
NaCl	17.5	g
H ₂ O	ad 1000	ml

Adjusted to pH 8.0 with NaOH.

Cell disruption by sonication

Volumes less than 2 ml were treated six times with the ultrasonic probe at 60 W for 10 s. The cycle was adjusted to 0.6. Between each sonication step the samples were kept on ice for 10 s. The samples were centrifuged (30 min, 13,000 rpm, 4°C) to remove remaining membrane compounds.

For larger volumes the samples were treated ten times with the ultrasonic probe at 85 W for 15 s. The cycle was adjusted to 0.5. Sonication steps were interrupted by 15 s breaks. The samples were kept on ice all the time. The samples were centrifuged two times (30 min, 8,000 rpm, 4 °C) to remove remaining membrane compounds.

Purification of His₆-tagged proteins

For protein purification, the described cell suspension was disrupted under denaturing conditions by sonication (see above). For purification of recombinant His₆-tagged proteins the supernatant fraction was loaded onto a 1 ml bed volume of Ni²⁺-NTA resin

in a chromatography column. The Ni^{2+} -NTA resin had been pre-equilibrated with 8 ml LEW buffer with 8 M urea and 10 mM imidazole. In a first 8 ml washing step a higher ionic stringency was applied (LEW buffer with 8 M urea, 10 mM imidazole and additional 700 mM NaCl). In a second 8 ml washing step the proteins were renatured (LEW buffer with 10 mM imidazole and additional 700 mM NaCl). Finally, the target protein was eluted in five steps with increasing imidazole concentration. 50, 100, 200, 300 and 500 mM imidazole were used in 3 ml elution fractions. Each fraction was analyzed by SDS-PAGE. The protein concentration was determined according to the method of Bradford using the Roti-Quant solution with bovine serum albumin as standard (Bradford, 1976). Protein solutions were kept on ice throughout. Purified proteins were used for immunizations of rabbits (Eurogentec, Seraing, Belgium) using an 87-day protocol with Freund's adjuvant.

For purification of His₆-tagged MDH, Protino Ni-TED 2000 kit was used according to the manufacturer's instructions for denaturing conditions. Purified MDH was dialyzed against LEW buffer (see above) in relation 1 to 1000 in a dialysis tube at 8 °C for 36 h (12-14 kDa molecular weight cut off) to remove urea. LEW buffer was once renewed. Prior to dialysis, the dialysis tube was boiled in 1 l 5 mM EDTA in H₂O. This step was repeated in 1 l H₂O.

Western blot analysis

Proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane with a semi-dry blotting apparatus. The PVDF membrane was activated in methanol and equilibrated in transfer buffer. A stack of following layers was prepared (from bottom to top): 3 layers Whatman paper soaked with transfer buffer, activated and equilibrated PVDF membrane, SDS-PAGE washed in transfer buffer, 3 layers of Whatman paper soaked with transfer buffer. The stack was loaded on the blotting apparatus and proteins were transferred for 1 h at 0.8 mA/cm². Protein transfer to the membrane was controlled by the applied prestained protein molecular weight marker. The membrane was incubated under continuous shaking in Blotto overnight to block unspecific binding sites on the membrane. Antibodies were diluted in Blotto and incubated with the membrane for 3 h at RT, while shaking. The antibody solution was removed and the membrane was washed three times for 30 min with Blotto. Then, the membrane was

incubated in the solution with secondary antibodies (anti-rabbit IgG coupled with alkaline phosphatase, diluted 1:100,000 in Blotto) for 30 min at RT. Again, the membrane was washed 3 times for 30 min in Blotto, followed by a short rinse with water and by equilibration in Buffer III for 5 min. Proteins were detected and quantified by chemiluminescence using CDP-Star (diluted 1:100 in Buffer III) as a substrate for alkaline phosphatase.

Solutions for western blot analysis:

Blotto

TBS 10 x	100	ml
Skim milk	25	g
Tween 20	1	ml
H ₂ O	ad 1000	ml

Buffer III

Tris	12.1	g
NaCl	5.8	g
H ₂ O	ad 1000	ml

Adjusted to pH 9.5 with HCl.

TBS 10 x

Tris	60	g
NaCl	90	g
H ₂ O	ad 1000	ml

Adjusted to pH 7.6 with HCl.

Transfer buffer

Tris	15.2	g
Glycine	72.1	g
Methanol	750	ml
H ₂ O	Ad 5000	ml

2.3.6. Microscopy

Light microscopy

For light microscopic analysis phase contrast was applied. Experiments were performed with the Axioskop 40 FL fluorescence microscope, equipped with digital camera and AxioVision software for image processing and Neofluar series 10 x, 40 x and 100 x objectives. For microscopy of all suspensions, a 50 µl drop taken from liquid culture was applied to a microscopy slide and covered with a coverslip. Semithin sections of samples embedded in Lowicryl K4M resin (see below, thickness approximately 400 nm) were transferred to Formvar-coated Cu single slot grids (Hoppert and Holzenburg, 1998) and processed for light microscopy and immunofluorescence (cf. Wrede et al., 2008).

Fluorescence microscopy

For fluorescence microscopy, following filter sets were used: EGFP HC-Filterset (BP: 472/30, FT 495, LP 520/35) for AlexaFluor 488, Atto488 and 6-FAM fluorescence and filter set 43 (BP: 545/25, FT 570, LP: 605/70) for AlexaFluor 546 and CY3 visualization. DAPI signals were analyzed with filter set 49 (BP: 445/50, FT: 395, G: 365). Images were processed with Adobe Photoshop.

Transmission electron microscopy (TEM)

The analyses were performed with a Zeiss Transmission Electron Microscope 902A equipped with an in-column electron energy loss spectrometer at 80 kV accelerating voltage and calibrated with a cross-lined grating replica. Micrographs were taken with a $1,024 \times 1,024$ charge-coupled device detector in combination with iTEM software. Images were processed with Adobe Photoshop or analyzed with ImageJ for line-density scan (Hoppert and Holzenburg, 1998; Collins, 2007).

Grids

Sections were transferred to Formvar-coated grids (diameter: 3.05 mm), Ni G300 for TEM and Cu single slot for immunofluorescence (Hoppert and Holzenburg, 1998).

Support films (Mahl and Möldner, 1973)

Ahead of uptaking the specimen, grids were coated with Formvar film for stabilization of ultrathin sections. Therefore, Formvar (Poly [vinyl formal]) was dissolved to a final concentration of 0.5 % (w/v) in water-free chloroform. The solution was filtered and stored in the dark. A microscopy slide was cleaned with detergent and water causing a thin residual layer of detergent on the surface. The microscopy slide was placed inside a cylindrical glass funnel (fitted with a tap and drain tube), filled with Formvar solution so that the slide was submersed at maximum 3/4 of its length. The slide was incubated in the covered funnel for 30 s, then the solution was drained off. After another 30 s, the microscopy slide was removed from the glass funnel and dried in a dust-free vessel. The edges of the microscopy slides were scratched with a razor blade and the film was floated on a clean water surface at an angle of 30-45°. The grids were placed on the floating film with the dull site down. The backing paper of Parafilm was lowered onto the grids and gently pressed home. The whole construction was removed from the water surface and dried upside-down on filter paper. The coated grids were stored in dust-free Petri dishes.

Fixation of TEM samples

For embedding in Lowicryl K4M resin, which allows preservation of antigenic determinants in specimens, samples of the Black Sea cold seeps were cut into small cubes with approximate edge lengths of 2 mm. The cubes were washed several times in PBS (50 mM, pH 7.5) and fixed in 0.3 % (v/v) solution of glutardialdehyde and 0.5 % (w/v) formaldehyde in PBS for 2 h. Then free aldehyde groups were blocked by washing the cubes in PBS with 10 mM glycine.

Cells from cultures were harvested (20 min, 5,000 rpm, 4 °C) and washed in PBS. The cells were subsequently fixed as described above and 50-150 µl aliquots were embedded by mixing with 2.5 % (w/v) molten Bacto-Agar in PBS (kept at 60 °C). After gelling, small cubes were cut and treated as described for the cold seep microbial mats. Since osmium tetroxide staining destroys the specific antigenic binding sites, it was omitted during sample preparation. All steps were performed at 4 °C.

For embedding in Spurr resin, cultured cells were harvested (20 min, 5,000 rpm, 4 °C) and washed in PBS (according to Spurr, 1969; modified). After that, cells were prefixed in 3.5 % (v/v) glutardialdehyde for 1.5 h, washed with PBS and embedded in 2.5 % (w/v) molten Bacto-Agar (kept at 60 °C). The gelled agar was cut in small cubes as already described. Microbial mats from GHOSTDABS cold seeps were directly cut into small cubes, followed by prefixation and washing in PBS. Then, the cubes were incubated with 1 % (w/v) osmium tetroxide in PBS for 1 h and washed in water for three times.

Low temperature embedding in Lowicryl K4M resin (Roth et al., 1981; Armbruster et al., 1982)

Low temperature embedding preserves the native structure of proteins. For that reason the low temperature embedding was chosen to preserve the ultrastructure of cells and the antigenicity of proteins. For dehydration, an ascending ethanol series was used. The temperature was successively lowered down to –35 °C. Dehydrated samples were transferred to a dilution series of Lowicryl K4M resin in ethanol, followed by pure resin, detailed protocol below:

Solution	Incubation time	Incubation temperature
15 % Ethanol	15 min	0 °C
30 % Ethanol	30 min	0 °C
50 % Ethanol	30 min	-20 °C
70 % Ethanol	30 min	-35 °C
95 % Ethanol	30 min	-35 °C
100 % Ethanol	2 x 30 min	-35 °C
50 % Methanol/50 % Lowicryl K4M resin	60 min	-35 °C
33 % Ethanol/66 % Lowicryl K4M resin	120 min	-35 °C
100 % Lowicryl K4M resin	1 x short change, then over night	-35 °C

Subsequently, the samples were transferred to gelatin capsules, which were filled with Lowicryl K4M resin. Polymerization was catalyzed in the deep freezer at -35 °C with ultraviolet light (260 nm) for 60 h.

Lowicryl K4M resin

Crosslinker	6.75	g
Monomer	43.30	g
Initiator	0.25	g

Spurr resin embedding (Spurr, 1969; Hoppert, 2003)

For embedding, a dehydration protocol with an ascending acetone series was used. Dehydrated samples were transferred to a dilution series of Spurr resin in acetone, followed by pure resin (detailed protocol below).

Solution	Incubation time	Incubation temperature
10 % Acetone	10 min	0 °C
30 % Acetone	10 min	0 °C
50 % Acetone	10 min	0 °C
70 % Acetone with 2 % (w/v) uranyl acetate	2 h or overnight	0 °C
70 % Acetone	10 min	0 °C
90 % Acetone	10 min	0 °C
100 % Acetone	10 min	0 °C
66 % Acetone/33 % Spurr resin	30 min	RT
25 % Acetone/75 % Spurr resin	90 min	RT
100 % Spurr resin	16 h	RT
100 % Spurr resin	3 h	RT

Subsequently, the samples were transferred to gelatin capsules, which were filled with Lowicryl K4M resin. Capsules were de-gassed in a vacuum chamber for 10 min. Polymerization was performed at 70 °C for 24 h.

Spurr resin

Vinylcyclohexene dioxide	15	g
Diglycidyl ether of polypropylene glycol (D.E.R. 736)	9	g
Nonenyl succinic anhydride (NSA)	39	g
Dimethylaminoethanol	0.6	g

Ultramicrotomy

The embedded specimens were trimmed with a rotating milling cutter to a small flat-topped pyramid with a face of about 0.2-2 mm². For sectioning with an ultramicrotome glass knives were prepared from a glass strip of 25 mm width and 6.5 mm thickness. Prior to use, the glass strip was thoroughly cleaned. Glass squares (25 x 25 mm) were made with a special knife-maker. By breaking the squares slightly off the diagonal axis, two usable knives were obtained. With a counterpiece of 0.5 mm thickness a real angle of 55° at the cutting edge was obtained. Quality characteristics are well formed conchoidal fracture marks and a unbowed knife edge without flaws. Knives accomplishing these features obtained an plastic tray fixed with special wax.

Knives were stored dust-free. Glass knives were used for smoothing the trimmed specimen and semithin as well as ultrathin sectioning.

The preparation of semithin and ultrathin sections was performed with an ultramicrotome. The trimmed block was mounted and the glass knife was fixed at an angle of 5°. The cutting edge was adjusted parallel to the cut surface. The tray was filled with water until the water surface is almost flat and extends to the cutting edge. This allows an optimal floating of the sections on the water surface. The thickness of the sections was checked on the basis of interference colors (Tab. 2.4.). For TEM, 40-100 nm ultrathin sections were prepared. Sections were moved with a mounted eyelash on the water surface and picked up with Formvar-coated specimen grids. For this purpose the grid, orientated with the support film downwards, was slowly lowered

onto the water surface. There the sections were allowed to adsorb on the support film for some seconds. Grids were withdrawn, the excessive liquid was drained off with a piece of filter paper and the grid was stored dust-free upside-down on filter paper.

Table 2.4.: Section thicknesses.

Interference color	Section thickness
Grey to silver	60-90 nm
Gold	90-150 nm
Purple-blue	150-190 nm
blue	190-240 nm

Post-staining of sections

For post-staining of ultrathin sections, phosphotungstic acid (PWS) was used. Therefore, phosphotungstic acid (pH 7.0, 1 % [w/v]) was centrifuged for 10 min, 13,000 rpm to remove particles. The grids with adsorbed specimen sections were incubated for 3 min on drops of particle-free PWS applied on fixed Parafilm. The excessive liquid was drained off with a piece of filter paper. Grids were dried dust-free upside-down on filter paper.

Furthermore, staining with uranyl acetate was applied. Uranyl acetate (4 % [w/v]) was centrifuged for 10 min, 13,000 rpm to remove particles. The grids were stained as described for staining with PWS.

To enhance staining of membranes, uranyl acetate and lead citrate staining were applied (Venable and Coggesha, 1965). Therefore, grids were incubated for 2 min on 0.1 M EDTA solution, followed by uranyl acetate staining as described. Lead citrate solution was freshly prepared: 10 % (w/v) lead citrate in 0.1 M NaOH (solution in carbon dioxide-free [boiled] water), centrifuged for 10 min and 13,000 rpm. Then, staining was performed in carbon dioxide-free atmosphere obtained by moistened NaOH pellets in a Petri dish. Grids were incubated for 3 min on lead citrate drops and subsequently washed by repeated dipping in a series of three 10 ml beakers of carbon dioxide-free water.

Immunocytochemistry for TEM and fluorescence microscopy

Ultrathin sections (60–100 nm) were used for immunocytochemistry with TEM. Semithin sections (approximately 400 nm) were used for immunofluorescence analyses (Wrede et al., 2008). Sections were transferred to Formvar-coated grids (Hoppert and Holzenburg, 1998), Ni 300 mesh for TEM and Cu single slot for immunofluorescence. For all labeling procedures grids were placed on drops of antibody dilutions and washing solutions with section facing downwards.

For TEM immunocytochemistry of Black Sea cold seep microbial mats the following protocol was applied: 30 min blocking with 3 % (w/v) bovine serum albumin (BSA) in PBS, 2 h primary antibodies (anti-MDH [α -MDH] antibodies in 1 % [w/v] BSA, anti-AprB [α -AprB] antibodies in 0.2 μ g/ μ l *M. mazei* Gö1 crude extract with protease inhibitor, complete EDTA free) with appropriate dilutions (α -MDH antibodies 1:50-1:1000; α -AprB antibodies 1:50-1:400; Wrede et al., subm.), three 5 min washing steps with PBS containing 0.05 % (v/v) Tween 20, 5 min washing step with PBS, 1 h secondary antibodies (goat-anti-rabbit IgG-10 nm gold conjugate) 1:80 diluted in the same solutions as first antibodies, three 5 min washing steps with PBS containing 0.05 % (v/v) Tween 20, 5 min washing step with PBS, two times 10 s desalting in H₂O. Post-staining was performed with 4 % (w/v) uranyl acetate solution for 3 min. All steps were performed at room temperature. The applied polyclonal antibodies were used as original antiserum. For controls embedded mixture of *M. mazei* Gö1 (AprB: not present, MDH: not present, MCR: present) and *D. vulgaris* Hildenborough (AprB: present, MDH: not present, MCR: not present) was used, to simulate a situation in the microbial mat layers. In addition, *M. extorquens* AM1 and 5 methylotrophic isolates were used as positive controls for the MDH labeling. Labeling without primary antibodies was applied to check the specificity of secondary antibodies. Furthermore, the specificity of all applied antibodies was checked by Western blot analyses.

For immunofluorescence, the protocol for TEM immunocytochemistry was applied (see above) with following modifications: 20 min blocking with 10 % goat normal serum in PBS prior to blocking with BSA. As secondary antibody, a goat-anti-rabbit IgG AlexaFluor 546 conjugate, diluted 1:250, was applied.

After desalting, the specimens were mounted with DPX on a microscopy slide. Modifications for double labeling: In a first labeling approach anti-MCR [α -MCR] labeling was performed as described (cf. for antibody Heller et al., 2008), but with F(ab')₂ fragment of goat-anti-rabbit IgG (H+L) AlexaFluor 488 conjugate, diluted 1:100. After air drying the α -AprB labeling was performed as described.

Cell extraction from volcano fluids (Kallmeyer et al., 2008)

Due to low cell counts, the fixed samples from the mud volcano fluids (see 2.1.3.) were extracted, concentrated and filtered onto 0.2 mm polycarbonate filters according to Kallmeyer et al. (2008).

Therefore, the stored samples were thoroughly mixed by vortexing. 33.3 μ l fluids were mixed with 66.6 μ l NaCl solution (with 2 % formalin). The slurry (100 μ l) was added to 500 μ l acetate buffer and incubated for 2 h under occasional vortexing. All carbonates were usually dissolved within 2 h. Vials were opened several times during the carbonate dissolution to avoid CO₂ overpressure. Carbonate-free slurries were centrifuged for 5 min at 3,000 g. The clean supernatant was removed and kept for further analysis, as the subsequent supernatants from the detergent mix/methanol treatment. The remaining pellet was used for further processing. The acetate buffer was completely removed from the sediment, as it may contain enough calcium ions interfering with the detergent mix. The pellet was washed twice with NaCl solution (with azide; 5 min, 3,000 g). The rinsing solution was kept for further analysis. The pellet was diluted with 300 μ l NaCl solution (with azide) and 50 μ l each of the detergent mix and methanol were added. The mixture was vortexed for 60 min for suspension of cells. A cushion of 500 μ l 60 % (w/v) Nycodenz was layered below the slurry with a syringe and the sample was centrifuged at 3,000 g for 10 min for separation of cells from suspension. The supernatant was carefully removed and kept for further analysis. The remaining Nycodenz was discarded. The remaining pellet was resuspended in 400 μ l NaCl solution (with azide) and another 50 μ l each of detergent mix and methanol were added. Then, the vial was sonicated in an ice water bath for 3×10 s with 20 s between the cycles (20 ml beaker with ice water; 20 W, cycle 0.5). Addition of Nycodenz and density centrifugation was repeated as described above. The supernatants were kept at

4 °C and used for cell counting and FISH analyses (Supernatants of two extractions were applicated onto one filter).

Solutions used for the cell extraction:

NaCl solution (with 2 % formalin)

NaCl	0.39	g
H ₂ O	ad 100	ml

Autoclaved and 2 % formalin (sterile filtered) added. Stored at 4 °C. Salinity should be adapted to each habitat.

NaCl solution (with azide)

NaCl	0.39	g
H ₂ O	ad 100	ml

Autoclaved and 0.1 % NaN₃ added. Stored at 4 °C. Salinity should be adapted to each habitat.

Acetate buffer

Glacial acetic acid	2	ml
Sodium acetate	3.5	g
NaCl	0.39	g
H ₂ O	ad 100	ml

Autoclaved and 2 % formalin (sterile filtered) added. Stored at 4 °C. Salinity should be adapted to each habitat.

Detergent mix

Disodium EDTA dihydrate	3.7	g
Sodium pyrophosphate decahydrate	4.5	g
Tween 80	1	ml
NaCl	0.39	g
H ₂ O	ad 100	ml

After autoclaving, the solution was cooled while stirring to avoid separation of Tween 80. Then 2 % formalin (sterile filtered) was added. Stored at 4 °C. Salinity should be adapted to each habitat.

Methanol was sterilized by filtration and stored at 4 °C.

All materials were flamed or autoclaved.

Enumeration of total archaea and bacteria

The extracted cells of two batches (see above) were applicated onto one filter. For this purpose, a hand-operated vacuum pump in combination with a combusted filtration tower was used. The moistened cellulose nitrate support filter (0.45 µm pore size) and the polycarbonate filter (0.2 µm pore size) were placed into the filtration tower. The supernatants were applicated into the filter tower and filtered by applying a gentle vacuum of -200 mbar. Support filters were utilized for several samples. For washing, 5 ml sterile H₂O were filtered two times.

For enumeration of cells, the prepared filters were DAPI stained and counted using the protocol of Sherr et al. (2001). For this purpose, 2 ml DAPI solution with a concentration 2 µg/ml in H₂O were incubated for 10 min in the dark on the filter. The solution was removed by applying underpressure (-200 mbar). Subsequently, the filter was washed two times with 2 ml H₂O. The filter was put in a Petri dish and allowed to air-dry. Finally one half of a filter was mounted with DPX on a microscopy slide and analyzed by fluorescence microscopy.

Cells were counted per camera field of view with a 40 x objective. The camera field of view corresponds 140 x 105 µm (W x L). The net filter surface of 2.3 cm in diameter corresponds to 28,264 camera fields of view. Because of the low cell counts, three microscopy slides were counted. On each microscopy slide 20 randomly selected camera fields of view were counted. The calculated average was extrapolated to the filter area.

Solutions used for the cell enumeration:

DAPI stock solution

DAPI	1	mg
H ₂ O	ad 1	ml

Stored at -20 °C in the dark.

DAPI solution

DAPI stock solution	4	μl
H ₂ O	ad 2	ml

Prepared directly prior to use. Protected from light.

Fluorescence *in situ* hybridization (FISH)

The described protocol refers to FISH with monolabeled fluorescent probes on membrane filters and glass slides (according to Pernthaler et al., 2001; modified). The extracted cells were applied to a polycarbonate filter (0.2 μm pore size) as described for cell enumeration. The filter was air-dried, followed by mounting of the filter with molten 0.9 % (w/v) agarose in water on a microscopy slide. One half of a filter was used per microscopy slide. The molten agarose was applied as a very thin layer (spread with a coverslip).

2 ml of hybridization buffer were prepared in a microfuge tube. For the hybridization mixtures 4 μl of probe working solution (50 ng/μl) were added to 36 μl of hybridization buffer. The hybridization mixtures and the probe solutions were kept dark and on ice. A piece of blotting paper was placed in a 50 ml polyethylene tube and soaked with the remaining hybridization buffer. Then, the agarose layer on the microscopy slide was covered with the hybridization mix. The slide was placed into the polyethylene tube in a horizontal position. The samples were incubated at 46 °C for at least 90 min (maximum: 3 hours) in a hybridization oven. Subsequently the slides with the filter sections were quickly transferred into preheated washing buffer and incubated for 15 min at 48 °C. In a next step the slides were rinsed by placing them into a Petri dish with distilled H₂O for 5 s. Then, the slides with filter sections were air-dried on blotting paper.

For counterstaining, filter sections were covered with 50 μl of DAPI solution (see above) and were incubated for 3 minutes. Afterwards, filter sections were washed for 3 min with distilled H₂O to remove unspecific staining, followed by mounting with distilled H₂O or PBS in the case of applied fluorochrome 6-FAM (requires pH ≥ 7). Fluorescence of the hybridized probes fades much more rapidly than DAPI fluorescence, and UV excitation will also bleach the CY3 signal. Therefore, it is recommended to analyze specifically stained cells first and in a second step the DAPI fluorescence under excitation by UV light with filter set 49.

All working steps were performed in the dark and solutions were protected from light with aluminum foil. The applied probes with respective formamide concentrations and fluorochromes are given in Tab 2.6.

Solutions used for FISH analyses:

Hybridization buffer

5 M NaCl	360	μl
1 M Tris-HCl pH 7.4	40	μl
Formamide	% depending on probe	
H ₂ O	ad 2	ml
10 % (w/v) SDS	2	μl

To avoid precipitation SDS is added last.

Washing buffer

5 M NaCl	depending on % formamide in hybridization buffer (Tab. 2.5.)	
1 M Tris-HCl pH 7.4	1	ml
0.5 M EDTA	0.5	ml
H ₂ O	ad 50	ml
10 % (w/v) SDS	50	μl

To avoid precipitation SDS is added last.

Table 2.5.: Concentrations of NaCl in washing buffer (48 °C) at different concentrations of formamide in hybridization buffer (46 °C).

% formamide in hybridization buffer	mM NaCl in washing buffer
5	900
10	636
15	450
20	318
25	225
30	159

% formamide in hybridization buffer	mM NaCl in washing buffer
35	112
40	80
45	56
50	40
55	28
60	20
65	14
70	10
75	7
80	5
5	3.5

Table 2.6.: Oligonucleotide probes used in this work.

Probe	Probe sequence (5'-3') (Specificity)	Dye	% FA	Reference
Arch915	GTGCTCCCCGCCAATTCCT (most archaea)	CY3 or Atto 488	35	(Stahl and Amann, 1991; Lösekann et al., 2007)
DSS658	TCCACTTCCCTCTCCCAT (<i>Desulfosarcina/Desulfococcus</i> branch)	6-FAM	40	(Manz et al., 1998; Boetius et al., 2000; Lösekann et al., 2007)
EelMS932	AGCTCCACCCGTTGTAGT (ANME-2 archaea)	CY3	40	(Boetius et al., 2000)
EUB338 I	GCTGCCTCCCGTAGGAGT	CY3	35	(Daims et al., 1999)
EUB338 II	GCAGCCACCCGTAGGTGT	CY3	35	(Daims et al., 1999)
EUB338 III	GCTGCCACCCGTAGGTGT (EUB338 I-III most bacteria)	CY3	35	(Daims et al., 1999)

3. Results

3.1. Detections of metabolic key enzymes in anoxic GHOSTDABS cold seeps

3.1.1. Methanol dehydrogenase detection of active methano- and methylotrophic bacteria

Production and characterization of α -MDH antibodies

The MDH α -subunit from *Methylobacterium extorquens* AM1 was selected as a metabolic marker for aerobic methane oxidation in Proteobacteria. *M. extorquens* is a model methylotroph with a sequenced and annotated genome (Vuilleumier et al., 2009). The subunit is highly conserved within the group and will also include organisms with a methylotrophic lifestyle.

The *M. extorquens* AM1-MDH is an $\alpha_2\beta_2$ heterotetrameric enzyme consisting of two 66 kDa α -subunits (encoded in *mxoF*; Fig. 3.1.) and two 8 kDa β -subunits (encoded in *mxoI*; Fig. 3.1.; Williams et al., 2005). Since the enzyme catalyzes the oxidation of methanol, it is abundant in the metabolism of methylotrophic as well as methanotrophic bacteria. The MDH α -subunit contains highly conserved sequences, which allows detection of MDH in different methanotrophic or methylotrophic species. The MDH α -subunit from *M. extorquens* AM1 was used as antigen for the production of polyclonal antibodies. Due to its small size, the MDH β -subunit contains less immunodominant regions and was not considered as suitable for antibody production.

For immunocytochemical localizations, polyclonal antibodies have to be monospecific for its target. Cross reaction of other proteins by the antibodies would be difficult to discriminate in microscopic labeling.

Figure 3.1.: Aerobic methane oxidation pathway.

Methanol dehydrogenase is the key enzyme for the aerobic oxidation of C₁-compounds. Details of the gene region encoding the MDH in *M. extorquens* AM1 are depicted in the upper right part of the figure. The *M. extorquens* AM1-MDH is an α₂β₂ heterotetrameric enzyme consisting of the α-subunit 66 kDa (encoded in *mdhA*) and the β-subunit 8 kDa (encoded in *mdhB*; Williams et al., 2005).

The antibodies were produced as described in the Methods section (cf. 2.3.4., 2.3.5. and Krukenberg, 2009). In order to check cross-reactivity and specificity of the α-MDH antibodies, respective antisera were tested by Western blot analysis (cf. 2.3.5.). Five isolates of methylotrophs (all of genus *Methylobacterium*) were used for this approach (Fig. 3.2.). The methylotrophs were isolated from dicot plants (see section 2.3.3.) and characterized by 16S rDNA analysis. MDH was detected in each of the isolated strains. Background or unspecific labeling of other protein bands was completely absent or very low, which shows the sufficient specificity of the antibody. The experiment also showed, that the polyclonal antibody detects MDH in different host organisms. This is a necessary prerequisite for using the antibody as probe for unknown MDH-expressing organisms.

Figure 3.2.: Western blot analysis with α -MDH antibodies.

Detection of the MDH (66 kDa) in crude extracts of 5 methylotrophic environmental isolates and *M. extorquens* AM1. Methylotrophic isolates 3 and 4 show a somewhat lower detection signal, due to less cross reactions of the antibodies with the respective MDH.

MDH expression in microbial mats

In a first study, the α -MDH antibodies were applied to investigate the GHOSTDABS cold seep mats for expressed MDH, indicating the abundance of active methano- or methylotrophs. Samples were taken from the black (surface layer, exposed to sea water) and the pink layer (underlying the black layer) representing the major parts of the multilayered mat taken from carbonate towers of the GHOSTDABS field in the Black Sea Crimean shelf (Reitner et al., 2005b). Fig. 3.3. depicts the Western blot analysis of the separated proteins (Western blot analysis is described in section 2.3.5.). One distinct

band is visible in the lane of the black layer at the expected molecular weight of the MDH α -subunit, corresponding to the results of Western blot analysis of the methylotrophs (Fig. 3.2.).

Figure 3.3.: MDH detection in the GHOSTDABS cold seep mat.

Western blot analysis of MDH (66 kDa; black arrow) in crude extracts of the black and the pink layers (α -MDH antibodies). Only the black layer exhibits a positive signal.

The positive MDH-signal in the black layer shows that methano- or methylotrophic bacteria in this part of the microbial mat are present and, due to the expression of one key enzyme, the pathway is active. The underlying pink layer of the cold seep carbonate towers seems to lack MDH-expressing cells, since the MDH-signal is missing there. The abundance of active methano- or methylotrophic bacteria is very remarkable for this habitat that has been considered as dysoxic so far, and is here described for the first time.

Immunocytochemical localizations (α -MDH)

By using light and electron microscopic imaging, the question was addressed, in which microscale environment the target cells are metabolically active and if any kind of interplay with other organisms of the microbial community is detectable. Therefore, immunofluorescence localization was used to gain an overview of the distribution of MDH-expressing cells. Certain subcellular features, visible by electron microscopy of ultrathin sections are also helpful for distinction of different methylotrophic groups (see section 2.3.6. for details of microscopy). In this microscopical approach also a correlation between low resolution light microscopy and high resolution transmission electron microscopy was performed (cf. Wrede et al., 2008). The environmental samples were embedded with a low temperature embedding procedure in Lowicryl K4M resin (cf. section 2.3.6. for method; Armbruster et al., 1982). The low temperature and a minimized concentration of aldehyde fixatives ensure that the antigenicity of the sample proteins is preserved. As controls, the environmental methylotrophic isolates and *M. extorquens* AM1 were also embedded for immunocytochemistry.

The obtained labeling results show an intensive signal of the MDH in the immunocytochemistry approach (see section 2.3.6. for immunocytochemistry method), which is equivalent to the Western blot analysis (Fig. 3.2.). *M. extorquens* AM1 and the environmental isolates show the abundance of the MDH predominantly in the cell periphery, including the periplasm (Fig. 3.4.). This is according to previous data of MDH immunogold labeling (Fassel et al., 1992). The signal intensity is within the range of other metabolic key enzymes (cf. Rohde et al., 1988). Isolates 3 and 4 show a somewhat lower signal by Western blot analysis, which is also reflected in the immunogold labeling. Hence, for these bacteria higher antibody concentrations are applied (Fig. 3.4.c,d). These results suggest a weaker cross reaction of α -MDH antibodies with MDH of methylotrophic isolates 3 and 4. This finding shows that, though strain-dependent differences in the signal strengths are observable, the enzyme is conserved enough to be detected by the antibodies and the signal remains specific.

Figure 3.4.: TEM immunocytochemistry analysis with polyclonal α -MDH antibodies.

5 methylotrophic environmental isolates and *M. extorquens* AM1 were analyzed. 10 nm immunogold particles mark detected MDH. (a) methylotrophic isolate 1 (α -MDH antibodies diluted 1:1000). (b) methylotrophic isolate 2 (α -MDH 1:1000). (c) methylotrophic isolate 3 (α -MDH 1:100). (d) methylotrophic isolate 4 (α -MDH 1:100). (e) methylotrophic isolate 5 (α -MDH 1:1000). (f) *M. extorquens* AM1 (α -MDH 1:1000).

For immunofluorescence detection, semithin sections of about 400 nm were applied (cf. section 2.3.6. for immunofluorescence technique). Cells expressing MDH, were detected by orange fluorescence of AlexaFluor 546. The marked cells show a weak phase contrast and are located in interspaces between ANME-2/SRB aggregates in the black layer (Fig. 3.5.a). In order to obtain more details about the identified cells, we investigated the localized interspaces by TEM. This high resolution technique shows that the methano- or methylotrophic cells form microcolonies of some tens of cells (Fig. 3.5.b), embedded in the dense matrix of EPS (Fig. 3.5.b). In contrast to the ANME-2/SRB consortia, the bacteria are not in close contact to cells of other morphotypes.

Figure 3.5.: Microscopical analysis of black layer of GHOSTDABS cold seeps.

(a) immunofluorescence labeling (α -MDH antibodies; AlexaFluor 546 as marker) in interspaces between ANME-2/SRB aggregates (asterisks). MDH expressing cells are depicted in orange. (b) TEM micrograph with microcolonies of MDH expressing cells (black arrow; cf. Fig. 3.6.) in interspaces between ANME-2/SRB aggregates.

Two types of cell morphologies were found. Fig. 3.6.a-e show the first type of cells. These cells are rod like ($\sim 0.3 \mu\text{m} \times \sim 0.9 \mu\text{m}$), with several concentric membrane layers located in the cell periphery. These membranes show similarity to intracytoplasmatic membranes of type II methanotrophs. The membrane layers are oriented parallel to the cytoplasmic membrane. In Fig. 2.6.e the membranes are highlighted by a grayscale inversion, indicating four membrane layers. This feature is also shown with a line-density scan (see section 2.3.6.). In the densitogram (cf. Fig. 2.6.e) each peak points out one phospholipid double layer membrane. Since osmium tetroxide staining destroys the specific antigenic binding sites, it was omitted during sample preparation.

Figure 3.6.: TEM analysis of MDH expression in the black layer.

(a-d) cells with ICM, exhibiting MDH expression. Immunogold labeling demonstrates MDH localization in the cell periphery (periplasm, membranes). ICM are arranged parallel to the cytoplasmic membrane. (e) grayscale inversion highlights membranes inside one cell as depicted in (c). Densitogram (inserted) of the cell periphery (white bar) points out 4 membrane layers. Each peak represents one membrane double layer. (f,g) cells without ICM, which exhibit MDH expression. Immunogold labeling demonstrates MDH localization in the cell periphery, extending into the center, in contrast to the first cell type.

Thus, membranes do not show the typical triple layer feature (dark-bright-dark). Instead, the middle lipid layer shows a bright contrast, the dark layers, representing the phospholipid headgroups, are missing, which has to be considered in image analysis and interpretation (Hoppert and Holzenburg, 1998). The overall thickness of a bright layer (measured between both inflection points next to one peak) indicates that this layer represents a complete double membrane (cf. Hoppert and Holzenburg, 1998). The immunogold labeling reveals the localization of expressed MDH in the cell periphery, also at the membrane layers. The marked regions, periplasm and ICM, correspond to MDH localization from previous investigations (Fassel et al., 1992) and the control isolates (Fig. 3.4.).

The second cell morphotype of MDH expressing cells exposes an approximately isodiametric morphology with diameters ranging from 0.3 to 0.6 μm (Fig. 3.6.f,g). As well as the first type, the organisms are arranged in microcolonies in interspaces, embedded in the EPS matrix. The cells are localized in areas of ANME-2/SRB aggregates. They are not in close contact to neighboring cells, as known from the ANME-2 cells. The immunocytochemical localization shows a strong immunogold labeling in the cell periphery, extending into the center, close to the nucleoid. Some of the methylotrophic isolates show a similar distribution of the MDH (Fig. 3.4.).

Several cells in the microbial mats contain membrane stacks, as described for type I methanotrophs (cf. section 1.2.2.). For these cells the expression of the MDH was checked. Fig. 3.7.a,b shows two representative cell morphotypes with this type of ICM, but no immunogold labeling was detectible. In contrast, the morphotype shown in Fig. 3.7.b will be identified as SRB (see section 3.1.2.). For the detection and localization of MDH it can be concluded, that two metabolic active methano- or methylotrophic cell types are located in interspaces between ANME-2/SRB aggregates, while cells with typical methanotrophic membrane stacks turned out to be no methanotrophs.

Figure 3.7.: TEM immunocytochemistry of cells exhibiting ICM (α -MDH antibodies).

(a,b) cell types from the black layer, exhibiting ICM stacks (known for type 1 methanotrophs) without MDH labeling (no MDH expression). Insert highlights the membrane stacks of the cell in (b).

3.1.2. APS reductase detection for active SRB

Novel method for generation of α -AprB antibodies and testing of the antibodies

In a previous work, Heller et al. (2008) showed expression of the MCR in ANME-1 and ANME-2 archaea by immunocytochemistry. The MCR is a key enzyme of the reverse methanogenesis, representing the archaeal part of the AOM pathway (Krüger et al., 2003; Hallam et al., 2004). The necessary electron sink for reverse methanogenesis is the sulfate reduction pathway of the syntrophic SRB partner cells. To uncover cells with key enzymes of this pathway, antibodies were raised against the dissimilatory APS reductase, a key enzyme of SRB.

Figure 3.8.: Novel technique for antibody generation.

In brief: DNA is extracted from an environmental setting (e.g. a cold seep microbial mat). With degenerate primers a gene coding for a conserved enzyme is amplified and cloned with TOPO TA (e.g. the *aprB* gene). One distinct sequence is selected and fused to a hexahistidin-tag, heterologously overexpressed and affinity purified. The purified protein is used for immunization of e.g. rabbits. After checking the specificity, the raised antibodies can be applied for Western blot analysis and immunocytochemistry.

For the detection of active SRB, we used a novel method for generation of antibodies directed against an epitope derived from a microbial mat, cf. Fig. 3.8. By this way, the detection system is highly adapted to mat-inhabiting SRB. The dissimilatory APS reductase was chosen as metabolic key enzyme of SRB, catalyzing the second step of the sulfate reduction pathway (Fig. 3.9.). The dissimilatory APS reductase is an

$\alpha\beta$ heteromer, consisting of the α -subunit of 75-80 kDa and the 18-23 kDa β -subunit. The subunits are highly conserved (encoded in the *aprBA* gene; Meyer and Kuever, 2007). To obtain the protein stable and - as far as possible - in its native structure, the whole β -subunit and not just a fragment was used for heterologous overexpression.

Figure 3.9.: Sulfate reduction pathway.

The dissimilatory APS reductase is a key enzyme of sulfate reduction. Details of the gene region encoding the enzyme in *D. vulgaris* Hildenborough are depicted in the upper right part of the figure. The dissimilatory APS reductase is an $\alpha\beta$ heteromer, consisting of the α -subunit of 75-80 kDa and the 18-23 kDa β -subunit (encoded in the *aprBA* gene; Meyer and Kuever, 2007).

It was aimed at the dissimilatory APS reductase β -subunit, because the complete, smaller subunit was better extractable from the microbial mats than the bigger α -subunit, since the latter has just few suitable primer target sites and a generally low degree of conserved nucleotide positions in the 3' terminal region (Meyer and Kuever, 2007). Moreover, it is known from the quaternary structure of the enzyme, that major

parts of the β -subunit are exposed to the surface allowing antibodies to detect its targets (Fritz et al., 2002; Meyer and Kuever, 2008; Chiang et al., 2009).

In a first step, a region including the *aprB* gene was amplified in a nested PCR approach. In combination with an established reverse primer annealing in the *aprA* gene, new degenerate forward primers were designed to get the whole *aprB* gene sequence as PCR product (cf. 2.3.4. for primer design and PCR conditions; Fig. 3.9.). The amplicates were cloned with TOPO TA cloning in *E. coli* TOP10 and sequenced. All sequences showed hits to sequences of *Desulfovibrio* as nearest neighbors. One sequence, representing the largest group of clones, was ligated in the plasmid pWH844 for fusing with a hexahistidin-tag (cf. 2.3.4. for applied methods; Schirmer et al., 1997). For this step, specific primers were used, which amplified the *aprB* gene from start to stop codon. The resulting plasmid was used for AprB overexpression in *E. coli* DH5 α . The protein was purified by affinity chromatography and used for the immunization of rabbits (cf. 2.3.5. for details of overexpression, purification and immunization).

Figure 3.10.: Western blot analysis with α -AprB antibodies.

Detection of dissimilatory APS reductase β -subunit in crude extract of *D. vulgaris* Hildenborough with polyclonal α -AprB antibodies in Western blot analysis. Black arrow indicates the AprB band.

The generated α -AprB antibodies were tested for their specificity by Western blot analysis (Fig. 3.10.; cf. section 2.3.5.). The α -AprB antibodies detected specifically the dissimilatory APS reductase β -subunit (AprB) without any background. Thus, the antibodies can be used for immunocytochemical localizations without the risk of false positive signals, caused by other than AprB epitopes.

D. vulgaris Hildenborough was used as positive control for immunogold labeling of AprB (cf. 2.3.6. for labeling technique). Ultrathin sections of *D. vulgaris* Hildenborough cells show cytoplasmic localization of AprB (Fig. 3.11.a). This labeling pattern is also common to other *Desulfovibrio* species (Kremer et al., 1988), analyzed with antibodies directed against the holoenzyme AprBA. No loss in signal strength could be observed by using the smaller β -subunit as target protein.

Figure 3.11.: See next page for figure legend.

Figure 3.11.: Microscopical analysis with α -AprB antibodies.

(a) TEM micrograph depicts AprB immunogold labeling in *D. vulgaris* Hildenborough. (b) immunofluorescence shows in orange AprB expressing cells, indicating active SRB (α -AprB antibodies; AlexaFluor 546 as marker). Predominantly vibrioid, active SRB, and coccoid, unmarked cells, next to the SRB are visible. Red circles highlight three examples of active SRB with ANME-2 partner cells. (c) details of ANME-2/SRB aggregates, at higher magnification. Image shows in the upper part light microscopy without fluorescence and below the same area with the AprB detection (orange fluorescence). Vibrioid cells show fluorescence.

Immunocytochemical localizations (α -AprB)

Investigations of the microbial mats for SRB followed the same strategy as for the detection of active methano- and methylotrophs. Previous FISH analysis could show that in the black layer the phylogenetic groups of SRB are localized in direct proximity to the ANME-2 cells (Knittel et al., 2005; Reitner et al., 2005b).

For immunofluorescence detection, the α -AprB antibodies were applied to the semithin sections of embedded samples. AlexaFluor 546-coupled antibodies were used as marker, indicating active SRB with bright orange fluorescence (cf. 2.3.6. for detailed method). Fig. 3.11.b depicts an overview of the black layer. Fluorescence signals revealed that cells performing sulfate reduction are often vibrioid and are located close to cells with coccoidal morphology (ANME-2). These findings are in accordance to the previously described FISH analysis, suggesting the direct association of ANME-2 and SRB. The vibrioid cells were assumed to be SRB (Reitner et al., 2005b), while Heller et al. (2008) gave already evidence that coccoid like cells express the MCR and represent active ANME-2. In Fig. 3.11.c the mentioned morphologies are shown in higher magnification.

The sometimes patchy distribution of the fluorescence signal is due to the fact that the antibody detection takes place at the surface of the sections. Consequently, only exposed cell parts can be labeled (Hoppert and Holzenburg, 1998). Especially the vibrioid morphology causes situations in the semithin sections where parts of the cells are exposed, whereas other parts remain covered by resin. Moreover, the expression level of the dissimilatory APS reductase may vary between individual cells.

Fig. 3.12.a depicts a typical AOM aggregate formed by ANME-2 and SRB cells. The immunocytochemistry at TEM resolution gives evidence for the expressed sulfate reduction pathway of the SRB on cellular level, i.e. the organisms perform sulfate

respiration at significant level (cf. section 2.3.6. for description of immunocytochemistry).

Vibrioid cells containing magnetosomes were frequently found within the ANME-2/SRB aggregates. It was shown that these magnetosome chains consist of greigite (Reitner et al., 2005a; Reitner et al., 2005b). The α -AprB antibodies were applied to investigate if the magnetosome-bearing cells perform an active sulfate reduction pathway. Fig. 3.12.b,c depicts that this cell type shows indeed a strong AprB expression, revealing a high activity of sulfate reduction.

Cells with internal membrane stacks, typical for type I methanotrophs, are abundant in the microbial mats (cf. Fig. 3.7.). Evidence was already given, that the investigated cells are not active methano- or methylotrophs (see above). In contrast, Fig. 3.12.d,e reveals AprB expression in a vibrioid cell type with ICM stacks, corresponding the cell type depicted in Fig. 3.7.b. Thus ICM containing cells are largely active SRB and not methano- or methylotrophs. All detected types of SRB contain PHA-like granular inclusions.

In order to detect the catalyzed processes of reverse methanogenesis (ANME-2) and sulfate reduction (SRB) simultaneously, an α -MCR/ α -AprB double labeling was performed (cf. section 2.3.6. for detailed technique). In Fig. 3.12.f the MCR expression is depicted in green, marking the ANME-2 cells. The sulfate reducing partner is depicted in orange. AprB and MCR-expressing cells are in direct contact to each other. These immunofluorescence data prove that the reverse methanogenesis and the sulfate reduction pathway are catalyzed simultaneously and in directly neighbored cells.

Figure 3.12.: TEM immunocytochemistry analysis with α -AprB antibodies.

(a) typical AOM aggregate. The vibrioid cell exhibits the sulfate reduction pathway, designating the cell as active SRB. ANME-2 cells show low background labeling. (b,c) cells with internal magnetosomes show strong immunogold labeling, revealing high sulfate reducing activity. (d, e) immunogold labeling of cells containing ICM stacks, demonstrates that the cells are active SRB. (f) double immunolabeling with α -MCR (green)/ α -AprB (w) antibodies. Two image pairs (one at the top, one at the bottom) showing light microscopy without and with fluorescence signals. Both key enzymes are expressed simultaneously.

3.2. Aerobic and anaerobic methane oxidation in terrestrial mud volcanoes in the Northern Apennines

3.2.1. Geological setting and mud volcano fluid composition

The Nirano mud volcano field is located in an ellipsoidal depression at approximately 200 m above sea level. This caldera-like structure is surrounded by hills (approximately 260 m above sea level) with steep flanks (Bonini, 2008) cut in the marine claystones of the Argille Azzurre Formation (Fig. 1.9.a,c). At the time of sampling, the Nirano mud volcano field consisted of four main vents, each composed of a varying number of active cones and pools. Results described in this work focus to the mud cone NR9 as depicted in Fig. 1.9.b (44° 30.848' N, 10° 49.527 E).

For the analysis of the geochemical data, fluids were sampled from approximately 1.5 m depth (for details see section 2.1.3). This sampling depth was used to ensure, that no perturbation of the surface fluids takes effect on the measurement. Of course, an influence of dilution by shallow ground water of meteoric origin cannot be ruled out. The redox potential of samples taken from a depth of 10 cm below the fluid outlet was as low as the redox potential measured in 1.5 m depth (-110 mV). Hence, the fluids are rapidly anoxic beneath the surface.

For analysis of the dissolved anorganic ions in the mud volcano fluids, solid particles, mainly clay fractions, were removed by centrifugation. In Tab. 3.1., the ionic composition of the processed water from the mud volcano fluid is shown (for details see section 2.3.2.). The data are in accordance with similar investigations from other studies over the past years. The fluid may be classified as brackish, connate water (Boschetti et al., 2010). The pH value is slightly alkaline. Nitrate, nitrite as well as sulfate and iron are only present in low concentrations. These compounds represent electron acceptors for anaerobic respiration. Sulfide is not detectable, which corresponds to the low concentration of sulfate with ≤ 7 mg/ml. Due to the chemical instability of hydrogen sulfide, however, small traces of this compound are difficult to measure after sample transportation. It has a low solubility in water, is volatile and shows autoxidation in the presence of oxygen. The redox potential measured just after sampling is below -110 mV, providing an anaerobic surrounding for e.g. sulfate reducing bacteria and

methanogenic or methanotrophic archaea. The low amounts of sulfate, however, will restrict microbial sulfate reducing activity, though it must be taken into account that the low sulfate concentration may be a result of sulfate reducing activity. The measured temperature of the fluids was 21.3 °C.

Table 3.1.: Mud volcano fluid composition (all results in mg/l, except pH).

Measured compounds	2010 NR4	2010 NR9	2008 NR4	Detection limit
pH	7.9	8.1	8.0	0.1
Chloride	6390	6310	5440	0.5
Sulfate	3	6	7	1
Nitrate	< 0.5	< 0.5	<0.5	0.5
Nitrite	< 0.02	< 0.02	0.23	0.02
Ammonium	4.9	9.2	7.7	0.04
Hydrogen carbonate	854	1930	1310	3.0
Sulfide	< 0.03	< 0.03	<0.03	0.03
Sulfite	< 0.5	< 0.5	<0.5	0.5
Metals				
Calcium	85.1	26.0	8.5	0.5
Iron	0.40	0.20	0.03	0.01
Potassium	23.9	33.6	32.4	0.5
Magnesium	120	76.6	63.9	0.05
Sodium	4420	4670	3510	0.5
Nickel	< 0.005	< 0.005	<0.005	0.005
Phosphor	0.20	1.1	0.46	0.05
Phosphate	0.61	3.37	1.41	0.15

3.2.2. Detection of organisms in mud volcano fluids

Environmental samples

In comparison to marine habitats, there is only little knowledge about microbial diversity in terrestrial sites of methane release (cf. Alain et al., 2006). Therefore, aim of this work was to detect and to analyze methane catabolizing microbial communities of the volcanic fluids. By taking sterile samples from approximately 1.5 m depth, a contamination by organism of the volcano surface can be ruled out. A continuous upwelling of the volcanic fluids prevents contamination with surface organisms. However, contamination with organisms from shallow ground water cannot be excluded.

The suspended clay particles in the mud volcano fluids complicate detection of organisms. Due to the low cell densities in mud volcano fluids, direct counting of microbial cells turned out to be impossible. Also simple one step concentration methods like density centrifugation failed since microbial cells were not detectable in remaining clay particles. By the combined approach of a cell extraction method, the removal of sediment debris as well as a concentration of the cells according to Kallmeyer et al. (2008), countable cells could be obtained from the volcanic fluids (cf. section 2.3.6. for detailed method).

Enumeration of DAPI stained cells resulted in a total cell number of 0.7×10^6 prokaryotic morphotypes per ml fluid (cf. section 2.3.6. for method of cell counting). Most cells appeared as single, apparently planktonic organisms (Fig. 3.13.a). These cell counts are significantly lower as cell counts from the marine methane rich sediments from the Black Sea, where about 2.5×10^8 cells/ml were counted (Knab et al., 2009) or marine sediments with 0.25×10^8 to 4.4×10^9 cells/ml (Joergensen and Revsbech, 1989; Llobet-Brossa et al., 1998). To discriminate between bacteria and archaea, FISH analyses with EUBI-III and ARCH915 probes were conducted (cf. 2.3.6. for details of FISH analysis). These experiments revealed that the single organisms extracted and concentrated from the fluids were all bacterial phylotypes. No archaea could be detected. (Fig. 3.13.b).

Figure 3.13.: Microscopic analyses of fluids.

(a) cells extracted from fluids of mud volcano NR9; a prokaryotic morphotype is marked with a red circle (DAPI stain). (b) cells extracted from fluids of mud volcano NR9; no planktonic archaea could be detected (probe EUB338 I-III and probe ARCH915; image: Anne Dreier). Inset: control experiment with *E. coli* DH5α (orange) and *M. mazei* Gö1 (green).

Figure 3.14.: Microscopic analysis of a microbial flake.

DAPI stain of a microbial biofilm flake. Dark mineral precipitates are visible embedded in the hyaline extracellular matrix.

About 0.2-0.3 % of the microbial cells were found associated in distinct flakes, embedded in a hyaline extracellular matrix (Fig. 3.14.). The microbial flakes represent rather large cell aggregates of often some hundreds of organisms.

Inside the matrix of the flakes, dark mineral precipitates could be observed by brightfield light microscopy (Fig. 3.15.a). The precipitates resemble raspberry-shaped structures. TEM analysis of samples, adsorbed onto Formvar-coated carbon grids (cf. 2.3.6.), showed, that some precipitates were morphologically equivalent to pyrite framboids (Fig. 3.15.b; Ohfuji et al., 2005). A single particle is of approximately 500 nm in diameter, roughly corresponding to the size of framboidal pyrite of the black layer biofilm from GHOSTDABS cold seeps (Wrede, 2007).

Figure 3.15.: Mineral precipitates in microbial flakes.

(a) brightfield image of a flake with dark mineral precipitates. (b) electron micrograph of a preparation similar to (a) depicting precipitates which are morphologically identical to framboidal pyrite.

As revealed by FISH analysis, the aggregates in environmental samples mainly consist of archaea (probe ARCH915). Also a filamentous archaean cell type was found to form microbial flakes (3.16.).

Most cells in the biofilm flakes could be identified as ANME-2 archaea by hybridization with the probe EelMS932 (Fig. 3.17.a,b). It is known, that ANME-2 archaea are associated with SRB, representing in most cases *Desulfosarcina/Desulfococcus* branch (Schreiber et al., 2010). Consequently the abundance of this SRB group was investigated with the probe DSS658.

Figure 3.16.: Detection of archaeal microbial flakes by FISH analysis.

Filamentous archaea detected in microbial flake with the hybridization probe ARCH915.

Figure 3.17.: Detection of ANME archaea and SRB by FISH analysis of microbial flakes.

Orange: ANME archaea (probe EelMS932), green: SRB of *Desulfosarcina/ Desulfococcus* branch (probe DSS658). SRB detection only in (c).

Symbiotic associations with sulfate reducing bacteria were not observed. Sulfate reducers could be detected with the DSS658 probe only in a very low number and never in direct association with ANME archaea (Fig. 3.17.c).

Figure 3.18.: Archaeal phylogenetic tree.

Maximum parsimony phylogenetic tree based on archaeal 16S rRNA gene sequences derived from Nirano mud volcano field and nearest relatives. As indicated, sequences resulted from samples of 2008 and 2009. Only bootstrap values $\geq 50\%$ are indicated.

Diversity analysis from environmental DNA (contributed by Stephanie Rockstroh and Silja Brady) revealed a high abundance of groups involved in anaerobic oxidation of methane (Fig. 3.18.), conducting either iron or manganese dependent AOM, as well as sulfate dependent AOM (Beal et al., 2009). The nearest neighbors of the analyzed sequences derive from uncultured organisms, which were in most cases found in marine methane seepage habitats, in particular mud volcanoes (e.g. AJ579323, GQ356856, GQ356817, FJ649523, HM584831, FJ555678, HM584830, FJ712387, GU120484, GQ356919). One of the largest groups were Crenarchaeote sequences representing 68 % (Fig. 3.19). Most notably, the sequences affiliated to clone GQ356856 and GQ356817 represent a dominant group of Crenarchaeota (20 sequences), which were detected in iron or manganese dependent AOM cultures (Fig. 3.18.; Beal et al., 2009). Approximately 14 % of the sequences (Fig. 3.19.) represent ANME-2a-2b archaea, related to Methanosarcinales. The total percentage of sulfate dependent AOM archaea amounts to 22 %, consisting of ANME 2a-2b, ANME-1 and GoM Arc I.

The calculated Chao1 and ACE richness estimates, with 35 and 38 in comparison to 20 detected species or with 9 and 12 in comparison to 7 detected phyla, indicated that not all groups were covered with this analysis (cf. Fig 3.19.). However, the dominant archaeal groups should be detected. The archaeal Shannon-Weaver diversity index is low with 0.89 for phylotypes regarding to e.g. the marine Mediterranean Kazan mud volcano with an index between 2.63 and 2.57 (Heijs et al., 2007).

■ Methanosaetaceae
 ■ Methanolobus
 ■ GOM Arc1
 ■ ASC21
 ■ Soil Crenarchaeotic Group
 ■ Micellaneos Crenarchaeotic Group
 ■ ANME-2a-2b
 ■ Methermicoccaceae
 ■ ANME-1
 ■ South African Goldmine
 ■ Marine Benthic Group

Estimates				
Distance	Detected	Chao1 ¹	ACE ²	Shannon ³
OTUs				
0.01	24	44	50	2.45
0.03	20	35	38	2.3
0.2	7	9	12	0.89

Figure 3.19.: Distribution of phylogenetic groups and diversity estimates.

The pie chart depicts distributions of phylogenetic groups of archaeal 16S rRNA gene library. The table shows diversity estimates of the libraries. Estimates were calculated with DOTUR (Schloss and Handelsman, 2005). Footnotes: ¹nonparametric richness estimator based on distribution of singletons and doubletons; ²abundance-based coverage estimator (ACE), nonparametric richness estimator regarding distribution of abundant (>10) and rare (≤ 10) OTUs; ³Shannon-Weaver Index of diversity. More diversity is related to higher numbers.

Enrichment cultures

By FISH and 16s rRNA gene analyses the abundance of anaerobic methane oxidizers was shown in the fluid samples (see above). Complementary, long-term incubation experiments with anoxic methane-gassed cultures were performed to detect AOM activity. Since *Methanosarcina*, and accordingly ANME archaea, have a high oxygen sensitivity (Fetzer and Conrad, 1993), samples from approximately 1.5 m depth with a redox potential of below -110 mV were used for inoculation directly after sampling. By this way, anoxic conditions could be maintained. The incubation temperature was adjusted to 20 °C, i.e. the approximate temperature of the mud volcano fluid just after sampling (cf. sections 2.1.3., 2.2., and 2.3.3. for applied methods).

Figure 3.20.: AOM activity.

Formation of sulfide from sulfate with methane as electron donor in fluid cultures under strictly anaerobic conditions (indicating AOM activity).

The development of hydrogen sulfide was used as indicator for anaerobic methane consumption (cf. 2.3.3.; Nauhaus et al., 2002). Actually, an increase of hydrogen sulfide was measured (Fig. 3.20.). From eleven inoculated samples, however, just four showed the development of hydrogen sulfide. This finding reflects the fact that just a low number of organisms, or, in fact biofilm flakes, must have been transferred as single units to the enrichment cultures and were activated during culturing. These results obtained with cultivated samples from mud volcano fluids are consistent with the presence of (sulfate dependent) methane oxidizers as identified by FISH and phylogenetic analysis (see above).

Aerobic or nitrite dependent methanotrophy could not be expected to occur in the low redox environment (below -110 mV, see section 3.2.1.) of the mud volcano fluid. But as already described above, aerobic methano- or methylotrophic organisms were detected in anoxic biofilms from GHOSTDABS cold seeps (cf. section 3.1.). Due to the low cell counts, methylotrophic organisms could not be detected in environmental samples with techniques used for investigation of the cold seeps. For that reason, cultivation experiments were performed to detect MDH dependent methanotrophs in the oxic and the anoxic volcanic fluids (cf. sections 2.1.3., 2.2. and 2.3.3.).

Figure 3.21.: TEM analysis of methanotrophic enrichment cultures.

Electron microscopy of aerobic methanotrophic enrichment cultures. Samples were embedded in Spurr resin and stained with uranyl acetate and lead citrate. Membrane stacks indicating type I methanotrophs.

In cultivation experiments, inoculated with samples either taken from the aerobic fluid outlet or from a depth of 1.5 m, aerobic methanotrophs were successfully enriched, even in the anaerobic fluids. DGGE analysis revealed the diversity of methanotrophs in enrichment cultures (Tab. 3.2.), mainly *Methylophaga* and *Methylobacter*-species (cf. 2.3.4. for DGGE technique). In the enrichment cultures from anaerobic fluids the abundance of a Methylococcaceae bacterium ET-SHO and a *Methylophilus*-related strain was shown (cf. Heinzelmann, 2010).

In order to obtain more information about the morphology of the enriched cells, especially with respect to internal membranes, cells from enrichment cultures were embedded in Spurr resin and ultrathin sectioned (cf. section 2.3.6. for methods). TEM analysis showed rod-like and coccoid cells, containing stacks of internal membrane layers. This ICM stacks are typical for type I methanotrophs, which corresponds to the DGGE data (Fig. 3.21.; Hanson and Hanson, 1996).

Table 3.2.: Phylotypes of detected methylotrophic bacteria.

Phylogenetic class	Origin in cone	Closest phylotype	Maximal identity	Closest described relative	Maximal identity
γ -Proteobacteria, Methyloccoccales; Methyloccocaceae, <i>Methylobacter</i>	Aerobic surface	Uncultured bacterium clone 1H_2	91%	<i>Methylobacter</i> sp. LW1	90%
γ -Proteobacteria, Methyloccoccales; Methyloccocaceae, <i>Methylobacter</i>	Aerobic surface	Uncultured <i>Methylobacter</i> sp. isolate DGGE gel band RH2O-3	87%	<i>Methylobacter</i> sp. LW1	87%
γ -Proteobacteria, Thiotrichales; Piscirickettsiaceae, <i>Methylophaga</i>	Aerobic surface	Uncultured bacterium clone nbw779c04c1	93%	<i>Methylophaga</i> sp. JAM1	93%
γ -Proteobacteria, Thiotrichales; Piscirickettsiaceae, <i>Methylophaga</i>	Aerobic surface	Uncultured bacterium, clone: SsB15	91%	<i>Methylophaga</i> sp. JAM1	91%
γ -Proteobacteria, Thiotrichales; Piscirickettsiaceae, <i>Methylophaga</i>	Aerobic surface	Uncultured marine bacterium isolate DGGE gel band ANT-WZ13	84%	<i>Methylophaga</i> sp. JAM1	84%
γ -Proteobacteria, Methyloccoccales; Methyloccocaceae, <i>Methylobacter</i>	Anaerobic fluids	Uncultured bacterium clone 1H_2	99%	<i>Methylobacter</i> sp. LW1	99%
γ -Proteobacteria, Methyloccoccales; Methyloccocaceae, <i>Methylobacter</i>	Anaerobic fluids	Uncultured <i>Methylobacter</i> sp. isolate DGGE gel band RH2O-3	88%	<i>Methylobacter</i> sp. LW1	88%
γ -Proteobacteria, Methyloccoccales; Methyloccocaceae	Anaerobic fluids	Methyloccocaceae bacterium ET-SHO	85%	Methyloccocaceae bacterium ET-SHO	85%
β -Proteobacteria, Methylophilales; Methylophilaceae, <i>Methylophilus</i>	Anaerobic fluids	Uncultured beta proteobacterium clone 56S_1B_1	99%	<i>Methylophilus</i> sp. CS3	94%

4. Discussion

4.1. Anaerobic methane oxidation – a view on the protein level

4.1.1. Are syntrophic bacteria of ANME cells active SRB?

Considering the current knowledge about sulfate dependent AOM, most details of the postulated pathway remain still unknown. For the archaeal organisms, more information is already available, than for the bacterial AOM partners (cf. section 1.2.3). Genomic analyses were supported by the detection of a (reverse) methanogenic key enzyme, the MCR protein, on cellular level of ANME-1 and ANME-2 cells (cf. section 1.2.3.; Krüger et al., 2003; Hallam et al., 2004). Most recently, metagenomic and mRNA analyses of ANME-1 archaea gave further evidence for the reverse methanogenic pathway (Meyerdierks et al., 2010).

Concerning the bacterial symbionts the knowledge is hitherto even more fragmentary (cf. section 1.2.3.). Recently, extensive FISH analysis could show that up to 95 % of ANME-2 bacterial symbionts belong to the SEEP-SRB1a subcluster of the DSS group (Schreiber et al., 2010). In addition to these SRB, *Desulfobulbus* (so far only known for ANME-3) and a significant fraction of non sulfate reducing α - and β -Proteobacteria, related to *Sphingomonas* and Burkholderiaceae, respectively, were described in another study (Pernthaler et al., 2008).

Since no data on expressed metabolic pathways in these consortia were available, we used an *aprB* gene sequence, obtained from the microbial mat by degenerate primers, to overexpress the AprB protein heterologously in *E. coli*. The purified protein was used for immunization (see Fig. 3.8.). By this method, an antibody directed against APS reductases from the environmental sample was obtained.

The immunogold labeling of the APS reductase β -subunit provides evidence for the expression of this enzyme. Thus, the sulfate reducing pathway is shown *in situ* to be active (cf. section 3.1.2.; Figs. 3.11. and 3.12.). The labeling results of *D. vulgaris* Hildenborough (Fig. 3.11.a) and of course of the SRB in the cold seep biofilm demonstrate the applicability of this novel method. The detected active SRB exhibit mostly a vibrioid morphology localized in close contact to ANME-2 cells. These first

data on AprB expression of SRB coincide with FISH analysis of Reitner et al. (2005b) and Schreiber et al. (2010), suggesting that the vibrio-like cells are phylogenetically related to DSS, SEEP-SRB1a group.

An expressed sulfate reduction pathway was not self-evident for the bacterial partner of ANME cells. Firstly, bacteria of α - and β -Proteobacteria without reported sulfate reduction pathway were found in association with ANME (see above). Secondly, it cannot be ruled out, that bacteria of the SRB group use fermentative pathways as energy source. This could be the case under sulfate limitation. Since the organisms in the microbial mats show a very high density (cf. Fig. 3.11.b), similar to the density of pelleted cells, sulfate respiration may cause sulfate depleted microenvironments. Fermentation in absence of sulfate is a prevalent option of SRB for growth (Rabus et al., 2006). Some *Desulfovibrio* species (Widdel, 1988), *Desulfobacterium* species (Brysch et al., 1987) and *Desulfosarcina variabilis* (Widdel, 1980) are able to ferment fumarate and malate (exception *D. variabilis*), to succinate, acetate, carbon dioxide and propionate. Pyruvate is fermented by many SRB using lactate (Postgate, 1984; Rabus et al., 2006). For *Desulfobulbus* species, lactate or ethanol fermentation with carbon dioxide was described (Laanbroek et al., 1982; Widdel and Pfennig, 1982). In co-cultures with hydrogen-scavenging methanogenic archaea, *Desulfovibrio* strains were able to grow on ethanol or lactate, but without sulfate. Under these conditions, SRB serve as hydrogen-producing acetogenic bacteria in a syntrophic association (Bryant et al., 1977). However, the immunodetection of AprB at significant level, as shown here, excludes these alternatives and shows an active sulfate respiration of most ANME partner cells (Fig. 3.11.b).

It should be stressed here that in the microscopical analysis the SRB show a vibrioid morphology (cf. Fig. 3.11.c and 3.12.a,c,d), which was also reported in other studies (Reitner et al., 2005a; Reitner et al., 2005b; Schreiber et al., 2010). Though morphology is no criterion for phylogenetic classification, for *Desulfosarcina/Desulfococcus* strains no cultured species with distinct vibrioid morphology has been described so far.

Use of antibodies as markers for metabolic enzymes combined with high resolution TEM shed light on cell types with distinct cellular and subcellular features, which were not assigned to metabolic activities of cells, yet. One prevalent attribute of cells from GHOSTDABS microbial mats are ICM stacks, also described in other studies (Ivanov et

al., 1991; Reitner et al., 2005b). In this work evidence was given that at least one cell type synthesizing ICM stacks represents active SRB (cf. section 3.1.2. and Fig. 3.12.d,e), but no cell type with ICM stacks showed MDH expression. Though ICM stacks are typical for type I methanotrophs (cf. Fig. 1.3.a and Hanson and Hanson, 1996) they have been also described for some SRB (Rozanova et al., 1988; Mohn et al., 1990).

Another subcellular structure found in cells of GHOSTDABS cold seeps are internal greigite particles, arranged in chains as described for magnetosomes (Reitner et al., 2005a). Here, immunolocalization with α -AprB antibodies demonstrates that these cells perform sulfate reduction at significant level (cf. section 3.1.2. and Fig. 3.12.b,c). The function of the magnetosome-like structures has not been studied, yet. Since the cells are embedded in the EPS matrix of the biofilm, geomagnetic navigation as known from aquatic habitats, i.e. magnetotaxis, can be ruled out (Faivre and Schöler, 2008). Processes like storage, a metabolic function or detoxification appear more likely. These features have also been postulated as functions of magnetosomes from magnetotactic bacteria (cf. Vainshtein et al., 2002; Frankel and Bazylinski, 2004; Simmons et al., 2004; Faivre and Schöler, 2008). Here, the greigite particles may be products of intracellular detoxification of hydrogen sulfide. In previous investigations, it was postulated that the high content of authigenic pyrite in the Black Sea cold seeps was caused by extracellular mineralization of sulfide released from SRB (Peckmann et al., 2001b). Regarding the data of this work that sulfate respiration leads to internal greigite precipitates (cf. also Reitner et al., 2005a; Reitner et al., 2005b), it can be postulated that sulfate respiration has also direct influence on intracellular biomineralization.

4.1.2. AOM, syntrophy of reverse methanogenesis and sulfate reduction?

The reconstruction of the postulated pathway of reverse methanogenesis in syntrophy with sulfate respiration is mainly based on bulk analysis. Methane uptake and sulfate reduction is measured for the determination of overall AOM activity in enrichment cultures (e.g. Michaelis et al., 2002; Nauhaus et al., 2007; Treude et al., 2007; Wegener et al., 2008). Though, up to now, the single reaction steps in this pathway could not be proven, e.g. by detection of the relevant metabolites. Thermodynamics of the AOM require that reverse methanogenesis has to be simultaneously conducted in direct

proximity to sulfate reduction. The maximal distances between partner cells are strongly restricted, e.g. for hydrogen 8.4 nm, for methanol 0 nm, for acetate 13 nm and for formate 66 nm between electron carrier production and consumption result in a loss of 5 kJ/(mol CH₄) (Sørensen et al., 2001).

By performing a double label approach with α -MCR and α -AprB antibodies, see Fig. 3.12.f, it could be demonstrated, that in fact, methane oxidation and sulfate reduction are synchronized and in direct proximity. This result supports the hypothesized AOM mechanism (Hoehler et al., 1994; Knittel and Boetius, 2009) and the genomic analyses (Hallam et al., 2004; Meyerdierks et al., 2005; Pernthaler et al., 2008; Meyerdierks et al., 2010).

4.1.3. Aerobic methanotrophic pathway in anoxic cold seeps

The stratification of the Black Sea water column provides a permanently anoxic environment for the GHOSTDABS field in a depth of approximately 230 m (cf. section 1.3.1 and Michaelis et al., 2002). Accordingly, the detection of aerobic methano- or methylotrophic bacteria does not correspond to common expectations for this habitat. So far, methanotrophic bacteria in the Black Sea were only found above methane seeps up to a depth of approximately 150 m (Durisch-Kaiser et al., 2005). The abundance in this area is based on the availability of oxygen. In contrast, it is demonstrated in this work that cells in the anaerobic GHOSTDABS microbial mats express the MDH, the methano- and methylotrophic key enzyme (see section 3.1.1., Figs. 3.5. and 3.6.). Expression of this methanotrophic key enzyme indicates the metabolic activity of the cells. Therefore it can be excluded, that the cells are inactive remains from the aerobic part of the water column. This conclusion is also supported by cytological features; ICM parallel to the cytoplasmic membrane suggest a relation to type II methanotrophs (cf. Fig. 3.6.a-e). In the water column above methane seeps, methanotrophs were found to be nearly exclusively (at least ~90 %) of type I (Durisch-Kaiser et al., 2005; Schubert et al., 2006). Consequently, the described bacteria in the biofilms differ from planktonic methanotrophs of the oxic water column.

4.1.4. Electron acceptors and microniches

Investigations of the microscale environment in the microbial mats exhibited the presence of the MDH expressing cells in small colonies (cf. Fig. 3.5.). These colonies were embedded within the EPS matrix between ANME-2/SRB consortia revealing cell division inside the mat. Thus, the cells are actively growing and represent not inactive cell remnants. Instead of colony formation, sedimented and metabolic inactive cells deposits should be found as single cells on the mat's surface and get degraded over the time.

In fact, the aerobic methanotrophic pathway is expected to use oxygen as electron acceptor at high redox levels. This allows a high energy yield of dissimilatory methane oxidation with $\delta G^0 = -818 \text{ kJ/mol}$ (cf. section 1.2.2.; Eq. 11). The difficult issue in this environment is the source of electron acceptors for methanotrophs. The question is raised, if the abundance of molecular oxygen in this anaerobic environment is possible. One explanation is a temporal fluctuation of the oxic-anoxic interface zone of euxinic seawater. Perturbation of the stratified water could allow an infiltration of the bottom water with traces of oxygen. The high dynamic oxic-anoxic interface zone is located in a depth between approximately 130 and 180 m (Luth and Luth, 1997). Some references even assume only 80-100 m (Sinninghe Damsté et al., 1993 and references therein). Although repeated measurements showed only minor differences in the oxic-anoxic interface (Luth and Luth, 1998) up- or downwards shifts of the chemocline cannot be excluded (Luth et al., 1999). Regarding the investigated samples, reverse methanogenesis, sulfate reduction and methanotrophy were conducted simultaneously in the microbial mat (see section 3.1.1 and 3.1.2.). Thus, exposition to oxygen seems rather unlikely in consideration of the high susceptibility to oxygen and the dependency to low redox potentials of (reverse) methanogens (Fetzer and Conrad, 1993). Even small traces of oxygen should have inactivated the ANME metabolism (Fetzer and Conrad, 1993), though it is possible that microenvironments in the microbial mat protect the oxygen-sensitive cells by formation of steep redox gradients (Brusa et al., 1987; Paerl and Prufert, 1987; Belay et al., 1988; Schramm et al., 1999; Whitman et al., 2006).

A second explanation for the detected methanotrophic microcolonies rests may be on the coupling of methane oxidation to nitrate reduction (cf. section 1.2.4.). Bacteria exhibiting this metabolic feature were recently described (Raghoebarsing et al., 2006; Ettwig et al., 2008; Ettwig et al., 2009; Hu et al., 2009; Ettwig et al., 2010). Of course,

nitrate and nitrite concentrations are typically higher in freshwater environments than in sea water due to inputs from soil fertilization and other anthropogenic sources (Galloway et al., 2004; Seitzinger et al., 2006; Galloway et al., 2008). In marine waters nitrate concentration varies usually between 0.4 μM and 8 μM , whereas nitrate/nitrite dependent AOM experiments were performed at concentrations of 2 mM nitrate (sampled from water containing 0.1 mM nitrate; Morris and Riley, 1963; Raghoebarsing et al., 2006; Ettwig et al., 2010). Consequently, the limiting factor for these organisms will be the electron sink. Microcolonies with only some tens of cells may reflect a minor ecological role of the aerobic methano- or methylotrophs in the methane seeps (cf. section 3.1.1.). Since the nitrate/nitrite based AOM has a significantly higher energy yield of $\Delta G^{\circ} = -928 \text{ kJ}/(\text{mol CH}_4)$ compared to $\Delta G^{\circ} = -21 \text{ kJ}/(\text{mol CH}_4)$ for the sulfate based AOM (Thauer and Shima, 2008), ANME/SRB consortia should be otherwise out-competed by bacteria.

Nitrogen compounds can be provided by (a) the sea water, (b) marine snow (Diercks and Asper, 1997) and (c) organic sources of the microbial mats. Nitrogen cycling is known in microbial mats and biofilms (Joye and Paerl, 1994). Rapid recycling and transfer of metabolites within mats has been documented for many bioactive elements, including C, N, S and O (Joye and Paerl, 1994; Guerrero et al., 2002; Beer and Stoodley, 2006). Since no methanol dehydrogenase-expressing organisms could be detected in the pink layer, it could be speculated that nitrate/nitrite is provided from outside of the microbial mats (see section 3.1.1., Fig. 3.3.). A feasible explanation may be that in the black surface layer nitrogen or nitrite concentration is sufficient for bacterial methanotrophy, whereas in the underlying pink layer these compounds are already depleted (see Fig. 4.1.). However, the absence of evidence for MDH expressing cells that conduct nitrate/nitrite dependent AOM means not the evidence of their absence. In line with this assumption a few detections of NC10 related bacteria were reported even in marine habitats (Zhu et al., 2010).

Figure 4.1.: Hypothesis of nitrate/nitrite consumption in the black layer.

Illustration shows magnification of GHOSDABS cold seep microbial mats. Black layer is dominated by ANME-2/SRB (green/orange) aggregates with microcolonies of nitrate/nitrite dependent methanotrophs (yellow). Nitrate of the seawater (blue arrows) diffuses into the microbial mats and gets reduced by bacterial methanotrophs. Only very low concentrations of nitrate reach the pink layer. There, no MDH expression is detectable. Pink layer is dominated by ANME-1 matrix (green) with embedded SRB (orange) colonies.

Microbial mats of GHOSDABS cold seeps are highly structured and exhibit large amounts of EPS (see section 1.3.1. and Reitner et al., 2005b; Wrede et al., 2008), providing good conditions for distinct microniches. It is described that microniches can cause different redox levels which determine diverse microbial metabolic activities in close proximity. Metabolically similar microbial populations, so called ‘guilds’, may establish these redox niches and conduct interdependent physiological processes (Davey and O’Toole G, 2000). This is reported for sediments (e.g. Stockdale et al., 2009; Bertics and Ziebis, 2010) as well as for biofilms (e.g. Costerton et al., 1994; Schramm et al., 1999). Since methanotrophic bacteria need higher redox levels compared to ANME/SRB aggregates, their localization in interspaces can be dedicated to microniches in the GHOSDABS cold seeps (see Fig. 4.2.). Redox potentials should be

expected at approximately +100 to +250 mV or higher for nitrate/nitrite or oxygen utilization (Bailey and Beauchamp, 1971; Reeburgh, 1983; Vance, 1996). In comparison, for methanogenesis and sulfate reduction redox levels of approximately -150 mV and below are necessary (Connell and Patrick, 1968; Reeburgh, 1983; Wang et al., 1993). Aerobic methanotrophs may contribute to the decreased redox levels in ANME/SRB consortia by scavenging oxidized compounds. Also further microorganisms can reduce the redox level by respirations, like heterotrophic bacteria growing with organic compounds inside the microbial mats. This coincides with the observation of several cell types with yet unknown metabolisms between ANME/SRB consortia.

Figure 4.2.: Postulated microniches with different redox level conditions.

Microniches in GHOSTDABS cold seep microbial mats. Black layer is dominated by ANME-2/SRB (green/orange cells) aggregates with microcolonies of nitrate/nitrite dependent methanotrophs (yellow cells). MDH dependent AOM requires higher redox levels for oxygen or nitrate/nitrite respiration. At the same time this metabolism is conditioning the low redox level for ANME-2/SRB dependent AOM. White cells represent microorganisms with unknown metabolism.

In addition, AOM associated archaea coexisted over a distinct time period in nitrite reducing AOM enrichment cultures together with bacteria of NC10 phylum (Raghoebarsing et al., 2006; Ettwig et al., 2008). This observation suggests a certain tolerance of ANME cells to higher redox levels.

4.1.5. Co-occurrence of ANME and methanotrophic bacteria, a unique phenomenon?

With the detection of MDH expressing cells at anoxic methane seeps the question arises, if this phenomenon is restricted to samples analyzed in this work. In a study applying metagenomics with a direct cell capture method, Pernthaler et al. (2008) focused on the bacterial partner cells of ANME-2 archaea. They detected, besides the expected DSS group and members of *Desulfobulbus*, cells of α - and γ -Proteobacteria. These phyla include also type II and type I methanotrophs. Furthermore, bacteria affiliated to the *Cytophaga* group and β -Proteobacteria were found. Data from stable isotope probing imply that members of *Cytophaga* and β -Proteobacteria are also oxidize methane aerobically (Morris et al., 2002; Radajewski et al., 2002; McDonald et al., 2008), β -Proteobacteria even at hydrocarbon seeps (Redmond et al., 2010). In addition, genes for denitrification were reported in the study of Pernthaler et al. (2008) for the analyzed β -Proteobacteria. The α - and β -Proteobacterial cells were also described to be loosely associated on the periphery of the ANME-2c/DSS aggregates, similar to our findings. Also Knittel et al. (2005) and Schreiber et al. (2010) reported cells in the ANME-2 periphery, which were not identified by SRB FISH probes. The latter study identified in particular α - and β -Proteobacteria. The organisms do not belong to sulfate reducing groups and an affiliation with MDH-dependent methanotrophs has to be investigated. However, it is interesting to note certain parallels to our data.

4.1.6. Implications for biomarkers

The abundance of methano- or methylotrophic bacteria in anoxic cold seep habitats has also to be considered for biomarkers of these environments. It is the first *in situ* detection of MDH-dependent methylotrophy in anaerobic cold seeps. Furthermore it is demonstrated here, that these cells are metabolically active. This is in accordance with biomarker data from the GHOSTDABS-field microbial mats (Thiel et al., 2001; Thiel et al., 2003) and other cold seep deposits (e.g. Elvert et al., 2000; Pancost et al., 2000; Hinrichs, 2001). These studies reported biomarkers of aerobic methanotrophs with strong ^{13}C depletions in addition to biomarkers of sulfate dependent AOM (see section 1.2.3.). Isotopically depleted C_{30} hopanoids (diplopterol, diploptene, and their diagenetic derivatives) are known from aerobic methanotrophs, but were also detected

in some groups of anaerobic bacteria (Sinninghe Damsté et al., 2004; Härtner et al., 2005; Blumenberg et al., 2006). But in addition biomarkers were found at methane seep deposits which are specific for aerobic methanotrophs, namely ^{13}C depleted hopanoids (3 β -methylated hopanoids) and 4-methylated steroids (lanostanes; Birgel and Peckmann, 2008). With evidence for active methano- or methylotrophic bacteria in recent anoxic methane seeps, given in this work, it has to be considered that biomarker hints are not necessarily based on oxic conditions of methane seeps or anaerobic non-methanotrophic bacteria. The cell types detected in this work should be taken into account as possible biomarker source.

4.2. Terrestrial mud volcanoes in Nirano

4.2.1. Mud volcanoes as AOM habitat

The Nirano mud volcano system generates a complex microbial environment. The fluid geochemistry reveals a heterogeneous substrate, consisting of diverse hydrocarbons and limited amounts of appropriate electron acceptors (e.g. Martinelli and Rabbi, 1998; Boschetti et al., 2010). In a previous study, ^{13}C -depleted hydroxyarchaeol, detected in the mud volcano fluids, was a hint of AOM conducted by recent organisms in the mud volcano fluids (Heller et al., 2010). The results of this work show that AOM is active in the recent system (see section 3.2.2.).

At an estimated thermal gradient of approximately 20 °C per km in the Apennine foredeep (e.g. Teichmüller and Teichmüller, 1986; Harabaglia et al., 1997; Capozzi and Picotti, 2002), organisms in the deep subsurface at a depth of 2000 m would experience an ambient temperature of approximately 60 °C. In the shallow fluid reservoir, 200 m beneath the surface, approximately 4 °C above surface temperature may be expected (cf. Fig. 1.9.c and Fig. 4.3.). The fluid reservoirs along boundaries of different geological units provide an environment of reduced flow velocity, compared with narrower ducts in homogeneous material. Though in the mud cones formed by the Argille Azzurre marine clays, consolidated biofilms could never be observed, it is very well possible that these films can develop along the solid/liquid phase boundaries inside the fluid conduits along thrust faults or other discontinuities, especially, where the fluid velocity is low. Thus, the fluid reservoirs may be the preferred sites of colonization by

consolidated biofilms. The Marnoso Arenacea deposits and the Epi-Ligurian units provide porous material with wide grain boundaries, i.e. putatively suitable surfaces for colonization. In particular, the shallow fluid reservoir (see Fig. 1.9.c) may provide electron acceptors as sulfate, since Messinian deposits get in contact with the upwelling fluids (cf. Fig. 4.3.). Before final emission, there is a final mud accumulation forming a superficial reservoir approximately 25 m below the surface (Fig. 4.3.). For the gas-mud mixture a gas volume of 10 % was estimated with seismic data (Accaino et al., 2007).

Figure 4.3.: Schematic illustration of mud volcano.

Schematic illustration shows fluid reservoirs of a Nirano mud volcano. A deep fluid reservoir is approximately 2000 m under the surface with a temperature of approximately 60 °C. Beneath the Argille Azzurre formation a shallow fluid reservoir may be in contact to the Messinian deposits. Directly under the cone in approximately 25 m depth a superficial fluid reservoir is located.

The superficial fluid reservoir has also to be considered as AOM habitat, since sulfate and methane are available at a low fluid migration velocity, although methane solubility is already lower than 200 m below the surface.

Colonization along thrust faults can be expected, too. It is very likely that the observed biofilm flakes (cf. section 3.2.2.; Figs. 3.14., 3.16., 3.17.) have developed under these conditions as part of a biofilm, on a consolidated surface. Though in clay substrata development of small microbial aggregates was observed (approximately 10 μm in diameter; Lawrence et al., 2000), the narrow pore spaces in compacted clay (0.1-0.2 μm) will not allow the formation of biofilms. The alternative, i.e. the development of aggregations in a gas-pertubated fluid phase enriched with sedimentary material, may also be considered, but due to the migration of fluids through the system (Capozzi and Picotti, 2002; Bonini, 2008) the organisms experience a rather fast change of environmental conditions. Keeping the low growth rates of ANME and other archaea in mind (cf. Fig. 3.20.; Nauhaus et al., 2007), a long retention period of several weeks or months under constant environmental conditions is likely, accounting for a ‘fixed-bed’ biofilm. The observed pyrite framboids are likely to be a by-product of sulfate reduction and precipitated in the biofilm matrix (cf. section 3.2.2.; Fig. 3.15.).

4.2.2. Role of detected microorganisms

The archaean diversity is remarkable and higher as observed for the first characterized mud volcano community (Paclele Mici, Romania; Alain et al., 2006), which is indicative for an important, if not dominant role of diverse AOM in the system. The high number of sequences of Crenarchaeota, known from manganese and iron dependent AOM studies (section 3.2.2. Figs. 3.18 and 3.19.), suggest electron acceptors other than sulfate, which are, however, very limited (section 3.2.1.; Tab. 3.1.). The presence of ANME-2a-2b as shown by sequence and FISH analysis, give a strong hint for abundant sulfate dependent AOM (section 3.2.2.; Figs. 3.17., 3.18., 3.19.). In addition, GoM Arc I and ANME-1 archaea were detected with 16S rRNA gene analysis. The occurrence of sulfate dependent AOM is also supported by methane dependent sulfide production, measured in enrichment cultures (Fig. 3.20.). In the mud volcano system, sulfate-dependent AOM is of course limited by the low sulfate concentrations in the mud volcano fluids (section 3.2.1.; Tab. 3.1). However, the low sulfate

concentrations may be also a consequence of the high microbial activity in the fluid reservoirs (cf. Capozzi and Picotti, 2002; Boschetti et al., 2010). It has to be further kept in mind that the measured sulfate concentrations highly vary in time between 7 mg/l and 200 mg/l (Martinelli and Judd, 2004; Duchi et al., 2005; this work).

The morphology of the detected ANME-2 aggregates corresponds to the mixed-type consortia, known for this ANME group (cf. Figs. 1.5.c,d and 3.17.; Knittel and Boetius, 2009). This is in accordance with the phylogenetic 16S rRNA gene analysis, which showed ANME-2a-2b archaea (cf. Figs. 3.18. and 3.19.), but no ANME-2c archaea, which form shell-type consortia (Figs. 1.5.f,g and 3.18.). Nevertheless, syntrophic SRB of the DSS group were not observed. This occurrence of ANME cells without SRB partner cell is, however, frequently reported from further habitats (Orphan et al., 2001a, 2002; Durisch-Kaiser et al., 2005; Eller et al., 2005; Knittel et al., 2005; Schubert et al., 2006; Lösekann et al., 2007; Treude et al., 2007).

AOM, indeed, appears to be replaced by aerobic methane oxidation wherever possible: Though unexpected in anaerobic environmental samples, diverse methylotrophic bacterial genera could be enriched (cf. section 3.2.2.; Tab. 3.2.). Here, a low concentration of organisms may be present in the fluids and represent a ‘seed bank’, when, at higher redox potential and under availability of oxygen, aerobic methane oxidation becomes possible. Therefore, already low oxygen concentrations can be sufficient, since genome analysis of *Methylococcus capsulatus* revealed a good adaptation to microaerophilic conditions and lower redox levels than previously thought for methanotrophic bacteria (Ward et al., 2004). Especially, the phylotypes of the detected methanotrophs enriched from the anaerobic fluids, strongly correlate with a recently published study of methane incorporation of bacteria from a marine hydrocarbon seep (Redmond et al., 2010). In this study, Methylococcaceae, *Methylophaga* and Methylophilaceae were identified as primary methane assimilating bacteria. Moreover, the Methylococcaceae bacterium ET-SHO was detected in the marine habitat (cf. with section 3.2.2.; Tab. 3.2.). Also from other marine methane seeps the absence of type II methanotrophs, as in this work, has been described (Durisch-Kaiser et al., 2005; Schubert et al., 2006). Comparing these results, rather high similarities between the marine habitats and the terrestrial habitat of this work become obvious. This is also true for most archaeal sequences matching to nearest neighbors of marine methane seepage habitats (section 3.2.2.). Due to the ancient marine deposits,

dissolved in the migrating fluids of the Nirano mud volcano field, the correlations to marine conditions are reasonable. However, counts of AOM conducting organisms and also total counts of microbial cells are much lower than in marine environments (section 3.2.2.). Thus, the terrestrial methane seeps must be far less effective in oxidation of the greenhouse gas methane as marine methane seeps, which is possibly based on low amounts of available electron acceptors (Knittel and Boetius, 2009).

4.3. Perspectives

The results of this work prove that metabolic key enzymes of reverse methanogenesis and sulfate reduction are expressed in directly neighbored ANME-2 and SRB cells. ANME-1 cells were not analyzed in this work. In future studies, the α -AprB antibodies may also be suitable to localize sulfate reduction in other cell types. Since ANME-1 cells, in particular, show no direct proximity to SRB in the pink layer of GHOSTDABS microbial mats, they might be capable of sulfate reduction by themselves (Knittel et al., 2005; Reitner et al., 2005a; Reitner et al., 2005b; Treude et al., 2007). Although no hints for the APS reductase were reported from genomic and transcriptomic analyses of ANME-1 archaea, its abundance could not be excluded (Meyerdierks et al., 2010).

As already described (cf. section 3.1.2.), magnetosome-like structures composed of greigite are abundant in SRB of the black layer (Reitner et al., 2005a). It is assumed that formation of greigite magnetosomes is similar to the formation of magnetite magnetosomes (Bazylinski et al., 1995). Thus an immunocytochemistry approach with antibodies raised against magnetosome-specific proteins could shed light on the similarity of observed structures to magnetosomes. MamK is involved in filament formation, while MamJ mediates the interaction of the filaments to magnetosome vesicles (Komeili, 2007). Since MamK is a homolog of the prevalent cytoskeletal protein MreB, MamJ should be preferred as antibody target to avoid crossreaction with MreB (Komeili, 2007).

In this work evidence for MDH expressing cells in the anoxic methane seeps was given (cf. section 3.1.1.). Further information about these methane oxidizers (in particular, their phylogenetic affiliation) would be interesting. The pMMO and the MDH are conserved enzymes of methanotrophic bacteria allowing phylogenetic investigations by gene analysis (cf. 1.2.2. and McDonald et al., 2008). These enzymes could also be used

for detection of *M. oxyfera*, which uses nitrate and nitrite for AOM (Ettwig et al., 2010). Moreover, the 16S rRNA gene could be amplified by primers specific for methanotrophs or even bacteria of the NC10 phylum (McDonald et al., 2008; Ettwig et al., 2009). However, 16S rRNA gene analysis of bacteria from the NC10 phylum revealed that a detection is difficult even in enrichment cultures (Ettwig et al., 2009). Thus, screening for members of this group in GHOSTDABS microbial mats is challenging.

The terrestrial mud volcanoes in Nirano revealed low cell counts in comparison to marine methane seep habitats (cf. section 3.2.2.). Nevertheless, the observed microbial flakes suggests the presence of a consolidated biofilm in the fluid system. Therefore, investigations of conduits and fluid reservoirs would be necessary to understand methane turnover at these sites (cf. section 4.2.1.). Direct sampling of deep fluid reservoirs, however, is nearly impossible. Moreover, deep drilling of mud volcanoes may cause uncontrolled mud flow as discussed for the Lusi mud volcano in Indonesia (Sawolo et al., 2009). In addition most mud volcanoes are protected natural sites and should not be damaged.

The fluids of the mud volcanoes are enriched by compounds of underlying geological formations. Consequently, fluids can be composed of varying concentrations of electron acceptors as sulfate or iron (Martinelli and Judd, 2004; Duchi et al., 2005). Temperature and gas composition (e.g. carbon dioxide as main component) may extremely vary. These conditions cause different environments for microbial populations. Thus, comparative analysis with further mud volcano sites could reveal adaptations of microbial communities to available carbon sources and electron sinks.

5. Summary

Anaerobic oxidation of methane (AOM) is recognized as one key process of methane consumption in oceans. According to phylogenetic and metagenomic analyses, the process is carried out by syntrophic consortia of anaerobic methanotrophic archaea and sulfate reducing bacteria. Here, a more comprehensive view inside methanotrophic consortia from anoxic Black Sea cold seeps is shown. By using a set of antibodies as markers, the metabolic activity of the syntrophic partners could be identified. The key enzymes methyl coenzyme M reductase of the (reverse) methanogenic pathway and APS reductase (sulfate reduction pathway) were detected on cellular and subcellular level. Intriguingly, active methylotrophic bacteria, known for aerobic methane oxidation, could be detected in the anoxic environment by the expressed methanol dehydrogenase. It was shown that aerobic as well as anaerobic methane oxidation occur simultaneously, several tens of micrometers apart, in a complex, redox-stratified consortium. The results confirm the syntrophic AOM as dominant process in these anoxic environments and suggest, at the same time, the overall importance of aerobic methanotrophy, since it is not limited to oxic zones of the oceans.

Moreover, a terrestrial site of methane emission was focused in this work. The diversity of prokaryotic organisms involved in oxidation of methane was investigated in fluid samples from the Salse di Nirano mud volcano field situated in the Northern Apennines (Italy). Cell counts were at approximately 0.7×10^6 microbial cells/ml. By fluorescence *in situ* hybridization analysis a fraction of the microbial biomass was identified as ANME archaea associated in densely populated flakes, of some tens of μm in diameter, embedded in a hyaline matrix. Diversity analysis based on the 16S rDNA genes, revealed a high proportion of archaea, involved in sulfate-dependent as well as suggested iron-manganese-dependent AOM. Aerobic methane-oxidizing Proteobacteria could be highly enriched from mud volcano fluids, indicating the presence of aerobic methanotrophic bacteria, which may contribute to methane oxidation, whenever oxygen is readily available. The results imply that biofilms, dominated by ANME archaea, colonize deeply buried parts of the mud volcano venting system.

Zusammenfassung

Die anaerobe Oxidation von Methan (AOM) ist als Schlüsselprozess des marinen Methankonsums bekannt. Nach phylogenetischen und metagenomischen Analysen wird der Prozess von Konsortien aus syntrophen Archaeen und Sulfat reduzierenden Bakterien katalysiert. In dieser Arbeit wird ein umfassenderer Einblick in die methanotrophen Konsortien der Cold Seeps des Schwarzen Meeres gezeigt. Die Stoffwechselaktivität der syntrophen Partner konnte durch die Anwendung von Antikörpern identifiziert werden. Die Schlüsselenzyme Methyl Coenzym M Reduktase der (reversen) Methanogenese und APS Reduktase der Sulfatreduktion wurden auf zellulärer und subzellulärer Ebene detektiert. Interessanterweise konnten auch aktive methylorophe Bakterien, deren Gruppe für die aerobe Methanoxidation bekannt ist, an dem anaeroben Standort gefunden werden. Es wurde gezeigt, dass sowohl anaerobe als auch aerobe Methanoxidation gleichzeitig in einigen zehn Mikrometern Entfernung in einer komplexen mikrobiellen Gemeinschaft mit Redox-Gradienten stattfinden. Die Ergebnisse bestätigen die syntrophe AOM als dominierenden Prozess in diesem anoxischem Lebensraum und weisen zugleich auf die globale Bedeutung des aeroben Stoffwechselweges hin, der nicht auf die oxischen Lebensräume der Ozeane beschränkt zu sein scheint.

Weiterer Gegenstand dieser Arbeit war ein terrestrischer Standort, an dem Methan austritt. Das Schlammvulkan-Feld, Salse di Nirano im nördlichen Apennin (Italien), wurde auf Prokaryoten untersucht, die an der Oxidation von Methan beteiligt sind. Die Zellzahl lag bei ungefähr 0.7×10^6 Zellen/ml. Mit Fluoreszenz *in situ* Hybridisierung konnte ein Teil der Zellen als ANME-Archaea identifiziert werden, die in dicht besiedelten Flocken (einige zehn Mikrometer im Durchmesser) in einer hyalinen Matrix eingebettet sind. Eine Untersuchung der Diversität mit 16S rDNA-Analyse zeigte einen hohen Anteil an Archaeen, die an Sulfat abhängiger als auch Eisen oder Mangan abhängiger AOM beteiligt sind. Die Anreicherung von methanotrophen Bakterien aus aeroben als auch anaeroben Fluiden zeigt, dass sobald Sauerstoff verfügbar wird diese Bakterien an der Oxidation von Methan beteiligt sind. Die Ergebnisse lassen vermuten, dass in tieferen Regionen des vulkanischen Systems von Archaeen dominierte Biofilme angesiedelt sind.

6. References

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7. Appendix

7.1. Materials

7.1.1. Chemicals

Chemicals with the highest commercially available purity level were used for preparation of buffers and media.

2-Mercaptoethanol	Merck, Darmstadt, Germany
Acrylamide	Roth, Karlsruhe, Germany
Agar	Roth, Karlsruhe, Germany
Agarose	Biozym, Hessisch Oldendorf, Germany
Ammonium persulfate	Serva, Heidelberg, Germany
Ampicillin	Sigma, Munich, Germany
Bacto-Agar	DIFCO, Lawrence, USA
Bromophenol blue	Serva, Heidelberg, Germany
CDP-Star	Roche Applied Science, Basel, Switzerland
Chloroform	Roth, Karlsruhe, Germany
Coomassie Brilliant Blue, G250	Serva, Heidelberg, Germany
DAPI	AppliChem, Darmstadt, Germany
dNTPs mixture	MBI Fermentas, St. Leon-Rot, Germany
DPX	Sigma, Munich, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Merck, Darmstadt, Germany
Formvar (Polyvinylformaldehyd)	Plano, Wetzlar, Germany
Glutardialdehyde	Sigma, Munich, Germany
Imidazole	Serva, Heidelberg, Germany
IPTG	AppliChem, Darmstadt, Germany
Lowicryl K4M resin	Electron Microscopy Sciences, Hatfield, USA

Methanol	Roth, Karlsruhe, Germany
Ni ²⁺ -NTA	Qiagen, Hilden, Germany
Nycodenz	Serva, Heidelberg, Germany
Oktanol	Roth, Karlsruhe, Germany
Osmium tetroxide	Science Services, Munich, Germany
PCR water	AppliChem, Darmstadt, Germany
SDS	Roth, Karlsruhe, Germany
TEMED	Merck, Darmstadt, Germany
Tris	Roth, Karlsruhe, Germany
Tryptone	Oxoid, Heidelberg, Germany
Tween 20	Sigma, Munich, Germany
Tween 80	Sigma, Munich, Germany

Chemicals and biochemicals were purchased, unless specified otherwise, from the companies AppliChem (Darmstadt, Germany), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Sigma Aldrich (Munich, Germany).

7.1.2. Auxiliary material

0.2 µm polycarbonate filters	Millipore, Schwalbach, Germany
0.45 µm cellulose nitrate support filters	Millipore, Schwalbach, Germany
Centrifuge cups	Beckmann, Munich, Germany
Cuvettes (microlitre, plastic)	Greiner, Nürtingen, Germany
Dialysis tubes	Serva, Heidelberg, Germany
Gelatin capsules	Plano, Wetzlar, Germany
Gene Amp Reaction Tubes (PCR)	Perkin Elmer, Weiterstadt, Germany
Glas pipet	Brand, Wertheim, Germany
Glass strips	LKB, Bromma, Sweden
Grids (Cu or Ni)	Plano, Wetzlar, Germany
Microfuge tube	Greiner, Nürtingen, Germany
Microlitre pipets (10 µl, 20 µl, 200 µl, 1000 µl)	VWR, Darmstadt, Germany

non-returnable syringe	Terumo, Tokyo, Japan
Parafilm	Pechiney Plastic Packaging Company, Chicago, USA
Petri dishes	Greiner, Nürtingen, Germany
Pipet tip	Sarstedt, Nümbrecht, Germany
Plastic trays	Plano, Wetzlar, Germany
Protino Columns	Macherey-Nagel, Düren, Germany
PVDF membrane	AppliChem, Darmstadt, Germany

7.1.3. Instrumentation

1,024 × 1,024 charge-coupled device detector (HSS 512/1024) for TEM 902A	Proscan Electronic Systems, Lagerlechfeld, Germany
Axioskop 40 FL fluorescence microscope equipped with digital camera and AxioVision software	Carl Zeiss AG, Oberkochen, Germany
Biofuge	Heraeus Christ, Osterode, Germany
Biofuge fresco	Heraeus Christ, Osterode, Germany
Chemiluminescence imager	PEQLAB, Erlangen, Germany
Filter set 43 (BP: 545/25, FT 570, LP: 605/70)	Carl Zeiss AG, Oberkochen, Germany
Filter set 49 (G: 365, FT: 395, BP: 445/50)	Carl Zeiss AG, Oberkochen, Germany
Filter set EGFP HC	AHF Analysentechnik, Tübingen, Germany
FlexCycler (Thermocycler)	Analytik Jena, Jena, Germany
French pressure cell press	SLM Aminco, Lorch, Germany
Gel electrophoresis apparatus	PEQLAB, Erlangen, Germany
Horizontal shaker VXR basic	IKA, Staufen, Germany
Ice machine	Ziegra, Isernhagen, Germany
INGENYphorU system	Ingeny, Goes, Netherlands

Innova 44R (incubator shaker)	New Brunswick, Neu-Isenburg, Germany
InoLab 7200 (pH meter)	WTW, Weilheim, Germany
Knife-maker II LKB 7800	LKB, Bromma, Sweden
Magnetic stirrer Reo basic C	IKA, Staufen, Germany
Mikro-Dismembrator S	Sartorius, Göttingen, Germany
Minigel-Twin (gel electrophoresis)	Biometra, Göttingen, Germany
Orbital shaker G10	New Brunswick, Neu-Isenburg, Germany
Power pack	Biometra, Göttingen, Germany
Refrigerated centrifuge RC5C Sorvall Instruments	Kendro, Hanau, Germany
Rotating milling cutter TM 60	Reichert-Jung, Heidelberg, Germany
Scale	Sartorius, Göttingen, Germany
Special accuracy weighing machine	Sartorius, Göttingen, Germany
Spektralphotometer Ultraspec 3000	Amersham Pharmacia, Uppsala, Sweden
Steam autoclave	Thermo Scientific, Waltham, MA, USA
ThermoStat	Eppendorf, Hamburg, Germany
Transmission Electron Microscope 902A	Carl Zeiss AG, Oberkochen, Germany
Ultramicrotome FC 4	Reichert-Jung, Heidelberg, Germany
Ultrasonic unit UP200S	Dr. Hielscher GmbH, Teltow, Germany
UniSampler (fluid sampler)	Bürkle, Bad Bellingen, Germany
UV Transilluminator 2000	Bio-Rad, Munich, Germany
Vortex Genie 2	Bender & Hobeing, Zurich, Switzerland
Water desalination plant	Millipore, Schwalbach, Germany

7.1.4. Commercial systems

Goat normal serum	Jackson ImmunoResearch Europe, Suffolk, UK
goat-anti-rabbit IgG (H+L) AlexaFluor 488 conjugate	Cell Signaling Technology, Danvers, USA
goat-anti-rabbit IgG AlexaFluor 546 conjugate	Molecular Probes, Eugene, USA
goat-anti-rabbit IgG alkaline phosphatase conjugate	Promega, Madison, USA
Goat-anti-rabbit IgG-10 nm gold conjugate	British Biocell International, Cardiff, UK
High Pure PCR Template Preparation Kit	Roche Applied Science, Basel, Switzerland
Nanocolor [®] Sulfid Kit	Macherey-Nagel, Düren, Germany
NucleoSpin [®] Food Kit	Macherey-Nagel, Düren, Germany
peqGOLD Gel Extraction Kit	PEQLAB, Erlangen, Germany
peqGOLD MicroSpin Cycle-Pure Kit	PEQLAB, Erlangen, Germany
peqGOLD Plasmid Miniprep Kit I	PEQLAB, Erlangen, Germany
Prestained Protein Molecular Weight Marker	MBI Fermentas, St. Leon-Rot, Germany
Protease inhibitor complete EDTA free	Roche Applied Science, Basel, Switzerland
Protino Ni-TED 2000 Packed Columns	Macherey-Nagel, Düren, Germany
Quick-Load [®] 1 kb DNA Ladder	NEB Biolabs, Frankfurt am Main
Roti-Quant	Roth, Karlsruhe, Germany
SybrGold solution	Molecular Probes, Eugene, USA

7.1.5. Enzymes

Calf intestinal alkaline phosphatase (CIAP)	MBI Fermentas, St. Leon-Rot, Germany
Chicken Egg White Lysozyme (178000 U/mg)	Merck, Darmstadt, Germany
Phusion [®] High-Fidelity DNA Polymerase	NEB Biolabs, Frankfurt am Main, Germany
Proteinase K	AppliChem, Darmstadt, Germany
Restriction endonucleases	MBI Fermentas, St. Leon-Rot, Germany
T4 DNA Ligase	MBI Fermentas, St. Leon-Rot, Germany
<i>Taq</i> DNA Polymerase	NEB Biolabs, Frankfurt am Main, Germany

7.2. Oligonucleotides

All unlabeled oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). Oligonucleotides with fluorochromes were purchased from biomers (Ulm, Germany).

Table 7.1.: Oligonucleotides

Name	Sequence (5'→3') ¹	Restriction site or dye	Description
CWAprB1- fw	AGTTGGTATGCCGACTTWY GTNRAYCC		first step of nested PCR for <i>aprB</i> gene amplification
AprA-5-RV	GCGCCAACYGGRCCRTA		first step of nested PCR for <i>aprB</i> gene amplification (Meyer and Kuever, 2007)
CWAprB3- fw	CCGACTTATGTTGATCCGG CNAARTGYGAYGG		second step of nested PCR for <i>aprB</i> gene amplifica- tion

Name	Sequence (5'→3') ¹	Restriction site or dye	Description
APS-RV	GGGCCGTAACCGTCCTT GAA		second step of nested PCR for <i>aprB</i> gene amplification (Deplancke et al., 2000)
CWAprBKl 1-fw	AAAGGATCCATGCCGAC TTATGTTGATCCGGC	<i>Bam</i> HI	cloning <i>aprB</i> gene in pWH844
CWAprBKl 2-rev	TTTAAGCTTTCACGTGTCGC CTTGCTAGAC	<i>Hind</i> III	cloning <i>aprB</i> gene in pWH844
CWMDHa fw	AAAGGATCCATGAGCAG GTTTGTGACATCAG	<i>Bam</i> HI	cloning <i>mxoF</i> gene in pWH844
CWMDHa rev	TTTAAGCTTTTACTTGGCG GCTGACTTC	<i>Hind</i> III	cloning <i>mxoF</i> gene in pWH844
CWMDHa seq	CTCGACGCTCACGATCG		sequencing of <i>mxoF</i> gene
8f	AGAGTTTGATCATGGCT CAG		16S rDNA amplification (Alain et al., 2002)
1492r	GTTACCTTGTTACGACTT		16S rDNA amplification (Alain et al., 2002)
Arch21F	TTCCGGTTGATCCYGCC GGA		16S rDNA amplification (DeLong, 1992)
Arch958R	YCCGGCGTTGAMTCCAATT		16S rDNA amplification (DeLong, 1992)
341f	CCTACGGGAGGCAGCAG		DGGE, reamplification (Schäfer et al., 2001)

Name	Sequence (5'→3') ¹	Restriction site or dye	Description
341f-GC	CGCCCGCCGCGCCCCGCGC CCGGCCCGCCGCCCCCGCC CCCCTACGGGAGGCAGCAG		DGGE, with GC-clamp (Stadnitskaia et al., 2005)
907Rc	CCGTCAATTCCTTTGAGT		DGGE (Schäfer et al., 2001)
907Ra	CCGTCAATTCATTTGAGT		DGGE (Schäfer et al., 2001)
M13f	GTAAAACGACGGCCAG		checking and sequencing of pCR2.1-TOPO inserts
M13r	CAGGAAACAGCTATGAC		checking and sequencing of pCR2.1-TOPO inserts
JS39	TCTATCAACAGGAGTCCA AGC	-	checking and sequencing of pWH844 inserts
pWH844fw	TATGAGAGGATCGCATC ACCAT	-	checking and sequencing of pWH844 inserts
Arch915	GTGCTCCCCCGCCAATT CCT	CY3	specific for most archaea (Stahl and Amann, 1991; Lösekann et al., 2007)
DSS658	TCCACTTCCCTCTCCCAT	6-FAM	specific for <i>Desulfosarcina</i> / <i>Desulfococcus</i> branch (Manz et al., 1998; Boetius et al., 2000; Lösekann et al., 2007)

Name	Sequence (5'→3') ¹	Restriction site or dye	Description
EelMS932	AGCTCCACCCGTTGTAGT	CY3	Specific for ANME-2 archaea (Boetius et al., 2000)
EUB338 I	GCTGCCTCCCGTAGGAGT	CY3	EUB338 I-III specific for most bacteria (Daims et al., 1999)
EUB338 II	GCAGCCACCCGTTAGGTGT	CY3	
EUB338 III	GCTGCCACCCGTTAGGTGT	CY3	

¹ Restriction sites are underlined.

7.3. Sequences

***aprB* gene sequence used for heterologous overexpression:**

5'-ATGCCGACTTATGTTGATCCGGCTAAATGCGACGGCTGCAAGGGGGGAG
 ACAAACCGCTCGTATGTACATCTGCCCAAACGACCTTATGATTCTCGACCC
 CGATGAGATGAAGGCCTTCAACCAGGAACCCGACGCGTGCTGGGAGTGTTA
 TTCCTGCGTGAAGATCTGCCCCCAAGGCGCGATTACGGCCCGTCCGTATGCG
 GACTTCGCCCCCATGGGCGGAACTTCCATTCCGCTTCGCGGCTCCGACGACA
 TCATGTGGACCATTAATTCCGCAACGGCAGCGTCAAGCGGTTCAAATTCCC
 CATCCGCACCACGCCTGAGGGCTCGATCAAGCCTTACGAGGGCAAACCCGA
 ACCCGCGGACCTGGAAAACGAAATGTACTTCAACGAGACCGAACTTAAATC
 GCCCGCGGATGCCATGGGCAAGAAGTTCGACATCGCCGAGGCCGACAAGAC
 CGTGACCTCCATGGCCTCCGCAGTCTAGCAAGGCGACAGTGA-3'

Table 7.2.: Archaeal 16S rDNA sequences.

Sequences 2008	GenBank No.	Classification in ARB	GenBank No. NCBI nearest neighbor	Nearest neighbor	Identity [%]
NR9_08_001	HQ654786	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF367445	Uncultured archaeon clone ZES-7 16S ribosomal RNA gene	886/887 (99%)
NR9_08_002	HQ654787	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	927/931 (99%)
NR9_08_003	HQ654789	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	920/929 (99%)
NR9_08_004	HQ654791	Crenarchaeota/Miscellaneous Crenarchaeotic Group	AJ579323	Uncultured archaeon partial 16S rRNA gene, clone HMMVCen-26	920/940 (97%)
NR9_08_005	HQ654793	Euryarchaeota/Methanomicrobium/ Methanosarcinales/ANME2a-2b	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal RNA	890/904 (98%)
NR9_08_006	HQ654795	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	924/933 (99%)
NR9_08_007	HQ654797	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	933/946 (98%)
NR9_08_008	HQ654800	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	920/924 (99%)
NR9_08_009	HQ654801	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	915/929 (98%)
NR9_08_010	HQ654802	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	925/929 (99%)
NR9_08_011	HQ654804	Crenarchaeota/Miscellaneous Crenarchaeotic Group	AJ579323	Uncultured archaeon partial 16S rRNA gene, clone HMMVCen-26	911/936 (97%)
NR9_08_012	HQ654805	Euryarchaeota/Methanomicrobium/ Methanosarcinales/ANME2a-2b	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal RNA	900/910 (98%)
NR9_08_013	HQ654806	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ267174	Uncultured crenarchaeote clone Mothra_A2-5 16S ribosomal RNA	812/835 (97%)
NR9_08_014	HQ654812	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	871/882 (98%)
NR9_08_015	HQ654814	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	936/945 (99%)
NR9_08_016	HQ654815	Euryarchaeota/Methanomicrobium/ Methanosarcinales/Methanosetaeaceae	EU721747	Uncultured Methanoseta sp. clone D00301103 16S ribosomal RNA	900/918 (98%)
NR9_08_017	HQ654818	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EU681927	Uncultured archaeon clone SMTZArch9 16S ribosomal RNA gene	921/929 (99%)
NR9_08_018	HQ654820	Crenarchaeota/Miscellaneous Crenarchaeotic Group	AJ579323	Uncultured archaeon partial 16S rRNA gene, clone HMMVCen-26	898/926 (96%)
NR9_08_019	HQ654823	Crenarchaeota/Soil Crenarchaeotic Group	AJ831163	Unidentified archaeon 16S rRNA gene, clone 235	887/890 (99%)

Sequences 2008	GenBank No.	Classification in ARB	GenBank No. NCBI nearest neighbor	Nearest neighbor	Identity [%]
NR9_08_020	HQ654824	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF367518	Uncultured archaeon clone ZES-81 16S ribosomal RNA gene	908/916 (99%)
NR9_08_021	HQ654825	Crenarchaeota/Miscellaneous Crenarchaeotic Group	AJ579323	Uncultured archaeon partial 16S rRNA gene, clone HMMV/Cen-26	914/936 (97%)
NR9_08_022	HQ654827	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356856	Uncultured archaeon clone FeSO4_A_119 16S ribosomal RNA gene	887/932 (95%)
NR9_08_023	HQ654828	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	904/909 (99%)
NR9_08_024	HQ654782	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	920/924 (99%)
NR9_08_025	HQ654788	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	936/945 (99%)
NR9_08_026	HQ654790	Crenarchaeota/Miscellaneous Crenarchaeotic Group	AJ579323	Uncultured archaeon partial 16S rRNA gene, clone HMMV/Cen-26	923/946 (97%)
NR9_08_027	HQ654792	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	921/925 (99%)
NR9_08_028	HQ654794	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	932/945 (98%)
NR9_08_029	HQ654796	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	933/945 (98%)
NR9_08_030	HQ654798	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	936/945 (99%)
NR9_08_031	HQ654799	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	933/945 (98%)
NR9_08_032	HQ654803	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	924/935 (98%)
NR9_08_033	HQ654807	Euryarchaeota/Methanomicrobia/ Methanosarcinales/ANME2a-2b	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	934/945 (98%)
NR9_08_034	HQ654808	Euryarchaeota/Methanomicrobia/ Methanosarcinales/GOM Arc1	FJ907179	Archaeon enrichment culture clone LCB_A1C7 16S ribosomal RNA gene	912/915 (99%)
NR9_08_035	HQ654809	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	923/925 (99%)
NR9_08_036	HQ654810	Euryarchaeota/Methanomicrobia/ Methanosarcinales/GOM Arc1	FJ907179	Archaeon enrichment culture clone LCB_A1C7 16S ribosomal RNA gene	910/914 (99%)
NR9_08_037	HQ654811	Crenarchaeota/Miscellaneous Crenarchaeotic Group	AJ579323	Uncultured archaeon partial 16S rRNA gene, clone HMMV/Cen-26	923/947 (97%)
NR9_08_038	HQ654813	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	937/945 (99%)

Sequences 2008	GenBank No.	Classification in ARB	GenBank No. NCBI nearest neighbor	Nearest neighbor	Identity [%]
NR9_08_039	HQ654816	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF367518	Uncultured archaeon clone ZES-81 16S ribosomal RNA gene	934/945 (98%)
NR9_08_040	HQ654817	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	934/945 (98%)
NR9_08_041	HQ654819	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal RNA	870/879 (98%)
NR9_08_042	HQ654821	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356856	Uncultured archaeon clone FeSO4_A_119 16S ribosomal RNA gene	922/925 (99%)
NR9_08_043	HQ654822	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	921/925 (99%)
NR9_08_044	HQ654826	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	922/925 (99%)
NR9_08_045	HQ654829	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356856	Uncultured archaeon clone FeSO4_A_119 16S ribosomal RNA gene	921/924 (99%)
NR9_08_046	HQ654783	Euryarchaeota/Methanomicrobium/ Methanosarcinales/ANME2a-2b	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal	905/918 (99%)
NR9_08_047	HQ654784	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	935/945 (99%)
NR9_08_048	HQ654785	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	933/944 (99%)

Sequences 2009	GenBank No.	Classification in ARB	GenBank No. NCBI nearest neighbor	Nearest neighbor	Identity [%]
NR9_09_001	HQ654830	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF367669	Uncultured archaeon clone ZES-232 16S ribosomal RNA gene, partial sequence	934/945 (98%)
NR9_09_002	HQ654831	Crenarchaeota/Soil Crenarchaeotic Group	FJ790535	Uncultured archaeon clone VA52 16S ribosomal RNA gene, partial sequence	885/891 (99%)
NR9_09_003	HQ654832	Crenarchaeota/Miscellaneous Crenarchaeotic Group	AJ579323	Uncultured archaeon partial 16S rRNA gene, clone HMMVCen-26	923/947 (97%)
NR9_09_004	HQ654833	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF680211	Uncultured archaeon clone MidArch27 16S ribosomal RNA gene, partial	910/944 (96%)
NR9_09_005	HQ654834	Euryarchaeota/Methanomicrobia/Methanosarcinales/GOM Arc1	FJ907179	Archaeon enrichment culture clone LCB_A1C7 16S ribosomal RNA gene	911/914 (99%)
NR9_09_006	HQ654835	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF680211	Uncultured archaeon clone MidArch27 16S ribosomal RNA gene, partial	910/944 (96%)
NR9_09_007	HQ654836	Euryarchaeota/Methanomicrobia/Methanosarcinales/Methanosarcinaceae/Methanobolbus	AJ969778	Uncultured archaeon 16S rRNA gene, clone ss058b	909/910 (99%)
NR9_09_008	HQ654837	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	920/924 (99%)
NR9_09_009	HQ654838	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	935/944 (99%)
NR9_09_010	HQ654839	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356919	Uncultured archaeon clone FeOrig_A_7 16S ribosomal RNA gene, partial sequence	912/945 (96%)
NR9_09_011	HQ654840	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	934/945 (98%)
NR9_09_012	HQ654841	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	918/925 (99%)
NR9_09_013	HQ654842	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356856	Uncultured archaeon clone FeS04_A_119 16S ribosomal RNA gene	923/925 (99%)
NR9_09_014	HQ654843	Euryarchaeota/Methanomicrobia/Methanosarcinales/Methanosarcinaceae/Methanobolbus	AJ969778	uncultured archaeon 16S rRNA gene, clone ss058b	914/918 (99%)
NR9_09_015	HQ654844	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	924/926 (99%)

Sequences 2009	GenBank No.	Classification in ARB	GenBank No. NCBI nearest neighbor	Nearest neighbor	Identity [%]
NR9_09_016	HQ654845	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EU681927	Uncultured archaeon clone SMTZArch9 16S ribosomal RNA gene	912/916 (99%)
NR9_09_017	HQ654846	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EU681927	Uncultured archaeon clone SMTZArch9 16S ribosomal RNA gene	912/924 (98%)
NR9_09_018	HQ654847	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	920/925 (99%)
NR9_09_019	HQ654848	Euryarchaeota/Methanomicrobia/ Methanosarcinales/ANME2a-2b	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal RNA	907/917 (98%)
NR9_09_020	HQ654849	Euryarchaeota/Methanomicrobia/ Methanosarcinales/ANME2a-2b	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal RNA	863/872 (98%)
NR9_09_021	HQ654850	Euryarchaeota/Methanomicrobia/ Methanosarcinales/ANME2a-2b	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal RNA	860/870 (98%)
NR9_09_022	HQ654851	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	924/925 (99%)
NR9_09_023	HQ654852	Euryarchaeota/Methanomicrobia/ Methanosarcinales/GOM Arc1	FJ907179	Archaeon enrichment culture clone LCB_A1C7 16S ribosomal RNA gene	909/914 (99%)
NR9_09_024	HQ654853	Crenarchaeota/Marine Benthic Group	DQ363774	Uncultured archaeon clone MKCSM-A6 16S ribosomal RNA gene, partial	876/900 (97%)
NR9_09_025	HQ654854	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	923/933 (98%)
NR9_09_026	HQ654855	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	921/925 (99%)
NR9_09_027	HQ654856	Euryarchaeota/Methanomicrobia/ Methanosarcinales/ANME2a-2b	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal RNA	890/899 (98%)
NR9_09_028	HQ654857	Crenarchaeota/Miscellaneous Crenarchaeotic Group	AJ579323	Uncultured archaeon partial 16S rRNA gene, clone HMMVCen-26	900/926 (97%)
NR9_09_029	HQ654858	Euryarchaeota/Methanomicrobia/ Methanosarcinales/GOM Arc1	AB177276	Uncultured archaeon gene for 16S rRNA, clone: ODP1251A20.3.	882/918 (96%)
NR9_09_030	HQ654859	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF680211	Uncultured archaeon clone MidArch27 16S ribosomal RNA gene, partial	913/944 (96%)
NR9_09_031	HQ654860	Euryarchaeota/Thermoplasma/ Thermoplasmales/South African Goldmine	GQ356919	Uncultured archaeon clone FeOrig_A_7 16S ribosomal RNA gene, partial	923/929 (99%)
NR9_09_032	HQ654861	Euryarchaeota/Thermoplasma/ Thermoplasmales/South African Goldmine	GQ356919	Uncultured archaeon clone FeOrig_A_7 16S ribosomal RNA gene, partial	922/929 (99%)
NR9_09_033	HQ654862	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF680211	Uncultured archaeon clone MidArch27 16S ribosomal RNA gene, partial	896/930 (96%)
NR9_09_034	HQ654863	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EU681927	Uncultured archaeon clone SMTZArch9 16S ribosomal RNA gene	913/924 (98%)
NR9_09_035	HQ654864	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF680211	Uncultured archaeon clone MidArch27 16S ribosomal RNA gene, partial	912/944 (96%)

Sequences 2009	GenBank No.	Classification in ARB	GenBank No. NCBI nearest neighbor	Nearest neighbor	Identity [%]
NR9_09_036	HQ654865	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	935/945 (98%)
NR9_09_037	HQ654866	Euryarchaeota/Methanomicrobia/ Methanosarcinales/ANME2a-2b	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal RNA	868/879 (98%)
NR9_09_038	HQ654867	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF680211	Uncultured archaeon clone MidArch27 16S ribosomal RNA gene, partial	911/944 (96%)
NR9_09_039	HQ654868	Euryarchaeota/Methanomicrobia/ Methanosarcinales/ANME2a-2b	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal RNA	865/875 (98%)
NR9_09_040	HQ654869	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	913/923 (98%)
NR9_09_041	HQ654870	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	919/924 (99%)
NR9_09_042	HQ654871	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	926/945 (97%)
NR9_09_043	HQ654872	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	935/945 (98%)
NR9_09_044	HQ654873	Euryarchaeota/Methanomicrobia/ Methanosarcinales/ANME2a-2b	AB252424	Uncultured archaeon gene for 16S rRNA, partial sequence, clone: OT-A17.11	902/909 (99%)
NR9_09_045	HQ654874	Euryarchaeota/Methanomicrobia/ Methanosarcinales/GOM Arc1	AB177276	Uncultured archaeon gene for 16S rRNA, clone: ODP1251A20.3.	882/918 (96%)
NR9_09_046	HQ654875	Euryarchaeota/Methanomicrobia/ Methanosarcinales/Methermicrocaceae	FJ712387	Uncultured archaeon clone KZNMV-25-A18 16S ribosomal RNA gene	913/919 (99%)
NR9_09_047	HQ654876	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	933/945 (98%)
NR9_09_048	HQ654877	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF680211	Uncultured archaeon clone MidArch27 16S ribosomal RNA gene, partial	896/933 (96%)
NR9_09_049	HQ654878	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF680211	Uncultured archaeon clone MidArch27 16S ribosomal RNA gene, partial	909/945 (96%)
NR9_09_050	HQ654879	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ712384	Uncultured archaeon clone KZNMV-25-A3 16S ribosomal RNA gene	901/912 (98%)
NR9_09_051	HQ654880	Euryarchaeota/Thermoplasmatata/ Thermoplasmatatales/ASC21	FJ705114	Uncultured archaeon clone ACWCSP1A4 16S ribosomal RNA gene	888/899 (98%)
NR9_09_052	HQ654881	Euryarchaeota/Thermoplasmatata/ Thermoplasmatatales/South African Goldmine	GQ356919	Uncultured archaeon clone FeOrig_A_7 16S ribosomal RNA gene,partial sequence	923/930 (99%)
NR9_09_053	HQ654882	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	935/945 (98%)

Sequences 2009	GenBank No.	Classification in ARB	GenBank No. NCBI nearest neighbor	Nearest neighbor	Identity [%]
NR9_09_054	HQ654883	Euryarchaeota/Thermoplasmata/ Thermoplasmatales/South African Goldmine	GQ356919	Uncultured archaeon clone FeOrig_A_7 16S ribosomal RNA gene,partial sequence	900/908 (99%)
NR9_09_055	HQ654884	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	935/945 (98%)
NR9_09_056	HQ654885	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	922/925 (99%)
NR9_09_057	HQ654886	Euryarchaeota/Methanomicrobia/ Methanosarcinales/ANME2a-2b	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal RNA	877/887 (98%)
NR9_09_058	HQ654887	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	936/945 (99%)
NR9_09_059	HQ654888	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	934/945 (98%)
NR9_09_060	HQ654889	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF367566	Uncultured archaeon clone ZES-129 16S ribosomal RNA gene, partial	926/932 (99%)

Sequences 2008	GenBank No.	Classification in ARB	GenBank No. NCBI nearest neighbor	Nearest neighbor	Identity [%]
NR9_08_001	HQ654786	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF367445	Uncultured archaeon clone ZES-7 16S ribosomal RNA gene	886/887 (99%)
NR9_08_002	HQ654787	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	927/931 (99%)
NR9_08_003	HQ654789	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	920/929 (99%)
NR9_08_004	HQ654791	Crenarchaeota/Miscellaneous Crenarchaeotic Group	AJ579323	Uncultured archaeon partial 16S rRNA gene, clone HMMV/Cen-26	920/940 (97%)
NR9_08_005	HQ654793	Euryarchaeota/Methanomicrobium/ Methanosarcinales/ANME2a-2b	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal RNA	890/904 (98%)
NR9_08_006	HQ654795	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	924/933 (99%)
NR9_08_007	HQ654797	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	933/946 (98%)
NR9_08_008	HQ654800	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	920/924 (99%)
NR9_08_009	HQ654801	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	915/929 (98%)
NR9_08_010	HQ654802	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ356817	Uncultured archaeon clone Fe_A_3 16S ribosomal RNA gene	925/929 (99%)
NR9_08_011	HQ654804	Crenarchaeota/Miscellaneous Crenarchaeotic Group	AJ579323	Uncultured archaeon partial 16S rRNA gene, clone HMMV/Cen-26	911/936 (97%)
NR9_08_012	HQ654805	Euryarchaeota/Methanomicrobium/ Methanosarcinales/ANME2a-2b	FJ555678	Archaeon enrichment culture clone AOM-Clone-D10 16S ribosomal RNA	900/910 (98%)
NR9_08_013	HQ654806	Crenarchaeota/Miscellaneous Crenarchaeotic Group	GQ267174	Uncultured crenarchaeote clone Mothra_A2-5 16S ribosomal RNA	812/835 (97%)
NR9_08_014	HQ654812	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	871/882 (98%)
NR9_08_015	HQ654814	Crenarchaeota/Miscellaneous Crenarchaeotic Group	FJ649523	Uncultured archaeon clone AMSMV-S1-A30 16S ribosomal RNA gene	936/945 (99%)
NR9_08_016	HQ654815	Euryarchaeota/Methanomicrobium/ Methanosarcinales/Methanosetaeaceae	EU721747	Uncultured Methanoseta sp. clone D00301103 16S ribosomal RNA	900/918 (98%)
NR9_08_017	HQ654818	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EU681927	Uncultured archaeon clone SMTZArch9 16S ribosomal RNA gene	921/929 (99%)
NR9_08_018	HQ654820	Crenarchaeota/Miscellaneous Crenarchaeotic Group	AJ579323	Uncultured archaeon partial 16S rRNA gene, clone HMMV/Cen-26	898/926 (96%)
NR9_08_019	HQ654823	Crenarchaeota/Soil Crenarchaeotic Group	AJ831163	Unidentified archaeon 16S rRNA gene, clone 235	887/890 (99%)
NR9_08_020	HQ654824	Crenarchaeota/Miscellaneous Crenarchaeotic Group	EF367518	Uncultured archaeon clone ZES-81 16S ribosomal RNA gene	908/916 (99%)

7.4. Internet programs and software

Table 7.3.: Internet programs and software.

URL/Software	Provider	Application
http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi	The Institute for Genomic Research, Rockville, USA	sequence analysis
http://www.ncbi.nlm.nih.gov/	National Institutes of Health, Bethesda, USA	literature enquiry
http://portal.isiknowledge.com/portall.cgi	The Thomson Corporation, Toronto, Canada	literature enquiry
http://www.molecularcloning.com	Cold Spring Harbor Laboratory Press, New York, USA	Molecular protocols
http://tools.neb.com/NEBcutter2/	NEB Biolabs, Frankfurt am Main, Germany	restriction sites analysis
http://us.expasy.org/	Swiss Institute of Bioinformatics, Geneva, Switzerland	protein structure analysis
http://blocks.fhcrc.org/blocks/blockmkr/make_blocks.html	Fred Hutchinson Cancer Research Center, Seattle, USA	alignments of conserved sequences
http://bioinformatics.weizmann.ac.il/blocks/codehop.html	Fred Hutchinson Cancer Research Center, Seattle, USA	degenerate primer design

URL/Software	Provider	Application
ARB program package	The ARB Project, München, Germany (Ludwig et al., 2004)	phylogenetic tree construction
Bellerophon	Australian National University, Canberra, Australia (Huber et al., 2004)	chimera analysis
CHIMERA_CHECK program	Ribosomal Database Project, East Lansing, USA (Maidak et al., 2001)	chimera analysis
gap4 program	MRC Laboratory of Molecular Biology, Cambridge, UK (Staden Package; Bonfield et al., 1995)	sequence analysis
Geneious	Biomatters Ltd, Auckland, New Zealand	sequence analysis
iTEM	Olympus, Tokyo, Japan	camera control EM 902, image processing
ImageJ	National Institutes of Health, Bethesda, USA	image analyses

URL/Software	Provider	Application
Mallard	Cardiff University, Cardiff, UK (Ashelford et al., 2006)	chimera analysis
Microsoft Office 2007	Microsoft Inc., Redmond, USA	text and data processing
Photoshop 8.0	Adobe Corp., San Jose, USA	image processing
SeqMan TM II 5.07	DNASTAR Inc., Madison, USA	sequence analysis
VectorNTI [®]	Invitrogen, Carlsbad, USA	sequence analysis

Curriculum Vitae

Name	Christoph Wrede
Geburtsdatum	8. November 1981
Geburtsort	Norden

08/1988 bis 06/2001	Schulausbildung Abitur, Cuxhaven
07/2001 bis 04/2002	Zivildienst Senioren pension Meier & Wätjen GmbH, Cuxhaven
04/2002 bis 09/2002	Lehramtsstudium für die Sekundarstufe II, Georg-August-Universität Göttingen Fächerkombination: Chemie/Biologie
10/2002 bis 01/2007	Diplomstudium Biologie, Georg-August-Universität Göttingen Hauptfach: Mikrobiologie Nebenfächer: Immunologie, Org. Chemie
02/2007 bis 11/2007	Anfertigung der Diplomarbeit „Strukturelle und funktionelle <i>in situ</i> Charakterisierung von mikrobiellen Matten aus Methan-Seeps“ Arbeitsgruppe von PD Dr. Michael Hoppert, Inst. f. Mikrobiologie und Genetik, Georg-August-Universität Göttingen
12/2007 bis 04/2008	Vorbereitende experimentelle Versuche zur Promotion Posterpreis der GBM und VAAM im März 2008
05/2008	Promotion „Metabolismus und Biomineralisation in anaerob Methan-oxidierenden Lebensgemeinschaften“ Arbeitsgruppe von PD Dr. Michael Hoppert, Inst. f. Mikrobiologie und Genetik, Georg-August-Universität Göttingen