Investigation of microbial groups involved in the uptake of atmospheric trace gases in upland soils

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

dem Fachbereich Biologie der Philipps-Universität Marburg

vorgelegt von

Jennifer Pratscher
aus Jülich

Marburg/Lahn 2010

Die Untersuchungen zur folgenden Arbeit wurden von Januar 2008 bis Oktober 2010 am Max-Planck-Institut für terrestrische Mikrobiologie in Marburg unter Anleitung von Prof. Dr. Ralf Conrad durchgeführt.

Vom Fachbereich Biologie der Philipps-Universität Marburg als Dissertation angenommen am: 28.10.2010

Erstgutachter: Prof. Dr. Ralf Conrad
Zweitgutachter: Prof. Dr. Erhard Bremer

Tag der Disputation: 13.01.2011

Publication list

Die in dieser Dissertation beschriebenen Ergebnisse sind in folgenden Publikationen veröffentlicht bzw. zur Veröffentlichung vorgesehen:

1. **Pratscher, J., Dumont, M.G., Conrad, R. (2010)** Assimilation of acetate by the putative atmospheric methane oxidizing clade USCα. (submitted to *Nature Geoscience* in October 2010)

2. **Pratscher, J., Dumont, M.G., Conrad, R. (2010)** Ammonia oxidation coupled to CO_2 fixation by *Archaea* and *Bacteria* in an agricultural soil. (submitted to *Proceedings of the National Academy of Sciences of the United States of America* on 30th July 2010)

3. **Constant, P., Chowdhury, S.P., Pratscher, J., Conrad, R. (2010)** Streptomycetes contributing to atmospheric molecular hydrogen soil uptake are widespread and encode a putative high-affinity [NiFe]-hydrogenase. *Environ Microbiol* **12:** 821-829.

"There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. - There is another theory which states that this has already happened."

Douglas Adams - *The Restaurant at the End of the Universe*

Contents

Abbreviations

°C	degree Celsius
µ	micro (10^{-6})
AOA	ammonia oxidizing archaea
AOB	ammonia oxidizing bacteria
bp	base pairs
CARD-FISH	catalyzed reporter deposition - fluorescence *in situ* hybridization
CsCl	cesium chloride
CsTFA	cesium trifluoroacetate
CTAB	cetyl trimethylammonium bromide
CTP	cytidine triphosphate
Cy3	carbocyanine 3
DAPI	4', 6-diamidino-2-phenylindol-dihydrochloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DSM	DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
et al.	et alteri
FA	formamide
FAM	carboxyfluoresceine
Fig.	figure
FISH	fluorescence *in situ* hybridization
g	gram
GTP	guanosine triphosphate
kb	kilobase
l	litre
M	molar (mol/l)
m	milli (10^{-3})
MF	Marburg forest soil
min	minute
MOB	methane oxidizing bacteria
MW	molecular weight
n	nano (10^{-9})
NCIMB	The National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland, UK
nD-TC	refractive index
nt.	nucleotide
ON	over night

p	pico (10^{-12})
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	negative common logarithm of the molar concentration of dissolved hydronium ions
PLFA	phospholipid fatty acids
qPCR	quantitative polymerase chain reaction
RH	Rauischholzhausen agricultural soil
RING-FISH	recognition of individual genes – fluorescence *in situ* hybridization
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
rRNA	ribosomale RNA
RT	room temperature
SDS	sodium dodecyl sulfate
SIP	stable isotope probing
sp.	species
SSMS	soil substrate membrane system
Tab.	table
T_m	melting temperature of oligonucleotides
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
Tris	tris(hydroxymethyl)aminomethane
USCα	upland soil cluster α
UTP	uridine triphosphate
V	volt
v/v	volume per volume
Vol.	volume
w/v	weight per volume

Zusammenfassung

Atmosphärischen Spurengasen kommt eine Führungsrolle in den derzeitigen klimatischen Veränderungen zu. Ein signifikanter Anteil am Austausch von atmosphärischen Spurengasen findet an der Schnittstelle zwischen Atmosphäre und „upland soils“ statt. Allerdings sind die für diese Dynamiken verantwortlichen Mikroorganismen nicht vollständig identifiziert und erforscht. Diese Arbeit befasst sich deshalb mit der Untersuchung mikrobieller Gruppen in terrestrischen Habitaten, die in die Aufnahme von atmosphärischen Spurengasen (CH_4, CO_2, H_2) involviert sind, und zwar das potentiell atmosphärisches Methan oxidierende „upland soil cluster α“ (USCα), die autotrophen Ammonium-oxidierenden Archaeen (AOA) und die Wasserstoff-oxidierenden Streptomyzeten. Verschiedene Methoden wurden angewandt, um die Aufnahme von markiertem Substrat in diese mikrobiellen Gruppen zu untersuchen und die Expression ihrer funktionellen Marker-Gene zu analysieren. Dabei handelte es sich um *pmoA* für die hoch-affine membran-gebundene (partikuläre) Methan-Monooxygenase von USCα, *amoA* für die Ammonium-Monooxygenase der AOA und *hydB* für die hoch-affine [NiFe]-Hydrogenase von *Streptomyces* sp. PCB7.

Trotz der Annahme, dass es sich bei dem “upland soil cluster α“ (USCα) in Waldböden um methanotrophe Bakterien handelt, die an atmosphärische Methan-konzentrationen angepasst sind und denen demnach eine grundlegende Rolle in der Aufnahme und Beseitigung dieses Treibhausgases aus der Atmosphäre zuteil wird, war noch ungeklärt, ob diese Mikroorganismen ihren gesamten Energie- und Kohlenstoffbedarf mit Methan decken können oder zusätzlich auf andere Kohlenstoffquellen angewiesen sind. Stabile Isotopenbeprobung wurde angewandt, um den Einbau von markiertem CH_4 und Acetat in Nukleinsäuren von USCα zu untersuchen. Die Ergebnisse dieser Studie weisen darauf hin, dass USCα atmosphärisches CH_4 möglicherweise nur als zusätzliche Energiequelle oder Überlebensstrategie nutzt, und stattdessen andere Kohlenstoffverbindungen, z.B. Acetat, zum Wachstum verwendet. Somit repräsentiert USCα eher fakultative als obligate Methanotrophe. Die Anwendung von CARD-FISH, spezifisch für *pmoA* Transkripte, ermöglichte zudem die erste Visualisierung von USCα *in situ*. Diese Resultate erweitern unseren Wissensstand und das Verständnis in Bezug auf „upland soils“ als Senke für atmosphärisches Methan und die Mikroorganismen, die für diese Prozesse verantwortlich sind.

Autotrophe Bakterien wurden lange Zeit alleinverantwortlich für die Ammonium-Oxidation gehalten. Doch inzwischen liegen zunehmend Studien vor, die auf eine zusätzliche Beteiligung von *Archaea* an diesem Prozess hinweisen. Allerdings war bis heute unbekannt, ob Ammonium-oxidierende Archaeen im Boden CO_2 assimilieren können und zu welchem Grad sie funktionell aktiv sind. Stabile Isotopenbeprobung von Nukleinsäuren unter Verwendung von $^{13}CO_2$ demonstrierte eine aktive Beteiligung der Ammonium-oxidierenden Archaeen an der mikrobiellen Ammonium-Oxidation in einem Feldboden, verbunden mit autotropher CO_2-Fixierung, vermutlich über den Hydroxypropionat-Hydroxybutyrat-Zyklus.

CARD-FISH zeigte weiterhin den hohen Anteil und damit die große Bedeutung der archaeellen Ammonium-Oxidierer in der gesamten archaeellen Gemeinschaft in dieser Umgebung. Diese Resultate liefern neue Belege für die substanzielle Beteilung der nitrifizierenden *Archaea* an der Ammonium-Oxidation und CO_2-Fixierung in terrestrischen Habitaten.

Obwohl Wasserstoff als einer der wichtigsten Energieträger der Zukunft gilt, ist der globale biochemische Zyklus dieses Spurengases noch größtenteils unerforscht. Nach neueren Forschungsergebnissen scheinen eher Mikroorganismen als „freie“ Enzyme für die Aufnahme von atmosphärischem H_2 im Boden verantwortlich zu sein. Die CARD-FISH Analyse in dieser Arbeit belegte, dass die hoch-affine H_2 Aufnahme-Aktivität nicht im Myzelium, sondern in den Sporen von Streptomyzeten exprimiert wird. Dies zeigt die essentielle Bedeutung von H_2-oxidierenden Streptomyzeten, oder Aktinobakterien im Allgemeinen, für die Aufnahme von atmosphärischem H_2 in „upland soils“.

Summary

Atmospheric trace gases play a leading role in the changes occurring in the atmosphere at present, including climate change. A significant part of atmospheric trace gas fluxes occurs at the interface between atmosphere and upland soils. Unfortunately, the microorganisms in charge of these dynamics are not fully understood. This thesis therefore focuses on the investigation of microbial groups in terrestrial environments, responsible for or proposed to be involved in the uptake of atmospheric trace gases (CH_4, CO_2, H_2), namely the potential atmospheric methane oxidizer upland soil cluster α (USCα), the autotrophic ammonia oxidizing archaea (AOA), and the hydrogen oxidizing streptomycetes. Several methods were tested to investigate the incorporation of labeled substrate and to monitor the expression of their functional marker genes, *pmoA* for the high-affinity particulate methane monooxygenase of USCα, *amoA* for the ammonia monooxygenase of AOA, and *hydB* for the high-affinity [NiFe]-hydrogenase of *Streptomyces* sp. PCB7.

Although the upland soil cluster α (USCα) in forest soils is assumed to represent methanotrophic bacteria adapted to the trace level of atmospheric methane and to play an essential part in the removal of this greenhouse gas from the atmosphere, so far it is unclear whether these microorganisms are able to obtain all their energy and carbon solely from CH_4 or use additional carbon compounds. Stable isotope probing was applied to investigate incorporation of labeled CH_4 and acetate into nucleic acids of USCα. The results of this study indicate that USCα might only use atmospheric CH_4 as an additional energy source or survival strategy, but utilizes additional carbon compounds, such as acetate, for growth suggesting the USCα represents rather facultative than obligate methanotrophs. Furthermore, CARD-FISH of *pmoA* transcripts visualized USCα *in situ* for the first time. These findings promote the knowledge and understanding of upland soils as a sink for atmospheric methane and the microorganisms proposed to be responsible for this process.

While for a long time autotrophic bacteria were believed to be solely responsible for the process of ammonia oxidation, there is now increasing evidence that also *Archaea* are involved. But to date it remained elusive whether ammonia oxidizing archaea in soil can assimilate CO_2 and to what extent they are functionally active. Stable isotope probing of nucleic acids using $^{13}CO_2$ showed that ammonia oxidizing archaea were actively involved in microbial ammonia oxidation in an agricultural soil and did fix CO_2 autotrophically, presumably via the hydroxypropionate-hydroxybutyrate cycle. CARD-FISH further demonstrated the numerical importance of the archaeal ammonia oxidizers to the overall archaeal community in this environment. These results give novel evidence that the contribution of nitrifying *Archaea* to ammonia oxidation and CO_2 fixation in terrestrial environments might be substantial.

Although hydrogen is considered to be one of the most important future energy carriers, little is known about the global biogeochemical cycle of this trace gas. Previous findings indicate that microorganisms rather than free soil enzymes are responsible for the uptake of atmospheric H_2 in soils. In this thesis, CARD-FISH analyses demonstrated that

streptomyces spores instead of the mycelia expressed the high-affinity H_2 uptake activity. This suggests that H_2-oxidizing streptomycetes, or actinobacteria in general, are essential for the uptake of atmospheric H_2 in upland soils.

I. Introduction

I.1 General introduction

Dynamic processes shape the composition of Earth's atmosphere. A prominent example from the past is the dramatic change from an anoxic to an oxic atmosphere, caused by the evolution of oxygenic photosyntheses by cyanobacteria, thus shifting the dominance of anaerobic to aerobic life (Holland *et al.*, 1986). At present, the Earth's atmosphere mostly consists of nitrogen (78% by volume) and oxygen (21% by volume). The remaining 1% of the atmospheric gases are generally considered as trace gases because they present such small concentrations. In particular several of these atmospheric trace gases play a leading role in the changes occurring in the atmosphere at present, as their increasing concentrations accelerate global warming (Intergovernmental Panel on Climate Change, 2007). The increase of CO_2, CH_4, and N_2O since pre-industrial times furthermore initiates the destruction of the stratospheric ozone layer, the increase in the amount of tropospheric ozone, and changes in the density of clouds in the troposphere and of aerosol in the stratosphere due to changed emissions of dimethyl sulfate (DMS) and carbonyl sulfide (OCS), respectively (Conrad, 1996). Cycling of atmospheric trace gases mainly depends on biospheric processes. Therefore, a significant part of atmospheric trace gas fluxes occurs at the interface between atmosphere and upland soils. Upland soils are generally defined as non-water-saturated, well-aerated soils that are generally oxic, in contrast to wetland soils, which are water-saturated soils that are generally anoxic (Conrad, 1995). The upland soils generally provide a substantial sink for atmospheric trace gases, such as CH_4, CO_2, H_2, and CO (Conrad, 1996), and occasionally also for N_2O (Chapuis-Lardy *et al.*, 2007). Considering the threat of global climate change, the biospheric processes responsible for uptake of these trace gases in soils are hence of special interest. But so far, the microorganisms in charge of these dynamics and the respective pathways are neither completely identified nor fully understood.

This imposes a further challenge on the science of soil microbial ecology. However, the continuous development of advanced culture-independent molecular techniques supports the study of soil microbial communities. Biochemical markers, such as functional genes, ribosomal (rRNA) or messenger RNA (mRNA), can be analysed with respect to phylogeny and function, to gain more insight into the processes in the environment.

An opportunity to link phylogeny to function regarding specific microbial groups and communities is provided by stable isotope probing (SIP) of nucleic acids and PLFAs (figure I.1-1). This technique allows the specific identification of microorganisms assimilating labeled substances, most commonly carbon from a particular ^{13}C-labeled substrate (Manefield *et al.*, 2002; Dumont and Murrell, 2005; Neufeld *et al.*, 2007; Chen and Murrell, 2010).

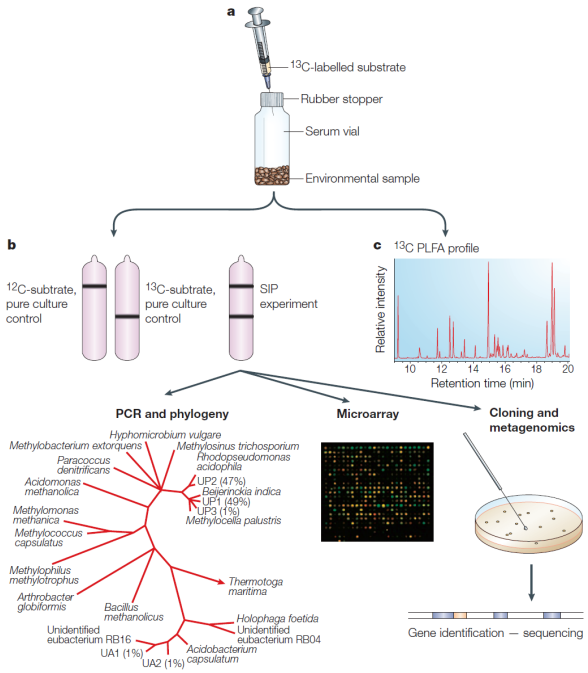


Figure I.1-1: DNA-based stable isotope probing (SIP) and ^{13}C-phospholipid fatty acids (PLFA) analyses. A labeled substrate is added to an environmental sample (a) and incorporated, which allows further analyses of DNA (b) or PLFA (c). Adopted from Dumont and Murrell (2005).

DNA and RNA are the most informative taxonomic biomarkers, and labeled molecules can be separated from unlabeled nucleic acids by density-gradient centrifugation. Once labeled DNA or RNA has been isolated, it can serve as a template in normal PCR or quantitative PCR (qPCR), using general primer sets that amplify rRNA genes of most known *Bacteria* and *Archaea*, or primer sets for specific functional genes. The analysis of the amplicons (e.g. by cloning, sequencing and subsequent phylogenetic analyses) allows the identification of microorganisms that have assimilated the labeled substrate.

A different methodological field to target and visualize functional genes, rRNA, or transcripts in environmental samples is displayed by fluorescence *in situ* hybridization (FISH) techniques. FISH is a cultivation-independent technique for the in situ identification of microorganisms, based on phylogenetic staining using labeled nucleotide probes to target rRNA, mRNA, or genes of microbial cells in fixed samples (Pernthaler, 2010). Fluorescence in situ hybridization (FISH) of bacteria was first described more than 20 years ago (Amann et al., 1990; DeLong et al., 1989) representing a significant progress in microbial ecology. However, since the fluorescence intensities of hybridized cells depend strongly on the concentration of the probe target, e.g. ribosomal RNA or mRNA, the hybridization intensities of cells in environmental samples are frequently below microscopic detection limits or lost in high background fluorescence. Therefore, an efficient way to improve the detection sensitivity is the use of horseradish peroxidase-labeled probes in combination with catalyzed reporter deposition (CARD) of fluorescently labeled tyramides (CARD-FISH) to detect rRNA or mRNA

in environmental samples (Pernthaler et al., 2002 and 2004). CARD–FISH combines CARD of fluorescently labeled tyramides with single-cell identification by FISH (figure I.1-2). The hybridization involves an oligo- or polynucleotice probe that is covalently crosslinked to one or more molecules which can be targeted with an antibody carrying a horseradish peroxidase (HRP) label or which are directly linked to HRP. Amplification of the signal relative to that achieved with probes that are labeled with a single fluorochrome is based on the radicalization of multiple tyramide molecules by a single horseradish peroxidase (Amann and Fuchs, 2008). Based on this enhanced sensitivity, CARD–FISH also enables the simultaneous detection of mRNA and rRNA in environmental bacteria, like the methanotrophs (Pernthaler *et al.*, 2004), thereby linking the identification of single cells to the expression of particular genes.

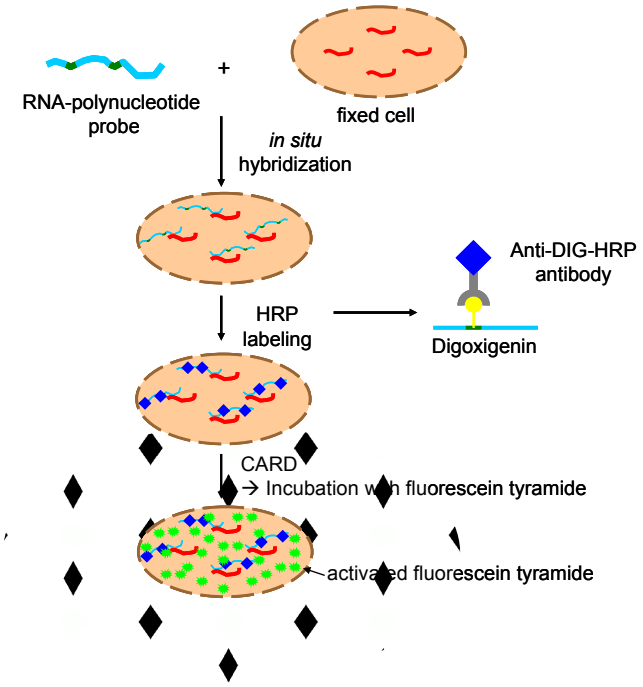


Figure I.1-2: Principle of catalyzed reporter deposition – fluorescence *in situ* hybridization (CARD-FISH).

A different approach to obtain broader insight into the phylogenetic affiliation of members of microbial communities in the environment is to use a special variant of fluorescence in situ hybridization called recognition of individual genes (RING)-FISH (Zwirglmaier *et al.*, 2004a) and to combine it with subsequent cell sorting (Zwirglmaier *et al.*, 2004b). RING-FISH involves using polyribonucleotide probes that are multiply labeled with several reporter molecules, and is characterized by typical halo-shaped fluorescence signals in the periphery of the cells (figure I.1-3). These halo-shaped signals are hypothesized to occur due to folding of the single-stranded RNA probe molecules into secondary structures (Zwirglmaier *et al.*, 2003), which results in the formation of a network of probes around the cells during whole-cell

hybridization. RING-FISH has previously been used to successfully detect genes of the nitrite reductase (*nirK*) in denitrifiers in pure culture and environmental samples (Pratscher *et al.*, 2009), and glycerol aldehyde 3-phosphate dehydrogenase (GAPDH) in *E. coli* (Zwirglmaier *et al.*, 2004a).

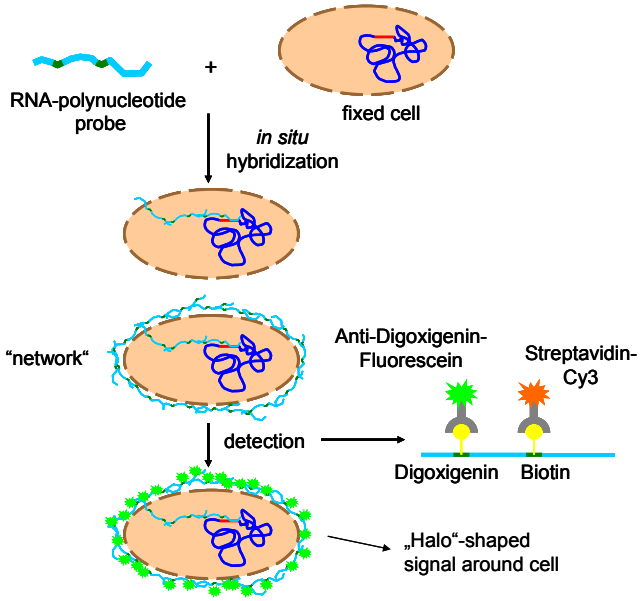


Figure I.1-3: Principle of recognition of individual genes – fluorescence *in situ* hybridization (CARD-FISH).

Most FISH methods can also be coupled to single-cell identification and quantification by either epifluorescence microscopy or flow cytometry, which also allows for fluorescence-activated cell sorting (FACS) (Amann and Fuchs, 2008) and further identification of cells.

These methods all open a wide range of possibilities to investigate the fundamentals underlying the uptake of atmospheric trace gases, such as CH_4, CO_2, and H_2, by microbial groups in upland soils.

I.2 Methane in the atmosphere and its uptake into upland soils

Methane (CH_4) is an important greenhouse gas with a global warming potential 20 times more effective than CO_2 and a current atmospheric concentration of 1.7 ppmv. About 40% of the heat trapped by anthropogenic greenhouse gases is due to gases other than carbon dioxide, primarily methane (Shine and Sturges, 2007). The mixing ratio in the atmosphere changed from a pre-industrial concentration of 715 ppb in 1750 to 1,774 ppb in 2005. Ice core studies indicated that consistently lower concentrations were present in the atmosphere over the last 650,000 years, varying between 400 ppb and 770 ppb (Spahni *et al.*, 2005). More than 70% of atmospheric methane originates from biogenic sources including natural wetlands, rice agriculture, livestock, landfills, termites and oceans. Natural wetlands represent the largest single source accounting for about 35% of total emissions. Non-biogenic sources include

burning and mining of fossil fuel, waste treatment, biomass burning, and geological sources such as geothermal or volcanic methane (Intergovernmental Panel on Climate Change, 2007). About 60% of the total emission can be attributed to anthropogenic activities. Most of the atmospheric CH_4 results from CH_4 production by methanogenic archaea as the final step in anaerobic degradation of organic matter (Conrad, 2009). These strictly anaerobic Euryarchaeota mainly use carbon dioxide and hydrogen or acetate as substrates for methane formation (Conrad, 1997; Thauer et al., 2008). However, only about 50% of the produced methane is finally emitted to the atmosphere, the remainder is oxidized by microbial groups in various environments.

Biological methane oxidation is performed by a diverse group, the methane oxidizing or methanotrophic bacteria. Aerobic methane oxidizing bacteria generally belong to 16 genera within the γ- (type I methanotrophs: 10 different genera) and α- (type II methanotrophs: genera *Methylocystis*, *Methylosinus*, *Methylocella*, *Methylocapsa*) proteobacteria. The filamentous bacteria *Crenothrix polyspora* and *Clonothrix fusca* were also found to be methanotrophic (Stoecker *et al.*, 2006; Vigliotta *et al.*, 2007), both belonging to the *Gammaproteobacteria* and closely related to the type I methanotrophs. Very recently, three obligate methanotrophs of the phylum *Verrucomicrobia* were discovered in hot and acidic environments (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008). The aerobic methanotrophs oxidize methane to carbon dioxide via the intermediates methanol, formaldehyde and formate. Type I methanotrophs assimilate C via the ribulose monophosphate pathway, while type II methanotrophs use the serine pathway (Trotsenko and Murrell, 2008). The key step in methane oxidation, the initial oxidation of CH_4 to methanol, is catalyzed via the methane monooxygenase enzyme which occurs as a particulate, membrane bound form (pMMO), and as a soluble, cytosolic form (sMMO) (Hanson and Hanson, 1996). The two enzymes are distinct and present the result of two evolutionary independent processes. The *pmoA* gene, which encodes the α-subunit of pMMO, has been generally used as a biomarker to investigate methanotrophic communities in various environments.

Besides the aerobic process, methane can also be oxidized anaerobically. Anaerobic methane oxidation is estimated to account for removing up to 90% of the methane produced in oceans by mobilization of gas hydrates, and thereby reducing the ocean contribution to 2% of the global methane sources. The microorganisms responsible for this oxidation are presumed to be three distinct clusters of methanotrophic archaea (ANME-1, ANME-2 and ANME-3), all related to *Methanosarcinales* and *Methanomicrobiales*, two orders of methanogens, found in cell aggregates together with sulfate reducing bacteria belonging to the δ-proteobacteria (Knittel and Boetius, 2009). But so far these microorganisms resisted cultivation.

Another pathway was recently discovered that couples anaerobic oxidation of methane with the reduction of nitrite to dinitrogen. The anaerobic, denitrifying bacterium ‘Candidatus *Methylomirabilis oxyfera*’ harbors the aerobic pathway for methane oxidation and

produces the O_2 required for the methane monooxygenase reaction by dismutating nitric oxide (Ettwig *et al.*, 2010).

Ammonia oxidizers are also able to convert methane to methanol by an enzyme homologous to the methane monooxygenase of methanotrophs, the ammonia monooxygenase (*amo*). It seems, however, that they cannot use this process for growth (Jones and Morita, 1983).

The major sink for atmospheric methane, accounting for >80% of the total, is the reaction with hydroxyl radicals in the troposphere. A small part also diffuses into the stratosphere. The most important biological sink for atmospheric methane is represented by upland soils (Intergovernmental Panel on Climate Change, 2007), with an uptake of approximately 30 Tg y^{-1} (Denman *et al.*, 2007). However, the identity of the microorganisms performing this uptake raises a question. Almost all of the cultured methane oxidizing bacteria can not utilize and grow on the low CH_4 concentrations in the atmosphere. Only several *Methylocystis* and *Methylosinus* species have been shown to utilize atmospheric CH_4. They contain two types of pMMO, the conventional enzyme pMMO1 and pMMO2, a second monooxygenase, which catalyses oxidation of CH_4 at atmospheric levels (Dunfield *et al.*, 2002; Baani and Liesack, 2008; Kravchenko *et al.*, 2010). These strains, however, are also not able to grow at atmospheric concentrations of methane and might require additional carbon sources (Baani and Liesack, 2008; Belova *et al.*, 2010; Dunfield *et al.*, 2010). Phylogenetic analyses of soils showing uptake of atmospheric methane revealed the presence of predominant, possible "high affinity" methanotrophic bacteria, which represent novel sequence lineages of *pmoA* and were therefore named upland soil cluster (USC) α and γ (Knief *et al.*, 2003; Ricke *et al.*, 2005; Kolb *et al*., 2005). Further studies showed that *pmoA* of USCα is most closely related to *Methylocapsa acidiphila* (Dedysh *et al.*, 2002; Ricke *et al.*, 2005) while USCγ exhibits next relation to the *Methylococcaceae* (Knief *et al.*, 2003). These findings all indicate the potential role of those upland soil clusters in the removal of methane from the atmosphere (Kolb *et al.*, 2009). But so far no successful isolation approach has been reported. Previous studies investigated the assimilation of CH_4 by USCα in soils showing uptake of atmospheric CH_4 by using stable isotope probing of PLFAs or DNA, and detected labeling of lipids characteristic for USCα but no incorporation of labeled carbon into DNA (Knief *et al.*, 2003; Chen *et al.*, 2008; Bengtson *et al.*, 2009; Menyailo *et al.*, 2010). Thus, unfortunately, only little is known about the molecular phylogeny and function of these microorganisms.

I.3 CO_2 fixation by ammonia oxidizing prokaryotes in terrestrial environments

CO_2 is a crucial greenhouse gas and represents the largest single contributor to radiative forcing, responsible for global warming. Human activity, primarily burning of fossil fuels and deforestation, has led to a steep increase of CO_2 in the atmosphere and shows to be responsible for more than 75% of the increase in atmospheric CO_2 concentration since pre-

industrial times. Thus, human activities are regarded as the single largest anthropogenic factor contributing to climate change (Denman *et al.*, 2007). On the other hand, the natural carbon sinks, such as photosynthesis, respiration, decay and sea surface gas exchange, process only a comparatively small net uptake of CO_2, approximately 3.3 GtC yr^{-1} over the last 15 years. Nevertheless, without these natural sinks atmospheric concentrations would have increased even more dramatically (Denman *et al.*, 2007). One of these sinks for atmospheric CO_2 is offered by the CO_2 fixation of autotrophic ammonia oxidizing microorganisms in upland soils.

Microbial ammonia oxidation, the first step in nitrification, is crucial for the global nitrogen cycle (see figure I.3-1). It is the only oxidative biological process linking reduced and oxidized pools of inorganic nitrogen in nature (Gruber and Galloway, 2008) and often shows to represent the rate-limiting step of nitrification in various environments, thus being critical to wastewater nitrogen removal and global N cycling (Kowalchuk and Stephen, 2001).

The first step of ammonia oxidation, the oxidation of ammonia to hydroxylamine, is catalyzed by the ammonia monooxygenase (AMO), the key enzyme of nitrification (Kowalchuk and Stephen, 2001). This enzyme is evolutionary related to the membrane bound methane monooxygenase pMMO (Holmes et al., 1995). The ammonia monooxygenase is not highly substrate specific and able to oxidize several apolar compounds such as carbon monoxide, phenol, and other hydrocarbons, but at much lower rates than ammonia (Hooper et al., 1997). It is furthermore able to oxidize methane to methanol, however, also at much lower rates than the methane monooxygenase (Bedard and Knowles, 1989).

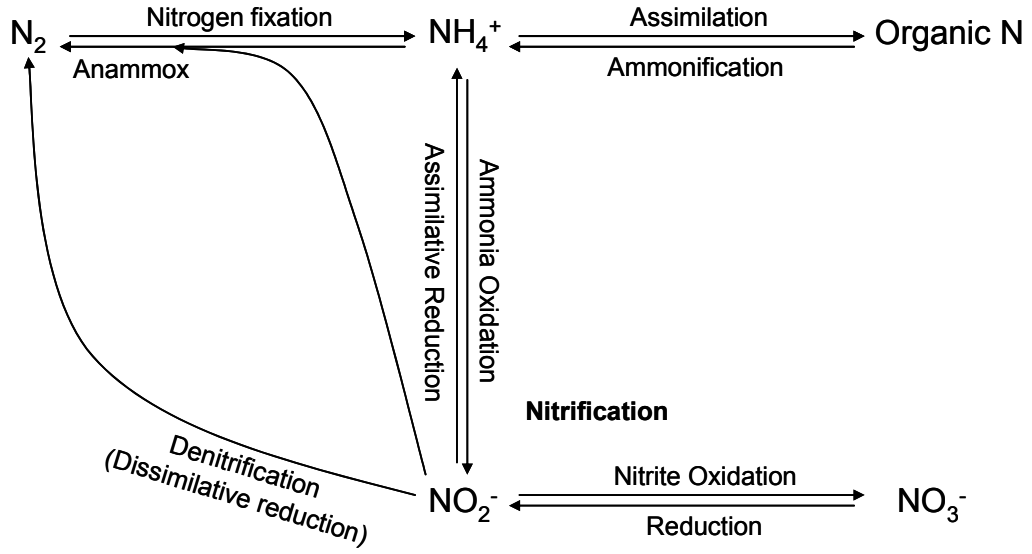


Figure I.3-1: The microbial nitrogen cycle. Modified after You *et al.* (2009).

For a long time, bacteria were believed to be solely responsible for this process and to exclusively possess the genes for the ammonia monooxygenase, but there is now increasing evidence that also *Archaea* are involved. *amoA* genes of *Archaea* encoding subunit A of ammonia monooxygenase have been found to occur in a wide variety of environments including marine systems, hot springs, and soils (Francis *et al.*, 2005; Leininger *et al.*, 2006; Wuchter *et al.*, 2006, Reigstadt *et al.*, 2008). Thus, the ammonia oxidizing prokaryotes can be divided into 2 groups: the ammonia oxidizing bacteria (AOB) and the ammonia oxidizing archaea (AOA).

The slow-growing, autotrophic ammonia oxidizing bacteria utilize ammonia oxidation as their sole source of energy, carbon dioxide as a carbon source using the Calvin Benson cycle, and molecular oxygen as an electron acceptor. The growth rates of AOB are thus directly linked to the availability of ammonium and the kinetics of its oxidation. The chemolithotrophic AOB commonly belong to three main genera: *Nitrosomonas* (*Betaproteobacteria*), *Nitrosospira* (*Betaproteobacteria*), and *Nitrosococcus* (*Gammaproteobacteria*) (Koops *et al.*, 2000). *Nitrosomonas/Nitrosospira* species seem to dominate natural and engineered systems, therefore AOB of the β-subclass Proteobacteria have been used as model organisms in microbial ecological studies (Kowalchuk and Stephen, 2001).

Ammonia can also be oxidized anaerobically. Anaerobic ammonium oxidation (anammox) bacteria, related to *Planctomycetales*, possess the unique metabolic ability to combine ammonium with nitrite (rather than O_2) as the electron acceptor, which results in N_2 production (Kuenen, 2008). Therefore, this process represents a denitrification pathway using ammonium as electron donor.

Molecular studies revealed that ammonia oxidizing archaea often outnumber the nitrifying *Bacteria* in most environments by orders of magnitude, especially in soils, where AOA showed to be 2- to 3000-fold more abundant than AOB (Leininger *et al.*, 2006; Wuchter *et al.*, 2006, Di *et al.*, 2009). These findings all demonstrate the potentially significant role of *Archaea* in the process of nitrification. New evidence also suggests the assignment of the ammonia oxidizing archaea to the new archaeal phylum *Thaumarchaeota* (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010). The first representative of the marine AOA was reported by Könneke *et al.* (2005), who isolated *Nitrosopumilus maritimus* from a marine aquarium. *Nitrosopumilus maritimus* belongs to the marine group I *Crenarchaeota*, grows autotrophically with ammonia as the sole energy source, and converts ammonia to nitrite with concomitant increase in cell number. Another representative of marine AOA, *Crenarchaeum symbiosum*, which was enriched from a hot spring, showed to belong to the group I.1b *Crenarchaeota*, mostly found in soil (Hatzenpichler *et al.*, 2008). Further isolates were all derived from mesophilic aquatic environments, such as *Nitrososphaera gargensis* (Hatzenpichler *et al*., 2008) and *Nitrosocaldus yellowstonii* (de la Torre *et al*., 2008). But so far, AOA in soils resisted isolation. Therefore, the actual contribution and importance of these microorganisms

to ammonia oxidation in soil remains elusive, since currently little is known about the exact ammonia oxidation pathways in archaea (Hallam *et al.*, 2006).

The AOA could also be of importance for the global carbon cycle. The ammonia oxidizing archaea isolated from aquatic environments were all shown to be autotrophs (Könneke *et al.*, 2005; de la Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008), like their bacterial counterparts, and analysis of ^{13}C-bicarbonate labeled lipid biomarkers of natural *Crenarchaeota* in the North Sea indicated an autotrophic metabolism (Wuchter *et al.*, 2003). However, it is still unclear whether this also applies to ammonia oxidizing archaea in soil and to what extent they are functionally active. So far, DNA-SIP analyses successfully showed autotrophy of ammonia oxidizing bacteria in sediments of a lake (Whitby *et al.*, 2001) and an estuary (Freitag *et al.*, 2006), but failed to detect CO_2 fixation of ammonia oxidizing archaea in agricultural soil (Jia and Conrad, 2009), although potential activity of these *Archaea* in soil was reported before (Schauss *et al.*, 2009). Furthermore, it remains elusive which pathway the AOA might use for fixation of CO_2. None of the chemolitoautotrophic archaea uses the Calvin cycle for CO_2 fixation (Berg et al., 2010). Instead, two new autotrophic carbon fixation cycles have been recently described in *Crenarchaeota*, the dicarboxylate-4-hydroxybutyrate cycle and the 3-hydroxypropionate-4- hydroxybutyrate cycle, and all *Crenarchaeota* studied so far use either the former or the latter cycle (Berg *et al.*, 2010). Thus, many questions concerning the physiological and functional traits of the ammonia oxidizing archaea still await to be answered.

I.4 Biological uptake of atmospheric H_2

Molecular hydrogen (H_2) is an indirect greenhouse gas present at trace level in the atmosphere (~530 ppbv). H_2 experiences a large and fast turnover in the troposphere, with an estimated total rate of 79 Tg yr^{-1} (Hauglustaine and Ehhalt, 2002). Sources of atmospheric H_2 are mainly methane and non methane hydrocarbon (NMHC) oxidation, industries and fossil fuels, biomass burning, nitrogen fixation by-products, and oceans (Constant *et al.*, 2009). Once in the troposphere, H_2 exhibits a lifetime of 1.4–2.0 years (Rhee *et al.*, 2006). Troposheric H_2 is taken up predominantly by upland soils, accounting for ~80% of the global loss of atmospheric H_2 (see table I.4-1). Although hydrogen is considered to be one of the most important future energy carriers (Turner *et al.*, 2004), little is known about the global biogeochemical cycle of this trace gas (Rhee *et al.* 2006). Also its impact on the atmosphere is discussed controversially, suggesting that a future H_2-based economy might change the oxidative capacity of the troposphere (Warwick *et al.*, 2004).

		NH fraction	Novelli et al. (1999)	This study		
			Global	Global	NH	SH
Sources	δD_i (‰)	χ_i				
Fossil fuel combustion	−270[a]	0.9	15±10	15±6	13	1.5
Biomass burning	−90	0.53	16±5	16±3	8.4	7.5
Photochemical production	190[b]	0.65	40±16	64±12	42	23
Biogenic N_2 fixation	−700[c]	0.6	3±1	6±5	3.6	2.4
Oceans	−700[c]	0.4	3±2	6±5	2.4	3.6
Total sources			77±16	107±15	69	38
Sinks	α_j	χ_j				
OH oxidation	0.58±0.07[d]	0.49	19±5	19±3	9.4	9.7
Soil uptake	0.943±0.007[e]	0.71	56±41	88±11	62	26
Total sinks			75±41	107±11	72	35
				δD_{QG}	δD_{QN}	δD_{QS}
Isotopic ratios of combined sources (‰)				−7.2	−2.6	−16
Lifetime (years)			2.1	1.4±0.2		

Table I.4-1: The global budget of atmospheric H_2 (Tg a^{-1}). NH and SH stand for the Northern and Southern Hemisphere, respectively. Adopted from Rhee *et al.* (2006).

In upland soils, H_2 oxidation activity is localized within the upper soil layers, showing a biphasic kinetic with two different affinities for H_2, a low- and a high-affinity activity (Schuler and Conrad, 1990; Häring and Conrad, 1994). The low-affinity activity is processed by a diverse group of aerobic H_2-oxdizing microorganisms, the Knallgas bacteria. However, these microorganisms are restricted to high concentrations of H_2 and are not able to consume atmospheric concentrations, due to their low affinity and high threshold for H_2 (Conrad *et al.*, 1983). Atmospheric uptake of H_2 in soils is catalyzed by a high-affinity activity, displaying a low $K_{m(app)}$ of 10-70 nM. Oxidation of atmospheric H_2 was long thought to be driven by abiontic high-affinity soil hydrogenases (Conrad, 1996). Recent studies, however, identified and isolated *Streptomyces* species showing a high-affinity H_2 uptake activity and possessing a [NiFe]-hydrogenase (Constant *et al.*, 2008). The gene encoding the large subunit of this enzyme (*hydB*-like gene sequence) can furthermore act as a functional marker gene. These observations indicate that microorganisms rather than free soil enzymes are responsible for the uptake of atmospheric hydrogen in soils.

I.6 Objectives of this study

Upland soils play an important role in the biological uptake and cycling of trace gases from the atmosphere. The physiological traits of many of the microbial groups involved in these processes are so far relatively uncharted, since most of them resisted isolation.

The aim of this study was to investigate microbial groups involved in the uptake of trace gases from the atmosphere in upland soils, namely the potential atmospheric methane oxidizer upland soil cluster α (USCα), the autotrophic ammonia oxidizing archaea (AOA), and the hydrogen oxidizing streptomycetes. Therefore, culture-independent molecular techniques were applied, such as stable isotope probing (SIP) of nucleic acids and CARD-FISH. The following questions were addressed:

Chapter III: Upland soil cluster α in Marburg forest soil

The upland soil cluster α (USCα), assumed to represent methanotrophic bacteria adapted to the trace level of atmospheric methane and to play an essential part in the removal of this greenhouse gas from the atmosphere, so far resisted isolation. Therefore questions regarding phylogenetic traits of these microorganisms still await to be answered.

- Does incorporation of CH_4 take place by USCα in the Marburg forest soil? (chapter III.1: RNA- and DNA-SIP using CH_4)
- Can USCα cells expressing *pmoA* be detected in this soil? (chapter III.1: mRNA CARD-FISH)
- Are USCα restricted to methane as sole carbon and energy source or also able to utilize other carbon compounds, such as acetate? (chapter III.1: RNA- and DNA-SIP using acetate)
- Can USCα cells be isolated from Marburg forest soil? (chapter III.2: RING-FISH coupled to cell sorting, and enrichment approaches)

Chapter IV: Ammonia oxidizing archaea and bacteria in an agricultural soil

Ammonia oxidation is an essential part of the global nitrogen cycling and was long thought to be driven only by *Bacteria* until recent findings expanded this pathway also to the *Archaea*. But most questions concerning the metabolism of ammonia oxidizing Archaea (AOA) yet remain open, especially for terrestrial environments.

- Are AOA in soil able to autotrophically fix CO_2, coupled to ammonia oxidation? Do they show the same response to different ammonia concentrations as the AOB? (RNA- and DNA-SIP using CO_2)
- Which pathway do AOA use to fix CO_2? (cloning of marker transcript/genes from gradient fractions)

- Can AOA cells expressing *amoA* be detected in the soil and what is their abundance?
  (mRNA CARD-FISH and qPCR)

Chapter V: H_2-oxidizing streptomycetes in soil

Previous findings suggest that actinobacteria could be responsible for atmospheric H_2 soil uptake. However, the ecological importance of H_2-oxidizing streptomycetes in soil awaits further investigation.

- Where is the H_2 uptake activity catalyzed within these streptomycetes? Where does expression of *hydB* take place in *Streptomyces* sp. PCB7?
  (mRNA CARD-FISH)

I.6 References

Amann RI, Krumholz L, Stahl DA (1990) Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol* 172: 762–770.

Amann R, Fuchs B (2008) Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. *Nature Rev* 6: 339-348.

Baani M, Liesack W (2008) Two isozymes of particulate methane monooxygenase with different methane oxidation kinetics are found in *Methylocystis* sp. strain SC2. *Proc Natl Acad Sci USA* 105: 10203–10208.

Bedard C, Knowles R (1989) Physiology, biochemistry, and specific inhibitors of Ch_4, Nh_4^+, and co oxidation by methanotrophs and nitrifiers. *Microbiol Rev* 53: 68-84.

Belova SE, Baani M, Suzina NE, Bodelier PLE, Liesack W, Dedysh SN (2010) Acetate utilization as a survival strategy of peat-inhabiting *Methylocystis* spp.. *Environ Microbiol Rep* (in press): doi:10.1111/j.1758-2229.2010.00180.x

Bengtson P, Basiliko N, Dumont MG, Hills M, Murrell JC, Roy R, Grayston SJ (2009) Links between methanotroph community composition and CH_4 oxidation in a pine forest soil. *FEMS Microbiol Ecol* 70: 356–366.

Berg IA, *et al.* (2010) Autotrophic carbon fixation in archaea. *Nat Rev Microbiol* 8: 447-460.

Brochier-Armanet C, *et al.* (2008) Mesophilic Crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. *Nat Rev Microbiol* 6: 245-252.

Chapuis-Lardy L, Wrage N, Metay A, Chotte J-L, Bernoux M (2007) Soils, a sink for N_2O? A review. *Glob Change Biol* 13: 1-17.

Chen Y, Dumont MG, McNamara NP, Chamberlain PM, Bodrossy L, Stralis-Pavese N, Murrell JC (2008) Diversity of the active methanotrophic community in acidic peatlands as assessed by mRNA and SIP-PLFA analyses. *Environ Microbiol* 10: 446–459.

Chen Y, Murrell JC (2010) When metagenomics meets stable-isotope probing: progress and perspectives. *Trends Microbiol* 18: 157-163.

Conrad R, Aragno M, Seiler W (1983) The inability of hydrogen bacteria to utilize atmospheric hydrogen is due to threshold and affinity for hydrogen. *FEMS Microbiol Lett* 18: 207–210.

Conrad R (1995) Soil microbial processes involved in production and consumption of atmospheric trace gases. *Adv Microb Ecol* 14: 207-250.

Conrad R (1996) Soil Microorganisms as Controllers of Atmospheric Trace Gases (H_2, CO, CH_4, OCS, N_2O, and NO). *Microbiol Rev* 60: 609-640.

Conrad R (1997) Production and consumption of methane in the terrestrial biosphere. Helas, G., Slanina, J., and Steinbrecher, R. (eds). Amsterdam: SBP Academic Publ., pp. 27-44.

Conrad R (2009) The global methane cycle: recent advances in understanding the microbial processes involved. *Environ Microbiol Rep* 1: 285–292.

Constant P, Poissant L, Villemur R (2008) Isolation of *Streptomyces* sp. PCB7, the first microorganism demonstrating high-affinity uptake of tropospheric H_2. *ISME J* 2: 1066–1076.

Constant P, Poissant L, Villemur R (2009) Tropospheric H_2 budget and the response of its soil uptake under the changing environment. *Sci Tot Environ* 407: 1809–1823.

de la Torre JR, Walker CB, Ingalls AE, Könneke M, Stahl DA (2008) Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. *Environ Microbiol* 10: 810–818.

DeLong EF, Wickham GS, Pace NR (1989) Phylogenetic stains: Ribosomal RNA-based probes for the identification of single cells. *Science* 243: 1360–1363.

Denman KL, Brasseur G, Chidthaisong A, Ciais P, Cox PM, Dickinson RE, *et al.* (2007) Couplings between changes in the climate system and biogeochemistry. In *Climate Change 2007: The Physical Science Basis*. Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, *et al*. (eds). Cambridge, UK: Cambridge University Press, pp. 499–587.

Di HJ, *et al.* (2009) Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nat Geosci* 2: 621-624.

Dumont MG, Murrell JC (2005) Stable isotope probing – linking microbial identity to function. *Nat Rev Microbiol* 3: 499-504.

Dunfield, P.F., Yimga, M.T., Dedysh, S.N., Berger, U., Liesack, W., and Heyer, J. (2002) Isolation of a *Methylocystis* strain containing a novel *pmoA*-like gene. *FEMS Microbiol Ecol* 41: 17–26.

Dunfield PF, Yuryev A, Senin P, Smirnova AV, Stott MB, Hou S *et al.* (2007) Methane oxidation by an extremely acidophilic bacterium of the phylum verrucomicrobia. *Nature* 450: 879-882.

Dunfield PF, Belova SE, Vorob'ev AV, Cornish SL, Dedysh SN (2010) *Methylocapsa aurea* sp. nov., a facultatively methanotrophic bacterium possessing a particulate methane monooxygenase. *Int J Syst Evol Microbiol* (in press): doi: 10.1099/ijs.0.020149-0.

Ettwig KF *et al.* (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* 464: 543-548.

Farquharson M, Harvie R, McNcol AM (1990) Detection of messenger RNA using a digoxigenin end-labeled oligonucleotide probe. *J Clin Pathol* 43: 423–428.

Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB (2005) Ubiquity and diversity of ammonia oxidizing archaea in water columns and sediments of the ocean. *Proc Natl Acad Sci USA* 102: 14683–14688.

Freitag TE, Chang L, Prosser JI (2006) Changes in the community structure and activity of betaproteobacterial ammonia-oxidizing sediment bacteria along a freshwater-marine gradient. *Environ Microbiol* 8: 684–696.

Gruber N, Galloway JN (2008) An Earth-system perspective of the global nitrogen cycle. *Nature* 451, 293–296.

Hallam SJ, *et al.* (2006) Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine *Crenarchaeota*. *PLoS Biol* 4: e95.

Hanson RS, Hanson TE (1996) Methanotrophic bacteria. *Microbiol Rev* 60: 439–471.

Häring V, Conrad R (1994) Demonstration of two different H2-oxidizing activities in soil using an H_2 consumption and a tritium exchange assay. *Biol Fertil Soils* 17: 125–128.

Hatzenpichler R, *et al.* (2008) A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proc Natl Acad Sci U S A* 105: 2134–2139.

Hauglustaine DA, Ehhalt DH (2002) A three-dimensional model of molecular hydrogen in the troposphere. *J Geophys Res* 107: 4330-4346.

Holland HD, Lazar B, McCaffrey M (1986) Evolution of the atmosphere and oceans. *Nature* 320: 27-33.

Holmes AJ, Costello A, Lidstrom ME, Murrell JC (1995) Evidence that particulate methane monooxygenase and ammonium monooxygenase may be evolutionarily related. *FEMS Microbiol Lett* 132: 203–208.

Hooper AB, Vannelli T, Bergmann DJ, Arciero DM (1997) Enzymology of the oxidation of ammonia to nitrite by bacteria. *Antonie van Leeuwenhoek* 71: 59-67.

Intergovernmental Panel on Climate Change (2007) Climate change 2007: The physical science basis. Summary for policymakers. Contribution of working group I to the fourth assessment report of the Intergovernmental Panel on Climate Change. Paris: Summary for policymakers formally approved at the 10th session of working group I of the IPCC.

Islam T, Jensen S, Reigstad LJ, Larsen O, Birkeland NK (2008) Methane oxidation at 55 degrees C and pH 2 by a thermoacidophilic bacterium belonging to the verrucomicrobia phylum. *Proc Natl Acad Sci USA* 105: 300-304.

Jia Z, Conrad R (2009) *Bacteria* rather than *Archaea* dominate microbial ammonia oxidation in an agricultural soil. *Environ Microbiol* 11: 1658-1671.

Jones RD, Morita RY (1983) Methane oxidation by nitrosococcus oceanus and nitrosomonas europaea. *Appl Environ Microbiol* 45: 401-410.

Knittel K, Boetius A (2009) Anaerobic oxidation of methane: progress with an unknown process. *Annu Rev Microbiol* 63: 311-334.

Kolb S, Knief C, Dunfield PF, Conrad R (2005) Abundance and activity of uncultured methanotrophic bacteria involved in the consumption of atmospheric methane in two forest soils. *Environ Microbiol* 7: 1150–1161.

Kolb S (2009) The quest for atmospheric methane oxidizers in forest soils. *Environ Microbiol Rep* 1: 336–346.

Könneke M, *et al.* (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437: 543–546.

Koops HP, Purkhold U, Pommerening-Röser A, Timmermann G, Wagner M (2000) The lithoautotrophic ammonia-oxidizing bacteria. In *The Prokaryotes*. Dworkin, M. (ed). New York: Springer

Kowalchuk GA, Stephen JR (2001) Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annu Rev Microbiol* 55: 485–529.

Knief C, Lipski A, Dunfield PF (2003) Diversity and activity of methanotrophic bacteria in different upland soils. *Appl Environ Microbiol* 69: 6703–6714.

Kravchenko IK, Kizilova AK, Bykova SA, Men'ko EV, Gal'chenko VF (2010) Molecular analysis of high-affinity methane-oxidizing enrichment cultures isolated from a forest biocenosis and agrocenoses. *Microbiology* 79: 106-114.

Kuenen JG (2008) Anammox bacteria: from discovery to application. *Nature* 6: 320-326.

Leininger S, *et al.* (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* 442: 806–809.

Manefield M, Whiteley AS, Griffiths RI, Bailey MJ (2002) RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Appl Environ Microbiol* 68: 5367–5373.

Menyailo OV, Abraham W-R, Conrad R (2010) Tree species affect atmospheric CH_4 oxidation without altering community composition of soil methanotrophs. *Soil Biol Biochem* 42: 101–107.

Neufeld J, Dumont M, Vohra J, Murrell J (2007) Methodological considerations for the use of stable isotope probing in microbial ecology. *Microb Ecol* 53: 435–442.

Pernthaler A, Pernthaler J, Amann R (2002) Fluorescence in situ hybridization and catalyzed reporter deposition (CARD) for the identification of marine bacteria. *Appl Environ Microbiol* 68: 3094–3101.

Pernthaler A, Amann R (2004) Simultaneous fluorescence in situ hybridization of mRNA and rRNA in environmental bacteria. *Appl Environ Microbiol* 70: 5426–5433.

Pernthaler A (2010) Identification of environmental microorganisms by fluorescence in situ hybridization. In: Handbook of Hydrocarbon and Lipid Microbiology. K. N. Timmis (ed.) DOI 10.1007/978-3-540-77587-4_322

Pratscher J, Stichternoth C, Fichtl K, Schleifer K-H, Braker G (2009) Application of recognition of individual genes-fluorescence in situ hybridization (RING-FISH) to detect nitrite reductase genes (*nirK*) of denitrifiers in pure cultures and environmental samples. *Appl Environ Microbiol* 75: 802-810.

Pol A, Heijmans K, Harhangi HR, Tedesco D, Jetten MS, Op den Camp HJ (2007) Methanotrophy below pH 1 by a new verrucomicrobia species. *Nature* 450: 874-878.

Reigstad LJ *et al.* (2008) Nitrification in terrestrial hot springs of Iceland and Kamchatka. *FEMS Microbiol Ecol* 64: 167–174.

Rhee TS, Brenninkmeijer CAM, Röckmann T (2006) The overwhelming role of soils in the global atmospheric hydrogen cycle. *Atmos Chem Phys* 6: 1611–1625.

Ricke P, Kube M, Nakagawa S, Erkel C, Reinhardt R, Liesack W (2005) First genome data from uncultured upland soil cluster alpha methanotrophs provide further evidence for a close phylogenetic relationship to *Methylocapsa acidiphila* B2 and for high-affinity methanotrophy involving particulate methane monooxygenase. *Appl Environ Microbiol* **71:** 7472–7482.

Schauss K, *et al*. (2009) Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils. *Environ Microbiol* 11: 446-456.

Schuler S, Conrad R (1990) Soils contain two different activities for oxidation of hydrogen. *FEMS Microbiol Lett* 73: 77–83.

Shine KP, Sturges WT (2007) CO_2 Is Not the Only Gas. *Science* 315: 1804-1805.

Spahni R, Chappellaz J, Stocker TF, Loulergue L, Hausammann G, Kawamura K. *et al.* (2005) Atmospheric methane and nitrous oxide of the late pleistocene from antarctic ice cores. *Science* 310: 1317-1321.

Spang A, *et al.* (2010) Distinct gene set in two different lineages of ammonia-oxidizing archaea supports the phylum Thaumarchaeota. *Trends Microbiol* doi:10.1016/j.tim.2010.06.003

Stoecker K, Bendinger B, Schoning B, Nielsen PH, Nielsen JL, Baranyi C *et al.* (2006) Cohn's crenothrix is a filamentous methane oxidizer with an unusual methane monooxygenase. *Proc Natl Acad Sci USA* 103: 2363-2367.

Thauer RK, Kaster AK, Seedorf H, Buckel W, Hedderich R (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat Rev Microbiol* 6: 579-591.

Trotsenko YA, Murrell JC (2008) Metabolic aspects of aerobic obligate methanotrophy. *Adv Appl Microbiol* 63: 183-229.

Turner JA (2004) Sustainable hydrogen production. *Science* 305: 972-974.

Vigliotta G, Nutricati E, Carata E, Tredici SM, De SM, Pontieri P *et al.* (2007) *Clonothrix fusca* Roze 1896, a filamentous, sheathed, methanotrophic gamma-proteobacterium. *Appl Environ Microbiol* 73: 3556-3565.

Warwick NJ, Bekki S, Nisbet EG, Pyle JA (2004) Impact of a hydrogen economy on the stratosphere and troposphere studied in a 2-D model. *Geophys Res Lett* 31: L05107.

Whitby CB, *et al.* (2001) ^{13}C incorporation into DNA as a means of identifying the active components of ammonia-oxidizer populations. *Lett Appl Microbiol* 32: 398–401.

Wuchter C, Schouten S, Boschker HTS, Sinninghe Damsté JS (2003) Bicarbonate uptake by marine Crenarchaeota. *FEMS Microbiol Lett* 219: 203–207.

Wuchter C, *et al.* (2006) Archaeal nitrification in the ocean. *Proc Natl Acad Sci USA* 103: 12317–12322.

Zwirglmaier K, Ludwig W, Schleifer K-H (2003) Improved fluorescence *in situ* hybridization of individual microbial cells using polynucleotide probes: The network hypothesis. *System Appl Microbiol* 26: 327-337.

Zwirglmaier K, Ludwig W, Schleifer K-H (2004a) Recognition of individual genes in a single bacterial cell by fluorescence *in situ* hybridization – RING-FISH. *Mol Microbiol* 51: 89-96.

Zwirglmaier K, Ludwig W, Schleifer K-H (2004b) Improved method for polynucleotide probe-based cell sorting, using DNA-coated microplates. *Appl Environ Microbiol* 70: 494-497.

II. Materials and methods

II.1 Chemicals and gases

All chemicals, unless otherwise noted, were purchased in p.A. quality from the following suppliers: Ambion (Darmstadt, Germany), Applied Biosystems (Darmstadt, Germany), GE Healthcare (Munich, Germany), Invitrogen (Darmstadt, Germany), Promega (Mannheim, Germany), Qiagen (Hilden, Germany), Roche (Grenzach-Wyhlen, Germany), Roth (Karlsruhe, Germany), Sigma Aldrich (Taufkirchen, Germany), and Thermo Fisher Scientific (Dreieich, Germany). The technical gases for GC were purchased from Air Liquide (Duesseldorf, Germany). The $^{13}CH_4$ (99 atom%) was purchased from Sigma Aldrich.

II.2 Cultures and media

The following pure cultures were used in this study: *Methylocapsa acidiphila* type strain (DSM 13967) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Methylocystis* sp. strain SC2 was provided by the group of Prof. Dr. Peter Frenzel at the MPI Marburg. *Streptomyces* sp. PCB7 and DNA of *Paracoccus denitrificans* were provided by Dr. Philippe Constant, also MPI Marburg.

Methylocapsa acidiphila was grown in 20 ml DSMZ medium 922 (Table II.2-1) (Dedysh *et al.*, 2002) in 120 ml serum bottles. 20% (v/v) methane was added to the gas phase and the culture was incubated on a rotary shaker (120 rpm) at 25°C in the dark.

Table II.2-1: Composition of medium 922 for growth of *Methylocapsa acidiphila*

KH_2PO_4	100.00	mg
$MgSO_4$ x 7 H_2O	50.00	mg
$CaCl_2$ x 2 H_2O	10.00	mg
Trace elements	1.00	ml
Distilled water	1000.00	ml
Trace elements:		
EDTA	5.00	g
$CuCl_2$ x 5 H_2O	0.10	g
$FeSO_4$ x 7 H_2O	2.00	g
$ZnSO_4$ x 7 H_2O	0.10	g
$NiCl_2$ x 6 H_2O	0.02	g
$CoCl_2$ x 6 H_2O	0.20	g
Na_2MoO_4	0.03	g
Distilled water	1000.00	ml
Final pH 4.5 – 5.8		

Methylocystis sp. strain SC2 was grown in 20 ml NMS medium (Table II.2-2) (Dalton and Whittenbury, 1976) in 120 ml serum bottles. 20% (v/v) methane was added to the gas phase and the culture was incubated on a rotary shaker (120 rpm) at 25°C in the dark.

Table II.2-2: Composition of medium NMS for growth of *Methylocystis* sp. strain SC2

$MgSO_4$ x 7 H_2O	1.00	g
$CaCl_2$ x 2 H_2O	200.00	mg
Fe(III)NH_4-EDTA	4.00	mg
KNO_3	1.00	g
Trace element solution	0.50	ml
KH_2PO_4	272.00	mg
Na_2HPO_4 x 12 H_2O	717.00	mg
Distilled water	1000.00	ml
Trace element solution:		
Na_2-EDTA	500.00	mg
$FeSO_4$ x 7 H_2O	200.00	mg
$ZnSO_4$ x 7 H_2O	10.00	mg
$MnCl_2$ x 4 H_2O	3.00	mg
H_3BO_3	30.00	mg
$CoCl_2$ x 6 H_2O	20.00	mg
$CaCl_2$ x 2 H_2O	1.00	mg
$NiCl_2$ x 6 H_2O	2.00	mg
Na_2MoO_4	3.00	mg
Distilled water	1000.00	ml
Adjust pH to 6.8		

Streptomyces sp. PCB7 was grown on GYM medium agar plates (DSMZ medium 65) (Table II.2-3). Plates were incubated at 30°C in the dark.

Table II.2-3: Composition of medium GYM for growth of *Streptomyces sp. PCB7*

Glucose	4.00	g
Yeast extract	4.00	g
Malt extract	10.00	g
$CaCO_3$	2.00	g
Agar	12.00	g
Distilled water	1000.00	ml
Adjust pH to 7.2 before adding agar		

All media were sterilized by autoclaving for 20 min at 121°C and 1 bar overpressure.

II.3 Environmental samples and sampling procedures

II.3.1 Marburg forest soil (MF)

For the experiments targeting methanotrophs, upland soil was sampled in an acidic forest in Marburg, Germany (N 50° 48‘ 30.2‘‘ E 08° 48‘ 02.2‘‘). Before sampling, the humus layer of the soil was removed. Soil was sampled from the upper mineral horizon (A_h) using 10 cm long soil cores in October 2008 (for CH_4 labeling, see II.7.1.1.1, and enrichments, see II.11) and in August 2009 (for acetate labeling, see II.7.1.1.2). Afterwards, the soil was homogenized, sieved through 3 mm mesh and stored at 4°C until further use. A small amount was also kept at -80°C for molecular analyses.

II.3.2 Rauischholzhausen agricultural soil (RH)

For the stable isotope incubations targeting ammonia oxidizing microorganisms in terrestrial environments (see II.7.1.2), soil was sampled using 40 cm long soil cores in April 2009 from maize plots at the long-term experiment field site of the University of Giessen in Rauischholzhausen, Germany (N 50° 45' 39.60" E 8° 52' 19.37"). Maize, wheat, and barley are annually rotated at the field site. The soil was air-dried, sieved through 1 mm mesh, homogenized, and stored at 4°C until further use. A small amount was also kept at -80°C for molecular analyses.

II.4 Chemical analyses

II.4.1 Determination of pH in soil samples

Soil (15 g) was mixed with 30 ml of distilled water and stirred until pH remained stable. A digital pH meter (Microprocessor pH meter 539, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) with an InLab Semi-Micro pH electrode (pH 0 to 12, Mettler Toledo, Gießen, Germany) was used to determine pH-values.

II.4.2 Gravimetric determination of soil moisture content

Soil samples (10 g) were weighed in aluminium tins and dried for 24 h or over night at 105°C until constant weight. After cooling the dry weight was determined by weighing the samples again. The moisture content in dry weight basis was calculated in percent from wet weight (Ww) and dry weight (Dw) using the following formula:

$$\theta = \frac{(Ww - Dw)}{Dw} x100$$

II.4.3 Determination of ammonium

An ammonium microassay was used to determine ammonium concentrations in soil samples and incubations (Murase *et al.*, 2006). Therefore, 0.15 g of soil or slurry was mixed with 1.5 ml of 2 N KCl, vortexed, and shaken horizontally for 2 h at 4°C. Soil was pelleted by centrifugation for 5 min at 4,000 xg at 4°C and supernatants were filtered (<0.2 µm, Whatman filter units, Whatman, Dassel, Germany). In each well of a 96-well microplate, 200 µl of filtrate and 50 µl of Rxn buffer (see table II.4-1) were added and incubated for 10 min at 63°C. After cooling down the plate for 2 min at room temperature, the fluorescent intensity was measured at wavelength 410 nm and emission wavelength 470 nm on a Tecan Safire microplate reader (Tecan, Mainz, Germany). A 10 mM NH_4Cl stock served as calibration standard.

Table II.4-1: Solutions for ammonium microassay

P-buffer:

1 M KH_2PO_4	13.61 g in 100 ml A.bidest
1 M K_2HPO_4	34.84 g in 200 ml A.bidest

Adjust pH to 6.8 and autoclave

Rxn buffer:

15 mM O-phthalaldehyde
50 mM 2-mercaptoethanol
in 500 mM purified P-buffer pH 6.8

1) Mix in a beaker:
   - 0.08 g O-phthalaldeyde ($C_8H_6O_2$)
   - 20 ml P-buffer pH 6.8
   - 20 ml A.bidest
2) sonicate to dissolve solution (15 min), cover beaker with Parafilm
3) Add 137 µl 2-mercaptoethanol (under fume hood)
4) Filter sterilize with 0.2 µm filter into transparent 50 ml Falcon tube
5) Prepare Rxn-buffer 1 day before and leave on the rack exposed to light

II.4.4 Analysis of nitrate and nitrite

Nitrate (NO_3^-) and nitrite (NO_2^-) in soil samples were analyzed using ion chromatography (IC). Soil samples (0.5 g) were mixed with 0.5 ml water, shaken for 1 h at 200 rpm and 4°C, and centrifuged for 10 min at maximum speed (14,000 rpm). Supernatants were filtered (REZIST

13/0.2 PTFE, Schleicher and Schuell, Dassel, Germany) and stored at -20°C until analysis. A solution of 1 mM nitrate and nitrite served as calibration standard.

Table II.4-2: Operating data for ion chromatograph

IC system	Solvent delivery system S1121, column oven S4260B, suppressor unit S4260A (all from Sykam, Fuerstenfeldbruck, Germany), sample injector S5200 (Schambeck SFD, Bad Honnef, Germany)
Column	6 cm stainless steel column, i.d. 4.6 mm
Eluant	5 mM Na_2CO_3, 1 ml l^{-1} modifier; flow rate: 1.5 ml min^{-1}
Detector	Conductivity detector S3111; UV detector Linear UVis 200, wavelength 220 nm
Oven temperature	70°C
Integration	Program Peak Simple (SRI-Instruments, Torrence, USA)
Detection limit	approx. 5 µM

II.4.5 Quantification of acetate

Quantification of acetate was performed on a high performance liquid chromatography (HPLC) system. Soil samples (0.5 g) were mixed with 0.5 ml water, shaken for 1 h at 200 rpm and 4°C, and centrifuged for 10 min at 4500 xg and 4°C. Supernatants were further purified through membrane filters (pore size 0,2 µm, PTFE membrane, Schleicher and Schuell GmbH, Dassel, Germany) and stored at -20°C until analysis. For HPLC analysis, 100 µl sample volume were injected and a 1 mM acetate standard was used for calibration.

Table II.4-3: Operating data for HPLC

HPLC system	Pump Spectra System P1000 (Thermo Finnigan, Jan Jose, CA, USA), column oven S4110 (Sykam, Gilching, Germany)
Sample injector	Jasco 851-AS (Jasco, Japan)
Column	30 cm stainless steel column, i.d. 7.8 mm
Carrier material	Sulfurized divinylbenzol styrene (Aminex HPX-87-H, BioRad)
Eluant	1 mM sulfuric acid, flow rate: 0.3 ml min^{-1}
Detector	Refraction Index (RI) detector ERC-7512 (ERMA CR. INC., Tokyo, Japan), operating temperature: 40°C; UV detector: UVis 200 (LINEAR Instruments, Reno, USA), wavelength: 208 nm
Oven temperature	65°C
Integration	Program Peak Simple (SRI-Instruments, Torrence, USA)
Standard	1 mM acetate

II.4.6 Analysis of methane

CH_4 in the soil incubations was analyzed using a gas chromatograph SRI 8610C equipped with a flame ionization detector (GC-FID) (SRI Instruments, Torrance, CA, USA). Gas samples were taken directly before the analysis using a 0.5 ml pressure lock syringe (VICI, Baton Rouge, LA, USA) and sampling through the septum. The sample volume was 0.2-0.5 ml. Defined CH_4 concentrations (2 ppmv, 5 ppmv, and 100 ppmv) in nitrogen served as calibration gases.

Table II.4-4: Operating data for gas chromatograph

Column	1.8 m stainless steel column, i.d. 7.8 mm, carrier material: Poropack Q 80/100 mesh
Carrier gas	Helium 5.0 (20 ml min^{-1})
Fuel gas	Hydrogen 5.0 (25 ml min^{-1}), synthetic air 5.0 (25 ml min^{-1}) (20.5% oxygen 5.0, 79.5% nitrogen 5.0)
Temperature	Oven: 100°C; detector: 140°C
Integration	Program Peak Simple (SRI-Instruments, Torrence, USA)
Detection limit	0.2 ppmv methane

II.4.7 Calculation of the atmospheric methane oxidation rate (V_{atm})

To investigate the ability of the Marburg forest soil (MF) to oxidize atmospheric methane, 10 g of sieved soil were incubated at 25°C in 120 ml serum bottles closed with butyl rubber septa. Triplicates were incubated under atmospheric CH_4 and the consumption of methane was followed over a period of 2 days depending on the activity of the sample and included 5-6 measurement points. Incubation under atmospheric CH_4 mixing ratios resulted in an exponential decrease in CH_4, from which the specific affinity a^0_s (first-order uptake rate constant) was calculated by using the least-squares iterative fitting procedure of Origin 6.1 (Microcal Software, Inc., Northampton, Maine).

$$y = y_0 + A \times e^{-R_0 \times x}$$

This equation shows the decrease in methane concentration (y) against time (x). While y_0 is the threshold until which methane is oxidized, A is the initial methane concentration. The first-order uptake rate constant R_0 equals the specific activity (a^0_s). The atmospheric methane oxidation rate (V_{atm}) was calculated by multiplying the specific activity with the atmospheric methane concentration.

II.4.8 Determination of $^{13}CO_2$

Analysis of $^{13}CO_2/^{12}CO_2$ in gas samples of the incubations with $^{13}CO_2$ for stable isotope probing of ammonia oxidizers (see II.7.1.2) was performed using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C–IRMS) system that was purchased from Finnigan (Thermo Fisher Scientific, Bremen, Germany). The principle operation was described by Brand (1996). The isotope reference gas was CO_2 (99.998% purity; Air Liquide, Duesseldorf, Germany), calibrated with the working standard methylstearate (Merck). The latter was intercalibrated at the Max Planck Institute for Biogeochemistry, Jena, Germany (courtesy of Dr. W.A. Brand) against NBS 22 and USGS 24.

Table II.4-5: Operating data for GC-C-IRMS

GC	Hewlett Packard 6890 (Waldbronn, Germany)
Injector	Split ratio 1:10; operating temperature: 150°C
Column	27.5 m Pora PLOT Q, i.d. 0.32 mm, 10 µm film thickness (Chrompack, Frankfurt, Germany)
Carrier gas	Helium 5.0; flow rate: 2.6 ml min^{-1}
GC/C-Interface	Standard GC Combustion Interface III (Thermo Electron, Bremen, Germany), oxidation reactor at 940°C, reduction reactor at 650°C
Detector	IRMS: Finnigan MAT delta plus (Thermo Electron)
Oven temperature	30°C
Integration	ISODAT™ NT 2.0 (Thermo Electron)

II.5 Nucleic acid extraction

II.5.1 Marburg forest soil

Nucleic acid extraction from Marburg forest soil was performed using a hexadecyltrimethylammonium bromide (CTAB) -based protocol (Griffith *et al.*, 2000). Soil (0.5 g) was sampled in 2 ml screw-cap tubes and either frozen immediately in liquid nitrogen and stored at -80°C until further processing or used directly for extraction. Extractions were performed by the addition of 0.5 ml of CTAB extraction buffer (see table II.5-1), 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0) and 200 µl of zirconia-silica beads (0.1 mm; Roth, Karlsruhe, Germany). Samples were vortexed, lysed in a FastPrep beat beating system for 30 s at 5.5 m s^{-1}, and the aqueous phase containing nucleic acids was separated by centrifugation (16,000 x g) for 5 min at 4°C. The aqueous phase was then extracted again with an equal volume (750 µl) of phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0), followed by centrifugation (16,000 x g) for 5 min at 4°C. The aqueous phase was transferred to a new tube and phenol was removed by mixing with an equal volume (600 µl) of chloroform-isoamyl alcohol (24:1) followed by repeated centrifugation (16,000 x g) for 5 min at

4°C. Total nucleic acids were subsequently precipitated from the extracted aqueous layer with 2 volumes of 30% (w/v) polyethylene glycol 6000 (Roth, Karlsruhe, Germany) – 1.6 M NaCl (see table II.5-1) for 2 h at room temperature, followed by centrifugation (18,000 x g) for 10 min at 4°C. Pelleted nucleic acids were then washed in 700 µl ice cold 70% (v/v) ethanol, centrifuged again, and air dried briefly prior to resuspension in 50-100 µl of nuclease-free water (Ambion, Darmstadt, Germany).

In case of the incubations with acetate, the samples showed a high release of fulvic and humic acids and had to be further purified using Illustra MicroSpin S-400 HR spin columns (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions (Wang *et al.*, 2009). Therefore, the resin in the column was resuspended by vortexing, the cap was loosened one quarter turn and the bottom closure was twisted of. The column was then centrifuged in a supplied collection tube for 1 min at 735 x g. The collection tube was discarded and replaced by a fresh nuclease-free 1.5 ml microcentrifuge tube, 50 µl of total nucleic acid extract were pipetted carefully on top of the resin and samples were centrifuged for 2 min at 735 x g. The column was discarded and purified extracts were kept at -80°C until further processing. Integrity of nucleic acids was checked on agarose gels (see II.6.6) and concentration was determined using a NanoDrop instrument (Thermo Fisher Scientific, Schwerte, Germany).

Table II.5-1: Solutions for CTAB-extraction

6% CTAB extraction buffer:	
1. 0.7 M NaCl	in A.bidest
2. 12% CTAB	3 g CTAB in 25 ml 0.7 M NaCl
3. 1 M K_2HPO_4	8.71 g 1M K_2HPO_4 (174.18 g mol^{-1}) in 50 ml A.bidest
4. 1 M KH_2PO_4	6.81 g 1M KH_2PO_4 (136.09 g mol^{-1}) in 50 ml A.bidest
5. 240 mM potassium phosphate buffer, pH 8.0	11.28 ml 1 M K_2HPO_4 + 72 µl 1 M KH_2PO_4 + 38 ml A.bidest
6. Extraction buffer	25 ml 12% CTAB – 0.7 M NaCl + 25 ml 240 mM potassium phosphate buffer pH 8.0
→ autoclave and filter sterilize	
30% PEG – 1.6 M NaCl:	
PEG 6000	30 g
NaCl	14.6 g
Distilled water	ad 100 ml
→ autoclave	

II.5.2 Rauischholzhausen agricultural soil (SDS)

Nucleic acid extraction from Rauischholzhausen agricultural soil was performed using a sodium dodecyl sulfate (SDS) -based protocol (Bürgmann *et al.*, 2003). Soil (0.5 g) was sampled in 2 ml screw-cap tubes and either frozen immediately in liquid nitrogen and stored at -80°C until further processing or used directly for extraction. Extractions were performed by the addition of 1 ml SDS extraction buffer (see table II.5-2) and 200 µl of zirconia-silica beads (0.1 mm; Roth, Karlsruhe, Germany). Samples were vortexed, lysed in a FastPrep beat beating system for 45 s at 6 m s^{-1}, and the aqueous phase containing nucleic acids were separated by centrifugation (18,000 x g) for 5 min at 4°C. The aqueous phase was then extracted twice with 850 µl of phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0), followed by centrifugation (18,000 x g) for 5 min at 4°C. The aqueous phase was transferred to a new tube and phenol was removed by mixing with 800 µl of chloroform-isoamyl alcohol (24:1) followed by repeated centrifugation (18,000 x g) for 5 min at 4°C. Total nucleic acids were subsequently precipitated from the extracted aqueous layer with 1 ml of 20% (w/v) polyethylene glycol 6000 (Roth, Karlsruhe, Germany) – 1.6 M NaCl precipitation solution (see table II.5-2) for 1 h at room temperature, followed by centrifugation (18,000 x g) for 30 min at 20°C. Pelleted nucleic acids were then washed in 800 µl ice cold 75% (v/v) ethanol, centrifuged again (18,000 x g) for 10 min at 4°C, and air dried briefly prior to resuspension in 50-100 µl of nuclease-free water (Ambion, Darmstadt, Germany). Extracts were stored at -80°C until further processing. Integrity of nucleic acids was checked on agarose gels (see II.6.6) and concentration was determined using a NanoDrop instrument (Thermo Fisher Scientific, Schwerte, Germany).

Table II.5-2: Solutions for SDS-extraction

SDS extraction buffer:	
SDS	2.5 g
1 M sodium phosphate (pH 8.0)	20 ml
5 M NaCl	2 ml
0.5 M EDTA (pH 8.0)	10 ml
Distilled water	ad 100 ml
→ filter through sterile filters	
Precipitation solution:	
PEG 6000	20 g
NaCl	14.6 g
Distilled water	ad 100 ml
→ autoclave	

II.5.3 Pure cultures

For nucleic acid extraction from pure cultures, a simple phenol-chloroform-isoamyl alcohol based protocol was used. In a 2 ml screw-cap tube, 2 ml of culture suspension were pelleted for 15 min at 10,000 x g and the pellet was resuspended in 500 µl of nuclease-free water. For extraction, the cell suspension was mixed with 200 µl of zirconia-silica beads (0.1 mm; Roth, Karlsruhe, Germany), and the samples were lysed twice in a FastPrep beat beating system for 45 s at 6.5 m s^{-1}. Between the two beat beating steps, the samples were cooled on ice for 2 min. The aqueous phase containing nucleic acids were separated by centrifugation (18,000 x g) for 15 min at 4°C. The aqueous phase was then extracted twice with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0), followed by centrifugation (18,000 x g) for 5 min at 4°C. The aqueous phase was transferred to a new tube and phenol was removed with 1 volume of chloroform-isoamyl alcohol (24:1) followed by repeated centrifugation (18,000 x g) for 5 min at 4°C. Total nucleic acids were subsequently precipitated from the extracted aqueous layer with 0.1 volume of sodium acetate (3 M, pH 5.2) and 3 volumes of ice cold 96% (v/v) ethanol for 1 h at -80°C, followed by centrifugation (18,000 x g) for 30 min at 4°C. Pelleted nucleic acids were then washed in 1 ml ice cold 70% (v/v) ethanol, centrifuged again (18,000 x g) for 10 min at 4°C, and air dried briefly prior to resuspension in 100 µl of nuclease-free water (Ambion, Darmstadt, Germany). Extracts were stored at -80°C until further processing. Integrity of nucleic acids was checked on agarose gels (see II.6.6) and concentration was determined using a NanoDrop instrument (Thermo Fisher Scientific, Schwerte, Germany).

II.5.4 Purification of RNA

For stable isotope probing of RNA (see II.7.2), 50 µl of nucleic acid extract were treated with RNase-free DNase I (Qiagen, Hilden, Germany) for digestion of DNA. To 50 µl of extract, 37.5 µl of nuclease-free water, 10 µl of buffer RDD, and 2.5 µl of RNase-free DNase I were added and gently mixed. Tubes were incubated for 10 min at room temperature. RNA was then purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified RNA was stored at -80°C until further usage. Integrity of RNA was checked on agarose gels (see II.6.6) and concentration was determined using a NanoDrop instrument (Thermo Fisher Scientific, Schwerte, Germany).

II.6 Amplification of DNA and cDNA by PCR

DNA and cDNA fragments were amplified using PCR (polymerase chain reaction). The used primers are listed in Table II.6-1. For every amplification a negative control was performed by adding the appropriate amount of water instead of DNA template. DNA containing the target molecule for amplification served as positive control.

Table II.6-1: Oligonucleotide primers used for PCR

Target gene	Primer	Sequence (5' → 3')	Reference
pmoA	A189f	GGNGACTGGGACTTCTGG	Holmes *et al.*, 1995
	A682r	GAASGCNGAGAAGAASGC	Holmes *et al.*, 1995
	mb661	CCGGMGCAACGTCYTTACC	Costello *et al.*, 1999
	Gam643r	ACGAAGCGGATGTACTCGGG	Kolb *et al.*, 2005
	Forest675r	CCYACSACATCCTTACCGAA	Kolb *et al.*, 2003
	Forest 675r-T3	ATAGGTATTAACCCTCACTAAAGGGGC CYACSACATCCTTACCGAA	This study[a]
amoA archaea	amo111f	TTYTAYACHGAYTGGGCHTGGACATC	Treusch *et al.*, 2005
	amo643r	TCCCACTTWGACCARGCGGCCATCCA	Treusch *et al.*, 2005
	amo643r-T3	ATAGGTATTAACCCTCACTAAAGGGG TCCCACTTWGACCARGCGGCCATCCA	This study[a]
amoA bacteria	amoA-1F	GGGGTTTCTACTGGTGGT	Avrahami *et al.*, 2003
	amoA-2R	CCCCTCGGGAAAGCCTTCTTC	Avrahami *et al.*, 2003
accA general	PcB_388F	GGBGGBGCMMGWATWCARGARGG	Yakimov *et al.*, 2009
	PcB_1271R	GGCCAHGCRTARTTNAYRTC	Yakimov *et al.*, 2009
accA	Crena_529F	GCWATGACWGAYTTTGTYRTAATG	Yakimov *et al.*, 2009
Marine group I	Crena_981R	TGGWTKRYTTGCAAYTATWCC	Yakimov *et al.*, 2009
hydB	NiFe-1129f	CCGCGGTGGTTCGACGGCAA	Constant *et al.*, 2010
	NiFe-1640r	TGCACGGCGTCCTCGTACGG	Vignais *et al.*, 2001
	NiFe-1640r-T3	ATAGGTATTAACCCTCACTAAAGGGG TGCACGGCGTCCTCGTACGG	This study[a]
16S rRNA	A109f	ACKGCTCAGTAACACGT	Grosskopf *et al.*, 1998
archaea	A934b	GTGCTCCCCCGCCAATTCCT	Grosskopf *et al.*, 1998
16S rRNA	Eub8-27F	AGAGTTTGATCMTGGCTCAG	Amman *et al.*, 1995
bacteria	Eub1392-1407R	ACGGGCGGTGTGTACA	Amman *et al.*, 1995
Vector	T7f	TAATACGACTCACTATAGGG	Promega
pGEM-T easy	M13r	CAGGAAACAGCTATGAC	Promega
Vector	pBAD forward	ATGCCATAGCATTTTTATCC	Invitrogen
pBAD-TOPO	pBAD reverse	GATTTAATCTGTATCAGG	pBAD TOPO® TA Expression Kit

[a] For generation of RNA polynucleotide probes for RING-FISH and CARD-FISH (see II.10.1)

'Wobble' positions: K = G/T; H = A/C/T; M = A/C ; N = A/C/G/T; R = A/G; S = G/C, W = A/T, Y = C/T

PCR reactions were performed in thermocyclers from Perkin Elmer (Tokyo, Japan) and Applied Biosystems (Darmstadt, Germany). The PCR conditions are listed in Table II.6-2. PCR for functional genes was performed using the Red Accu Taq® Polymerase (Sigma Aldrich, Taufkirchen, Germany). PCR targeting 16S rRNA genes and vectors was performed using Recombinant Taq DNA Polymerase (Invitrogen, Darmstadt, Germany).

Table II.6-2: PCR reaction mixtures

For functional genes:

Component	Stock conc.	Final conc.	Volume
Forward primer	10 pmol/µl	0.2 pmol/µl	0.5 µl
Reverse primer	10 pmol/µl	0.2 pmol/µl	0.5 µl
dNTPs	10 pmol/µl	0.2 pmol/µl	0.5 µl
BSA	4 µg/µl	0.4 µg/µl	2.5 µl
10x Red Accu Taq® buffer	10x	1x	2.5 µl
Red Accu Taq® Polymerase	1 U/µl	0.05 U/µl	1.25 µl
DNA or cDNA-Template	25 ng/µl	1 ng/µl	1 µl
A. bidest			ad 25 µl

For 16S rRNA genes and transcripts, and vectors:

Component	Stock conc.	Final conc.	Volume
Forward primer	10 pmol/µl	0.2 pmol/µl	1 µl
Reverse primer	10 pmol/µl	0.2 pmol/µl	1 µl
dNTPs	10 pmol/µl	0.2 pmol/µl	1 µl
$MgCl_2$	50 mM	1.5 mM	3 µl
10x Invitrogen Taq buffer	10x	1x	5 µl
Invitrogen Taq Polymerase	5 U/µl	0.05 U/µl	0.5 µl
DNA or cDNA-Template	25 ng/µl	0.5 ng/µl	1 µl
A. bidest			ad 50 µl

II.6.1 Amplification of *pmoA*

For amplification of *pmoA* from pure cultures, soil samples, DNA and cDNA, the following conditions were used. PCR temperature profiles consisted of an initial denaturation for 3-5 min at 94°C, a specific amplification step (see table II.6-3), and a final elongation for 7 min at 72°C, before samples were cooled down to 4°C.

Table II.6-3: Temperature profiles for PCR of *pmoA*

Target gene	Primer pair	Temperature program	Cycles
pmoA general	A189f/A682r	94°C, 60 s	35
	A189f/mb661	62-55°C[a], 45 s	
		72°C, 60 s	
pmoA USCα	A189f/Forest675r	94°C, 60 s	40
	A189f/Forest 675r-T3	73.8 – 66.8°C[a], 45 s	
pmoA USCγ	A189f/Gam643r	72°C, 60 s	

[a] Touchdown PCR: Annealing temperature was decreased by 0.5°C in every cycle until final temperature was reached.

II.6.2 Amplification of *amoA*

For amplification of *amoA* from pure cultures, soil samples, DNA and cDNA, the following conditions were used. PCR temperature profiles consisted of an initial denaturation for 4-5 min at 94°C, a specific amplification step (see table II.6-4), and a final elongation for 10 min at 72°C, before samples were cooled down to 4°C.

Table II.6-4: Temperature profiles for PCR of *amoA*

Target gene	Primer pair	Temperature program	Cycles
amoA archaea	amo111f/amo643r	94°C, 45 s	40
	amo111f/amo643r-T3	57°C, 60 s	
		72°C, 60 s	
amoA bacteria	amoA-1F/amoA-2R	94°C, 45 s	30
		57°C, 30 s	
		72°C, 60 s	

II.6.3 Amplification of *accA*

To amplify and detect *accA* transcripts in cDNA fractions from stable isotope probing of Rauischholzhausen agricultural soil (RH), labeled with $^{13}CO_2$ (see II.7.1.2), the following conditions were used. PCR temperature profiles consisted of an initial denaturation for 5 min at 94°C, a specific amplification step (see table II.6-5), and a final elongation for 10 min at 72°C, before samples were cooled down to 4°C.

Table II.6-5: Temperature profiles for PCR of *accA*

Target gene	Primer pair	Temperature program	Cycles
accA general	PcB_388f/PcB_1271R	94°C, 60 s	35 for DNA
Marine group I	Crena_529f/Crena_981R	53.5°C, 60 s	40 for cDNA
		72°C, 120 s	

II.6.4 Amplification of *hydB*

To generate a template for the RNA polynucleotide probe used for CARD-FISH of *hydB* mRNA, the following conditions were used. PCR temperature profiles consisted of an initial denaturation for 5 min at 94°C, a specific amplification step (see table II.6-6), and a final elongation for 10 min at 72°C, before samples were cooled down to 4°C.

Table II.6-6: Temperature profiles for PCR of *hydB*

Target gene	Primer pair	Temperature program	Cycles
hydB	NiFe-1129f/NiFe-1640r	94°C, 45 s	30
	NiFe-1129f/NiFe-1640r-T3	55°C, 45 s	
		72°C, 45 s	

II.6.5 Amplification of archaeal and bacterial 16S rRNA genes and transcripts

For amplification of archaeal and bacterial 16S rRNA genes and transcripts from pure cultures, soil samples, DNA and cDNA, the following conditions were used. PCR temperature profiles consisted of an initial denaturation for 5 min at 94°C, a specific amplification step (see table II.6-7), and a final elongation for 5 min at 72°C, before samples were cooled down to 4°C.

Table II.6-7: Temperature profiles for PCR of 16S rRNA genes and transcripts

Target gene	Primer pair	Temperature program	Cycles
16S archaea	A109f/A934b	94°C, 30 s	25
16S bacteria	Eub8-27F/Eub1392-1407R	55°C, 45 s	
		72°C, 60 s	

II.6.6 Gelelectrophoresis and purification

Gel electrophoresis was carried out as a visual control for a successful amplification. Therefore, 3-5 µl of PCR product or nucleic acid extraction was mixed with 3 µl of loading buffer (Gel loading solution, Sigma Aldrich), loaded onto a 1.5% (w/v) 1× TAE agarose gel (SeaKem LE, Biozym; in TAE-buffer), and separated for 35 min at 120V. A 1kb DNA Extension ladder (Invitrogen, Darmstadt, Germany) was used as size standard. Gels were stained in an ethidium bromide solution and documented using a Gel Jet Imager (Intas, Goettingen, Germany). If multiple bands appeared on a gel after amplification, the band matching the size of the desired amplicon was cut out of the gel. Afterwards, the Wizard SV Gel and PCR Clean-Up System (Promega, Mannheim, Germany) was used to purify the PCR product or gel fragments by following the preparation instructions of the manufacturer. The DNA was finally eluted in 30 µl of nuclease-free water and stored at -20°C.

II.6.7 Quantification

Purified PCR products, nucleic acid extractions and polynucleotide probes for FISH were quantified using a NanoDrop ND-1000 instrument (Thermo Fisher Scientific, Schwerte, Germany) and 1.5 µl of respective sample.

II.6.8 T-RFLP analysis

Enrichment incubations with acetate for USCα (see II.11.2) were analyzed with T-RFLP (terminal restriction fragment length polymorphism) to monitor potential changes in bacterial population. Amplification of bacterial 16S rRNA genes from DNA extracts was carried out with the primers Eub8F and EuB1392R (see table II.6-7), the former was carboxyfluoresceine (= FAM) labeled (5' end). The purified PCR products were digested over night at 37°C using 5 µl of PCR, 1.1 µl of incubation buffer Tango (Fermentas), and 0.3 µl of restriction enzyme *Msp*I (10 U μl^{-1}). The batch was filled up with sterile H_2O to a total volume of 10 µl. The restriction digestion was purified via the 'Sigma Spin™ Post Reaction Clean-Up Columns Kit' according to the preparation instructions. To prepare the samples for the T-RFLP analysis, 3 µl of the purified restriction digestions were mixed with 0.3 µl of an internal lane standard (MapMarker® 1000, 50 to 1000 bp, x-rhodamine labeled, BioVentures Inc., USA) and 11 µl HiDi™ formamide (Applied Biosystems, Weiterstadt, Germany) and denatured for 3 min at 95°C. The analysis of the digested PCR products was performed by separation using capillary electrophoresis with an automatic sequencer (3130 Genetic Analyzer, Applied Biosystems) for 30 min at 15 kV and 9 µA. The injection time per sample was 10 s. After capillary electrophoresis, the lengths of the fluorescently labeled T-RF's were identified by comparison to the internal standard using the GeneMapper software (version 4.0, Applied Biosystems). The areas of the measured peaks were used to determine the relative abundances of the terminal restriction fragments.

II.7 Stable isotope probing (SIP) of nucleic acids

Stable isotope probing (SIP) is a method used for labeling microorganisms in environmental samples or directly in field studies using substrate enriched with a stable isotope (e.g., ^{13}C). After consumption of the substrate, the cells that consumed the substrate become enriched in the isotope. Separation of labeled nucleic acids is performed by ultracentrifugation in either cesium trifluoroacetate (CsTFA) for RNA or cesium chloride (CsCl) for DNA. Labeled biomarkers, such as ribosomal RNA, mRNA, and functional genes can be analyzed with a range of molecular and analytical techniques, and used to identify and characterize the organisms that incorporated the substrate.

II.7.1 Incubation for SIP

The following incubations were performed for stable isotope probing of RNA and DNA.

II.7.1.1 Incubation of Marburg forest soil (MF) for SIP of Upland Soil Cluster α

II.7.1.1.1 Labeling with CH_4

Incubation for stable isotope probing of Upland Soil Cluster α (USCα) with CH_4 was performed in duplicates for each treatment. Marburg forest soil (5 g *d.w.s.*, see II.3.1) was amended with 1 ml H_2O and incubated at 25°C and darkness in 120 ml serum bottles capped with butyl stoppers. The methane concentration in the headspace was adjusted to and maintained at 20 ppmv, 200 ppmv, and 10% of CH_4 ($^{12}CH_4$ or $^{13}CH_4$), respectively. An additional incubation with 1000 ppmv $^{13}CO_2$ was set up as a control for secondary labeling. Consumption of CH_4 was monitored by GC (see II.4.6). Every week, 0.5 g of soil of each bottle were sampled, frozen immediately in liquid nitrogen and stored at -80°C until further processing. Nucleic acids were extracted from soil for SIP using a CTAB-based protocol (see II.5.1). RNA- and DNA-SIP were performed (see II.7.2 and II.7.3, respectively) and *pmoA* and 16S rRNA genes and transcript were quantified from gradient fractions using quantitative PCR (see II.8).

II.7.1.1.2 Labeling with acetate

Incubation for stable isotope probing of Upland Soil Cluster α (USCα) with acetate was performed in duplicates for each treatment. Marburg forest soil (20 g *d.w.s.*, see II.3.1) was amended with a final concentration of 10 mM acetate (^{12}C or ^{13}C), dissolved in 6 ml H_2O, 20 ml H_2O, or 40 ml of medium DSMZ 922 (designed for *Methylocapsa acidiphila*; Dedysh *et al.*, 2002), respectively. Fully labeled $^{13}C_2$ sodium acetate (99 atom%) was purchased from Campro Scientific (Berlin, Germany). Incubations were performed on a shaker (200 rpm) at 25°C and darkness in 250 ml Schott bottles capped loosely to allow incubations to stay oxic. An additional incubation with 5% $^{13}CO_2$ was set up as a control for secondary labeling. Acetate-free controls received an equal amount of distilled water. Every week, acetate treatments were renewed. For chemical analysis of pH (see II.4.1) and acetate, aliquots of the soil were removed from each treatment every week. Concentrations of acetate in the microcosms were analyzed by HPLC (see II.4.5). Every week, 0.5 g of soil of each bottle were sampled, frozen immediately in liquid nitrogen and stored at -80°C until further processing. Nucleic acids were extracted from soil for SIP using a CTAB-based protocol (see II.5.1). RNA- and DNA-SIP were performed (see II.7.2 and II.7.3, respectively) and *pmoA* and 16S rRNA genes and transcript were quantified from gradient fractions using quantitative PCR (see II.8).

II.7.1.2 Incubation of Rauischholzhausen agricultural soil (RH) with CO_2 for SIP of ammonia oxidizing microorganisms

Incubation for stable isotope probing of ammonia-oxidizing prokaryotes with 5% CO_2 was performed in triplicates for each treatment. Rauischholzhausen agricultural soil (10 g *d.w.s*, see II.3.2) was incubated at 60% maximum water holding capacity (WHC), 25°C and darkness in 120 ml serum bottles capped with butyl stoppers. Five percent (~ 6 ml) of CO_2 ($^{12}CO_2$ or $^{13}CO_2$) were added to the headspace and the soil was fertilized with either 100 µg or 15 µg $(NH_4)_2SO_4$-N g^{-1} *d.w.s.*, respectively, dissolved in distilled water. Nitrogen-free controls received an equal amount of distilled water. Every week, bottles were flushed with synthetic air (20% O_2, 80% N_2), 5% of CO_2 was added, and fertilization treatments were renewed. For chemical analysis of pH (see II.4.1), ammonium (II.4.3), nitrite, and nitrate (both see II.4.4), aliquots of the soil were removed from each treatment every week and analyzed. Additionally, CO_2 concentration in the headspace was measured by GC-IRMS (see II.4.8). Also every week, 0.5 g of soil of each bottle were sampled, frozen immediately in liquid nitrogen and stored at -80°C until further processing. Nucleic acids were extracted from soil for SIP using an SDS-based protocol (see II.5.2). RNA- and DNA-SIP were performed for 8- and 12-week incubations (see II.7.2 and II.7.3, respectively) and *amoA* and 16S rRNA genes and transcript were quantified from gradient fractions and untreated soil using quantitative PCR (see II.8). Complementary DNA (cDNA) of the heavy and light fractions of the $^{13}CO_2$ treatments with 15 µg $(NH_4)_2SO_4$-N g^{-1} *d.w.s.* was also used for PCR amplification (see II.6.) and phylogenetic analysis (see II.9.3) of acetyl-CoA carboxylase alpha subunit (*accA*) cDNA fragments and archaeal 16S rRNA transcripts. RNA- and DNA-SIP were performed (see II.7.2 and II.7.3, respectively) and *pmoA* and 16S rRNA genes and transcript were quantified from gradient fractions using quantitative PCR (see II.8).

II.7.2 RNA stable isotope probing

II.7.2.1 Solutions for RNA-SIP

The following solutions were prepared for stable isotope probing of RNA.

Table II.7-1: Solutions for RNA-SIP

Gradient Buffer (GB):	Final conc.	Volume
0.2 M EDTA	1 mM	0.5 ml
1 M Tris-HCl (pH 8.0)	0.1 M	10 ml
1 M KCl	0.1 M	10 ml
Distilled water		79.5 ml

→ Prepare with RNase-free reagents in nuclease-free water, filter sterilize (0.2 µm), and autoclave in baked glassware.

II.7.2.2 RNA-SIP protocol

For the preparation of one RNA gradient, 4.5 ml of CsTFA (~ 2 g ml^{-1}, GE Healthcare, Munich, Germany) was mixed with 1 ml of gradient buffer (see table II.7-1), and 3.59% of formamide was added. The refractive index of this solution was measured (50 µl aliquot) on a refractometer (Reichert, Depew, NY, USA) to control pre-centrifugation average density. Refractive index (nD-TC) of this solution should be 1.3725 ± 0.0002 (≈ 1.79 g ml^{-1} CsTFA). If necessary, nD-TC was adjusted by adding 100 µl aliquots of GB or CsTFA. The medium was transferred into 6 ml polyallomer UltraCrimp tubes (Thermo Scientific, Dreieich, Germany) and mixed with up to 500 ng of purified RNA (see II.5.4). Tubes were balanced for rotor to ± 0.01 g and sealed with UltraCrimp stoppers (Thermo Scientific, Dreieich, Germany). Ultracentrifugation was performed with a TV865 vertical rotor in a Sorvall Discovery 90 ultracentrifuge (Thermo Scientific, Dreieich, Germany) for ~65 h (over weekend) at 20°C and 39 krpm (130,000 x g). Deceleration was set to 0 to keep gradients intact. After centrifugation, tubes were carefully removed from the rotor and adjusted within the fractionation device. A sterile 0.4-mm needle was fit to the tubing of a syringe pump (Kent Scientific, Torrington, CT, USA), filled with nuclease-free water, and carefully poked into the centrifugation tube slightly above the centrifugation medium. With another sterile 0.4-mm needle, a hole was carefully poked into bottom of tube. The syringe pump was started at a flow rate of 0.45 ml min^{-1}, displacing the gradient medium with nuclease free water at the top of the tube, and 12 fractions (450 µl) in nuclease-free 2 ml cups were collected by manually shifting every 60 seconds. nD-TC of fractions was measured (50 µl from each fraction), starting with the lightest (= 12^{th}) fraction. Due to fractionation, the 12^{th} fraction may contain some water and the refractive index might be lower than expected. In this case, the true density of the last fraction was estimated from the decreasing densities of the other fractions. Densities were calculated from refractive indices by the following equation:

$$y = 384.4406x^2-1031.00836x+692.65494$$

RNA was precipitated from fractions with 0.1 volume (40 µl) sodium acetate (3 M, pH 5.2), 20 µg (1 µl) glycogen (from mussels, Sigma Aldrich), and 2 volumes (1102 µl) of cold 96% (v/v) ethanol for 1 h or over night at -20°C. Precipitated RNA was pelleted by centrifugation (18.000 x g) for 30 min at 4°C, washed with 150 µl of ice cold 70% (v/v) ethanol, and centrifuged again. Pellets were air-dried briefly and resuspended in 5-15 µl of nuclease free water.

II.7.2.3 Reverse transcription of RNA fractions

Before fractions could be analyzed by qPCR, RNA had to be reverse transcribed (RT) into cDNA. The following protocols were used for this purpose. cDNA was stored at -20°C.

Table II.7-2: Conditions for reverse transcription of RNA

a.) Protocol for RT of RNA fractions from Marburg forest soil (MF):

1. Reaction set-up (on ice)	1x
Water	2.4 µl
50 ng/µl random hexamer primer (Invitrogen, diluted 1:60)	5 µl
2 mM dNTP	5 µl
	12.4 µl/tube + 1 µl RNA
> 65°C 5 min	Remove tubes and place on ice

2. For reactions	1x
5x reaction buffer	4 µl
DTT	1 µl
Superscript III RT (all SuperScript® III reverse transcriptase kit, Invitrogen)	0.1 µl
SUPERase-In (20 U/µl, A) (Applied Biosystems)	1 µl
BSA (20 µg/µl, Roche)	0.5 µl
	6.6 µl/tube and mix

3. Continue thermocycler program:

> 25°C, 5 min
> 50°C, 60 min
> 70°C, 15 min
> heated lid off and hold at 8°C

b.) Protocol for RT of RNA fractions from Rauischholzhausen agricultural soil (RH):

1. Reaction set-up (on ice)	1x
Water	4 µl
Random hexamer primer (Roche, diluted 1:50)	1 µl
RNA (up to 2 µg)	5 µl
> 75°C, 5 min	Remove tubes and place on ice

2. For reactions	1x
dNTPs	1 µl
M-MLV 5x reaction buffer (Promega)	4 µl
RNAse-Inhibitor(40 U/µl, Roche)	0.4 µl
M-MLV reverse transcriptase (200 U/µl, Promega)	0.8 µl
Water	3.8 µl
	10 µl/tube and mix
> 37°C, 1 h	

II.7.3 DNA stable isotope probing

II.7.3.1 Solutions for DNA-SIP

The following solutions were prepared for stable isotope probing of DNA.

Table II.7-3: Solutions for RNA-SIP

Gradient Buffer (GB) → see table II.7-1

CsCl solution [1.85 g ml^{-1}]:

CsCl (Sigma Aldrich)	50 g
Gradient buffer (GB)	30 ml

→ Autoclave in baked glassware. nD-TC should be 1.4153

30% PEG – 1.6 M NaCl → see table II.5-1

II.7.3.2 DNA-SIP protocol

For the preparation of one DNA gradient, 4.86 ml of CsCl (~ 1.85 g ml^{-1}, see table II.7-3) was mixed with 1 ml of gradient buffer (see table II.7-1). The refractive index of this solution was measured (50 µl aliquot) on a refractometer (Reichert, Depew, NY, USA) to control pre-centrifugation average density. Refractive index (nD-TC) of this solution should be 1.4029 ± 0.0002 (≈ 1.72 g ml^{-1} CsCl). If necessary, nD-TC was adjusted by adding 100 µl aliquots of GB or CsTFA. The medium was transferred into 6 ml polyallomer UltraCrimp tubes (Thermo Scientific, Dreieich, Germany) and mixed with up to 5 µg of purified DNA (see II.5). Tubes were balanced for rotor to ± 0.01 g and sealed with UltraCrimp stoppers (Thermo Scientific, Dreieich, Germany). Ultracentrifugation was performed with a TV865 vertical rotor in a Sorvall Discovery 90 ultracentrifuge (Thermo Scientific, Dreieich, Germany) for ~36 h at 20°C and 45 krpm (177,000 x g). Deceleration was set to 0 to keep gradients intact. After centrifugation, tubes were carefully removed from rotor and adjusted within the fractionation device. A sterile 0.4-mm needle was fit to the tubing of a syringe pump (Kent Scientific, Torrington, CT, USA), filled with nuclease-free water, and carefully poked into the centrifugation tube slightly above the centrifugation medium. With another sterile 0.4-mm needle, a hole was carefully poked into bottom of tube. The syringe pump was started at a flow rate of 0.45 ml min^{-1}, displacing the gradient medium with nuclease free water at the top of the tube, and 12 fractions (450 µl) in nuclease-free 2 ml cups were collected by manually shifting every 60 seconds. Refractive index (nD-TC) of fractions was measured (50 µl from each fraction), starting with the lightest (= 12th) fraction. Densities were calculated from refractive indices by the following equation:

$y = 17.0066x^2-36.8684x+19.9749$

DNA was precipitated from fractions with 2 volumes (800 µl) of 30% PEG (see table II.5-1) and 20 µg (1 µl) glycogen (from mussels, Sigma Aldrich) for 1 h at room temperature. Precipitated DNA was pelleted by centrifugation (18.000 x g) for 30 min at 4°C, washed with 150 µl of ice cold 70% (v/v) ethanol, and centrifuged again. Pellets were air-dried briefly and resuspended in 30 µl of nuclease free water. Tubes were shaken (1,400 rpm) for 1 min at 30°C in an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany) to dissolve DNA-pellets.

II.8 Quantitative PCR (qPCR)

DNA and cDNA fragments were quantified using quantitative PCR (qPCR). Reactions were performed on iCycler IQ thermocyclers (Bio-Rad, Munich, Germany) in 96-well microplates (Bio-Rad). Solutions and oligonucelotide primers used for qPCR are listed in II.8.1 and II.8.3. Samples and standards were quantified in duplicates. After addition of the reaction mix, plates were sealed with optical tape (Bio-Rad, Munich, Germany). Fluorescein solution (FITC, 1mM, Biorad) was added to the reaction mix as calibration dye to optimize camera adjustments. To detect the amplification kinetics during qPCR, the ready-mix contained the DNA stain SybrGreen I (Sigma Aldrich) which leads to an increase in fluorescence intensity simultaneous to amplification of dsDNA during cycles. Data analysis was carried out with iCycler software (Bio-Rad). The cycle at which the fluorescence of a certain target molecule number exceeded the background fluorescence (threshold cycle [C_T]) was determined from dilution series of target DNA with defined target molecule amounts. C_T was proportional to the logarithm of the target molecule number. Thus, a C_T measured in a sample could be converted to a target molecule number.

II.8.1 Solutions for qPCR

The following solutions were used for quantitative PCR.

Table II.8-1: Solutions for qPCR

Component:	Source:
Sybr Green Jumpstart Taq Ready-Mix	Sigma Aldrich, Taufkirchen, Germany
$MgCl_2$ 50 mM	Provided with Ready-Mix (see above)
Fluorescein (FITC) calibration dye → dilute 1:1000 in nuclease-free water	Bio-Rad, Munich, Germany
BSA 20 µg/µl	Roche, Grenzach-Wyhlen, Germany

II.8.2 qPCR assays

The used primers are listed in Table II.8-2. For every quantification a negative control was performed by adding the appropriate amount of water instead of DNA or cDNA template.

Table II.8-2: Oligonucelotide primers used for qPCR

Target gene	Primer	Sequence (5' → 3')	Reference
pmoA	A189f	GGNGACTGGGACTTCTGG	Holmes *et al.*, 1995
	mb661	CCGGMGCAACGTCYTTACC	Costello *et al.*, 1999
	Forest675r	CCYACSACATCCTTACCGAA	Kolb *et al.*, 2003
amoA archaea	amo196f	GGWGTKCCRGGRACWGCMAC	Treusch *et al.*, 2005
	amo277r	CRATGAAGTCRTAHGGRTADCC	Treusch *et al.*, 2005
amoA bacteria	amoA-1F	GGGGTTTCTACTGGTGGT	Avrahami *et al.*, 2003
	amoA-2R	CCCCTCGGGAAAGCCTTCTTC	Avrahami *et al.*, 2003
16S rRNA	A364aF	CGGGGYGCASCAGGCGCGAA	Burggraf *et al.*, 1997
archaea	A934b	GTGCTCCCCCGCCAATTCCT	Grosskopf *et al.*, 1998
16S rRNA	Ba519f	CAGCMGCCGCGGTAANWC	Lane, 1991
bacteria	Ba907r	CCGTCAATTCMTTTRAGTT	Lane, 1991

'Wobble' positions: K = G/T; H = A/C/T; M = A/C ; N = A/C/G/T; R = A/G; S = G/C, W = A/T, Y = C/T

II.8.2.1 Quantification of USCα *pmoA*

For quantification of *pmoA* genes and transcripts of USCα from DNA and cDNA SIP-fractions, the following conditions and temperature profile were used. USCα *pmoA* amplified from Marburg forest soil (MF) and cloned into *E. coli* Top10 competent cells using primers A189f/Forest675r (see II.6.1) was used as standard in dilution series (10^1-10^7 copies).

Table II.8-3: qPCR conditions for quantification of USCα *pmoA*

qPCR reaction mixture:			**Thermal profile:**	
Component	Final conc.	Volume	Temperature program	Cycles
2x SybrGreen Ready-Mix	1x	12.5 µl	94°C, 6 min	1
50 mM $MgCl_2$	4 mM	2 µl	94°C, 25 s	45 (plate read)
50 µM A189f	1 µM	0.5 µl	67°C, 20 s	
50 µM Forest675r	1 µM	0.5 µl	72°C, 45 s	
20 µg/µl BSA	0.5 µg/µl	0.625 µl	82°C, 10 s	
1:1000 FITC		0.25 µl	75.0 -94.8°C[a], 6 s	100 (melting curve)
DNA or cDNA template		1 µl	4°C, 5 min	1
Distilled water		ad 25 µl		

[a] Melting curve: Set point temperature decreased after cycle 2 by 0.2°C for each cycle.

II.8.2.2 Quantification of general *pmoA*

For quantification of *pmoA* genes and transcripts of methanotrophs from DNA and cDNA SIP-fractions, the following condition and temperature profile was used. *pmoA* amplified from methanotroph type I pure culture *Methylomonas* sp., cloned into *E. coli* Top10 competent cells was used as standard in dilution series (10^1-10^7 copies).

Table II.8-4: qPCR conditions for quantification of general *pmoA*

qPCR reaction mixture:			**Thermal profile:**	
Component	Final conc.	Volume	Temperature program	Cycles
2x SybrGreen Ready-Mix	1x	12.5 µl	94°C, 6 min	1
50 mM $MgCl_2$	4 mM	2 µl	94°C, 25 s	45 (plate read)
50 µM A189f	0.667 µM	0.33 µl	65.5°C, 20 s	
50 µM mb661	0.667 µM	0.33 µl	72°C, 35 s	
1:1000 FITC		0.25 µl	72°C, 10 s	
DNA or cDNA template		1 µl	75.0 -94.8°C[a], 6 s	100 (melting curve)
Distilled water		ad 25 µl	4°C, 5 min	1

[a] Melting curve: Set point temperature decreased after cycle 2 by 0.2°C for each cycle.

II.8.2.3 Quantification of archaeal *amoA*

For quantification of *amoA* genes and transcripts of *Archaea* from DNA and cDNA SIP-fractions, and soil extracts, the following condition and temperature profile was used. Archaeal *amoA* amplified from Rauischholzhausen agricultural soil (RH) and cloned into *E. coli* Top10 competent cells using primers amo111F/amo643R (see II.6.2) was used as standard in dilution series (10^1-10^7 copies).

Table II.8-5: qPCR conditions for quantification of archaeal *amoA*

qPCR reaction mixture:			**Thermal profile:**	
Component	Final conc.	Volume	Temperature program	Cycles
2x SybrGreen Ready-Mix	1x	12.5 µl	95°C, 3 min	1
50 mM $MgCl_2$	4 mM	2 µl	95°C, 15 s	45 (plate read)
50 µM amo196f	0.5 µM	0.5 µl	55°C, 45 s	
50 µM amo277r	0.5 µM	0.5 µl	95°C, 60 s	1
20 µg/µl BSA	0.5 µg/µl	0.625 µl	55°C, 60 s	1
1:1000 FITC		0.25 µl	68.0 -97.7°C[a], 10 s	100 (melting curve)
DNA or cDNA template		1 µl	72°C, 10 min	1
Distilled water		ad 25 µl	4°C, 5 min	1

[a] Melting curve: Set point temperature decreased after cycle 2 by 0.3°C for each cycle.

II.8.2.4 Quantification of bacterial *amoA*

For quantification of *amoA* genes and transcripts of *Bacteria* from DNA and cDNA SIP-fractions, and soil extracts, the following condition and temperature profile was used. Bacterial *amoA* amplified from Rauischholzhausen agricultural soil (RH) and cloned into *E. coli* Top10 competent cells using primers amoA-1F/amoA-2R (see II.6.2) was used as standard in dilution series (10^1-10^7 copies).

Table II.8-6: qPCR conditions for quantification of bacterial *amoA*

qPCR reaction mixture:			**Thermal profile:**	
Component	Final conc.	Volume	Temperature program	Cycles
2x SybrGreen Ready-Mix	1x	12.5 µl	94°C, 15 min	1
50 mM $MgCl_2$	3 mM	1.5 µl	94°C, 45 s	40 (plate read)
50 µM amoA-1F	0.5 µM	0.25 µl	57°C, 30 s	
50 µM amoA-2R	0.5 µM	0.25 µl	72°C, 3 min	
20 µg/µl BSA	0.2 µg/µl	0.25 µl	83°C, 10 s	
1:1000 FITC		0.25 µl	78.0 -97.8°C[a], 6 s	100 (melting curve)
DNA or cDNA template		1 µl	72°C,10 min	1
Distilled water		ad 25 µl	4°C, 5 min	1

[a] Melting curve: Set point temperature decreased after cycle 2 by 0.2°C for each cycle.

II.8.2.5 Quantification of archaeal 16S rRNA genes and transcripts

For quantification of 16S rRNA genes and transcripts of *Archaea* from DNA and cDNA SIP-fractions, and soil extracts, the following condition and temperature profile was used. 16S rRNA gene amplified from *Methanosarcina barkeri*, amplified using primers A109f/A934b (see II.6.5), was provided by Melanie Klose and used as standard in dilution series ($7.83x10^1$-$7.83x10^7$ copies).

Table II.8-7: qPCR conditions for quantification of archaeal 16S rRNA genes and transcripts

qPCR reaction mixture:			**Thermal profile:**	
Component	Final conc.	Volume	Temperature program	Cycles
2x SybrGreen Ready-Mix	1x	12.5 µl	94°C, 6 min	1
50 mM $MgCl_2$	3 mM	1.5 µl	94°C, 35 s	45 (plate read)
50 µM A346aF	0.3 µM	0.15 µl	66°C, 30 s	
50 µM A934b	0.3 µM	0.15 µl	72°C, 45 s	
1:1000 FITC		0.25 µl	86.5°C, 10 s	
DNA or cDNA template		1 µl	75.0 -94.8°C[a], 6 s	100 (melting curve)
Distilled water		ad 25 µl	4°C, 5 min	1

[a] Melting curve: Set point temperature decreased after cycle 2 by 0.2°C for each cycle.

II.8.2.6 Quantification of bacterial 16S rRNA genes and transcripts

For quantification of 16S rRNA genes and transcripts of *Bacteria* from DNA and cDNA SIP-fractions, and soil extracts, the following condition and temperature profile was used. 16S rRNA gene amplified from *Escherichia coli* strain K12, amplified using primers Eub8F/Eub1392R (see II.6.5), was used as standard in dilution series (10^1-10^7 copies).

Table II.8-8: qPCR conditions for quantification of bacterial 16S rRNA genes and transcripts

qPCR reaction mixture:			**Thermal profile:**	
Component	Final conc.	Volume	Temperature program	Cycles
2x SybrGreen Ready-Mix	1x	12.5 µl	94°C, 8 min	1
50 mM $MgCl_2$	4 mM	2 µl	94°C, 20 s	35 (plate read)
50 µM Ba519f	0.25 µM	0.125 µl	50°C, 20 s	
50 µM Ba907r	0.25 µM	0.125 µl	72°C, 50 s	
20 µg/µl BSA	0.2 µg/µl	0.25 µl	75.0 -94.8°C[a], 6 s	100 (melting curve)
1:1000 FITC		0.25 µl	4°C, 5 min	1
DNA or cDNA template		1 µl		
Distilled water		ad 25 µl		

[a] Melting curve: Set point temperature decreased after cycle 2 by 0.2°C for each cycle.

II.9 Phylogenetic analyses

For phylogenetic analyses of USCα *pmoA* and bacterial 16S rRNA sequences from Marburg forest soil, and of *accA* and archaeal 16S rRNA sequences from RNA- and DNA-SIP fractions from Rauischholzhausen agricultural soil, respective PCR products had to be cloned and sequenced.

II.9.1 Cloning

Purified PCR products from functional genes had to undergo an additional A-tailing prior to cloning. Conditions for A-tailing are listed in table II.9-1. A-tailed samples were cleaned up using the Wizard SV Gel and PCR Clean-Up System (Promega, Mannheim, Germany) (see II.6.6).

Table II.9-1: A-tailing for blunt ended PCR products

Sequencing reaction mixture:		**Thermal profile:**	
Component	Volume	Temperature program	Cycles
dNTPs	1 µl	72°C, 10 min	1
$MgCl_2$	1.5 µl	4°C, ∞	1
10x Invitrogen Taq buffer	2.5 µl		
Invitrogen Taq Polymerase	0.3 µl		
Purified PCR	27-25 µl		

The pGEM-T Vector System kit (Promega) was used for ligation. Therefore, 3 µl of PCR product were mixed with 5 µl Ligation buffer, 1 µl of pGEM-T vector, and 1 µl of T4 DNA Ligase. Reactions were shortly vortexed and incubated overnight at 4°C.

For transformation, two LB-ampicillin-IPTG-XGal-agar plates (see table II.9-2) for each ligation reaction were equilibrated to room temperature prior plating (X-Gal = 5-Bromo-4-chloro-3-indolyl-β-D-Galactopyranosid). Of each ligation, 2 µl were added to 50 µl of JM109 High Efficiency Competent Cells (Promega) (from storage at -80°C, thawed on ice) and incubated on ice for 20 min. Cells were heat-shocked for 45 seconds in a water bath at 42°C and incubated on ice for 2 min. Pre-warmed SOC medium (950 µl; Sigma Aldrich) was added to the tubes and samples were shaken (400 rpm) for 1.5 hours at 37°C. Transformed cells were plated onto duplicate LB-ampicillin-IPTG-XGal-agar plates and plates were incubated overnight at 37°C.

Table II.9-2: Solutions for LB-Amp-IPTG-XGal-agar plates

IPTG stock solution (0.1 M)	
IPTG	1.2 g
Distilled water	50 ml
→ Filter sterilize and store at 4°C	
X-Gal (50 mg ml^{-1})	
X-Gal	100 mg
N,N'-dimethyl-formamide	2 ml
→ Cover with aluminium foil and store at -20°C	
LB medium	
Tryptone	5 g
Yeast extract	2.5 g
NaCl	2.5 g
Agar	7.5
Distilled water	500 ml
→ Adjust to pH 7.0 and autoclave	
After autoclaving, cool down to 45°C and add:	
100 mM IPTG	1.7 ml
50 mg ml^{-1} X-Gal	333 µl
0.1 g ml^{-1} Ampicillin	500 µl
→ Stir and pour 30-35 ml into 85 mm Petri dishes	

After incubation, plates were stored at 4°C, positive clones (blue-white selection) were screened by PCR (see table II.9-3), transferred to a fresh master plate, and PCR products

were checked on agarose gels. Products showing the right size were purified and used for sequencing (see II.9.2).

Table II.9-3: Conditions and thermal profile for screening of clones

Sequencing reaction mixture:		**Thermal profile:**	
Component	Volume	Temperature program	Cycles
T7f or other forward primer	0.3 µl	94°C, 5 min	1
M13r or other reverse primer	0.3 µl	94°C, 30 s	
dNTPs	0. 25 µl	55°C, 40 s	30
50 mM $MgCl_2$	0.375 µl	72°C, 1 min	
10x Invitrogen Taq buffer	1.25 µl	72°C, 7 min	1
Invitrogen Taq Polymerase	0.1 µl	4°C, ∞	1
Distilled water	ad 12.5 µl		
Add colony with sterile toothpick			

II.9.2 Sequencing

Sequencing of purified PCR products was performed following the chain termination method (Sanger *et al.*, 1977) using 4 different fluorescently labeled dideoxynucelotide triphosphates. The reaction containing these terminator dyes was set up using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit™ v.3.1 (Applied Biosystems). See table II.9-4 for conditions of PCR sequencing reaction and thermal profile.

Table II.9-4: Conditions for sequencing reaction

Sequencing reaction mixture:		**Thermal profile:**	
Component	Volume	Temperature program	Cycles
BigDye Terminator Ready Mix	2 µl	96°C, 30 s	1
BigDye Terminator 5x buffer	3 µl	94°C, 10 s	
T7f or M13r primer	0.125 µl	50°C, 5 s	25
DNA template ~10-40 ng	x µl	60°C, 4 min	
Distilled water	ad 20 µl	4°C, ∞	1

Sequencing reactions were purified using AutoSeq™ G-50 spin columns (GE Healthcare, Little Chalfont, UK) or the Spin™ Post Reaction Clean-Up Columns (Sigma Aldrich) according to the manufacturer's instructions. For samples with low concentration of DNA, the reactions were dried in a SpeedVac (DNA 110, Savant) for 20 min at 30°C and medium speed, and each pellet was resuspended in 20 µl of HiDi™ formamide (Applied Biosystems). For samples with higher concentrations of DNA, 10 µl of each reaction were directly mixed with 10 µl of HiDi™ formamide. All samples were denatured for 3 min at 90°C and kept at 4°C until sequencing. The sequencing was performed on an automatic sequencer (3130 Genetic Analyzer, Applied Biosystems).

II.9.3 Phylogenetic analyses

Sequences derived from the automatic sequencer were controlled and extracted using the SeqMan 4.05 and EditSeq Software (DNASTAR, Madison, Wis, USA). The identities of the gene and transcript sequences were confirmed by searching the sequence databases using nucleotide and protein blast (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analyses of the *pmoA* sequences from Marburg forest soil and the *accA* and archaeal 16S rRNA sequences from RNA- and DNA-SIP fractions from Rauischholzhausen agricultural soil, and deduced amino acid sequences were carried out using the ARB software package (Ludwig *et al*., 2004). Sequences were manually aligned with the related sequences obtained from the GenBank and RDP (http://rdp.cme.msu.edu/) database. Phylogenetic trees were reconstructed from sequence data by neighbor-joining approach, using 1000 bootstrap replicates, and maximum likelihood analyses of the data sets.

II.10 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) provides a cultivation-independent method for the *in situ* identification of single cells and the analysis of the composition of microbial communities and their dynamics. Microbial cells are first treated with appropriate chemical fixatives and then hybridized under stringent conditions on a glass slide or in solution with specific probes. These probes are either labeled directly with a fluorescent dye or, in case of longer polynucleotide probes, are labeled with a component that can be detected using antibodies. After stringent washing, specifically stained cells are detected via epifluorescence microscopy.

II.10.1 Generation of RNA polynucleotide probes – *in vitro* transcription

For both RING-FISH and CARD-FISH of mRNA, long RNA polynucleotide probes are needed to guarantee sufficient signal amplification. This can be done by *in vitro* transcription of a DNA template using T3 or T7 RNA-polymerases of bacteriophages. For this purpose RNA polynucleotide probes were generated for *pmoA* of USCα, archaeal *amoA* and *hydB* of *Streptomyces* sp. PCB7. The genes were amplified by PCR as described in II.6.1, II.6.2 and II.6.4, respectively, with the only difference that a T3 RNA-polymerase promoter was attached to each reverse primer. After amplification, PCR products were purified and used for *in vitro* transcription as described in table II.10-1. Probes were labeled with either Digoxigenin-11-UTP or Biotin-16-UTP.

Table II.10-1: Conditions and solutions for *in vitro* transcription of RNA polynucleotide probes

DIG- or Biotin-Mix			
Component	Initial conc.	Final conc.	Volume
ATP	100 nmol μl^{-1}	10.24 nmol μl^{-1}	3.9 µl
CTP	100 nmol μl^{-1}	10.24 nmol μl^{-1}	3.9 µl
GTP	100 nmol μl^{-1}	10.24 nmol μl^{-1}	3.9 µl
UTP	100 nmol μl^{-1}	3.68 nmol μl^{-1}	1.4 µl
DIG-11-UTP	10 nmol μl^{-1}	6.56 nmol μl^{-1}	25 µl
or Biotin-16-UTP (all purchased from Roche)			

Set-up for *in vitro* transcription			
Component	Initial conc.	Final conc.	Volume
DNA template	400 ng	ca. 50 ng μl^{-1}	4 µl
T3-polymerase (Roche)	20 U μl^{-1}	2 U μl^{-1}	3 µl
T3-buffer (Roche)	10x	1x	3 µl
RNase-Inhibitor (Roche)	40 U μl^{-1}	2 U μl^{-1}	1.5 µl
DIG-/Biotin-NTP-Mix	10.24 nmol μl^{-1}	1.71 nmol μl^{-1}	5 µl
Distilled water			ad 30 µl

Transcription was performed for 3-4 h at 37°C. To digest the DNA template, 3 µl of DNase I (RNase-free, 10 U μl^{-1}; Roche) were added and the sample was incubated for 15 min at 37°C. Addition of 3 µl EDTA (0.2 M) was used to stop the reaction. To precipitate the RNA probe, 0.1 volume of NaAc (3 M, pH 5.2) and 3 volumes of ice cold 96% (v/v) ethanol were added to the tube and the sample was incubated for 1 h at -80°C (or alternatively overnight at -20°C). The precipitated RNA was pelleted (18,000 x g) for 15 min at 4°C, washed with 1 ml of ice cold 70% (v/v) ethanol, and centrifuged again. The pellet was air dried for 5-10 min and was then resuspended in 30 µl of distilled water and 1 µl of RNase-Inhibitor (40 U μl^{-1}, Roche). Integrity of probes was checked on agarose gels and concentration was determined using a NanoDrop instrument (Thermo Fisher Scientific, Schwerte, Germany) (see II.6.6 and II.6.7).

II.10.2 Generation of expression clones as controls for FISH

To produce controls for CARD-FISH of mRNA, expression clones of *pmoA* (USCα from Marburg forest soil, *Methylocapsa acidiphila*, *Methylocystis* sp. strain SC2), *amoA* (ammonia-oxidizing archaea and bacteria from Rauischholzhausen agricultural soil), and *hydB* (*Streptomyces* sp. PCB7, *Paracoccus denitrificans*) were generated. Therefore, the respective genes were amplified from pure cultures and soil samples (see II.6) and cloned and expressed by using the pBAD TOPO® TA Expression Kit (Invitrogen, Karlsruhe, Germany). Fresh PCR product (2 µl) was mixed with 1 µl of Salt solution (1.2 M NaCl, 0.06 M

$MgCl_2$), 1 µl of pBAD-TOPO® vector, and 2 µl of sterile water. Reactions were carefully mixed and incubated for 5 min at room temperature. For transformation, two LB-ampicillin-agar plates (see table II.9-2) for each ligation reaction were equilibrated to room temperature prior plating. Of each ligation, 2 µl were added to one tube of *E. coli* Top10 competent cells (Invitrogen) (from storage at -80°C, thawed on ice) and incubated on ice for 20 min. Cells were heat-shocked for 30 seconds in a water bath at 42°C and incubated on ice for 2 min. Pre-warmed SOC medium (250 µl; Invitrogen) was added to the tubes and samples were shaken (400 rpm) for 1 h at 37°C. Transformed cells were plated onto duplicate LB-ampicillin-agar plates and plates were incubated overnight at 37°C. Clones were screened using primers pBADforward and pBADreverse (Invitrogen) (see table II.6-1), sequenced, and clones containing the right insert were chosen for induction. For expression of insert, clones were first inoculated in 2 ml of LB-ampicillin-medium and shaken overnight at 37°C. The next morning, 0.1 ml of overnight culture was transferred to fresh 10 ml of LB-Amp-medium and shaken for 2 h at 37°C. Expression was induced by adding 0.1 volume of 20% (w/v) L-arabinose (Invitrogen), dissolved in sterile water and filter sterilized. Cells were incubated for another 3 h at 37°C, and then 2x 2 ml of culture were used for cell fixation (see II.10.4). Uninduced *pmoA* clones were also fixed and used for RING-FISH.

II.10.3 Separation of microbial cells from soil

For FISH of Marburg forest soil, cells were separated from the soil using a Histodenz-density-gradient-centrifugation (Sigma Aldrich) to avoid disturbance of hybridization by autofluorescence of larger soil particles. First, the soil was mixed in a beaker with 1x PBS (see table II.10-2) and vortexed vigorously two times for 2 min. Tubes were placed on the bench for 10 min, to allow sedimentation of large soil particles and debris. From the supernatant, 40 ml were transferred to a 50 ml Greiner tube (Greiner Bio One, Frickenhausen, Germany) and centrifuged (5,000 rpm) for 10 min at 4°C (Universal 320 R, Hettich Zentrifugen). The supernatant was discarded and the pellet was resuspended in 10 ml of 1x PBS. The mixture was transferred to a sterile and transparent 50 ml Nalgene® centrifugation tube (VWR, Darmstadt, Germany) and 4 ml of Histodenz solution (80% w/v, in distilled water; density: ~ 1.3 g ml^{-1}) were injected underneath the slurry using a long needle. Mixture of both phases before centrifugation had to be avoided. Gradient-density-centrifugation was performed for 1.5 h at 4°C and 14,000 x g (Rotor SS-34, Sorvall RC B5 Plus centrifuge, Thermo Scientific). After the centrifugation the gradient showed 4 phases (from bottom to top): a pellet of remaining soil particles, the Histodenz-phase, a thin cell-layer, and a large PBS-phase. The δ-value of the cell layer (~ 1.12 g ml^{-1}) corresponds to the δ-value of most microorganisms. This allows the concentration of microbial cells in a fine layer. Cell- and PBS-layer of every gradient were transferred to a new 15 ml tube and centrifuged for 25 min at 5,000 rpm. The supernatant was discarded, the pellet was resuspended in 300-500 µl 1x PBS and subsequently used for cell fixation (see II.10.4).

II.10.4 Fixation of cells and environmental samples

Fixation treatment of samples for FISH that takes place before hybridization is crucial to preserve the integrity and shape of cells and prevent cell loss through lysis. Samples for fixation (2 ml cell- or soil- suspension) were first sedimented for 10 min at 16,000 x g and resuspended in 300-500 µl of 1x PBS. Solutions prepared for fixation are listed in table II.10-2.

Clones for RING-FISH were fixed in 4 volumes of 4% (vol/vol) paraformaldehyde (PFA, see table II.10-2) for 12-16 h at 4°C, centrifuged for 10 min at 16,000 x g, and washed once with 1ml of 1x PBS and twice with 1 ml of 50% ethanol in 1x PBS.

Expression clones, pure cultures, and filter pieces from SSMS incubations of Marburg forest soil (see II.11.1) for CARD-FISH were fixed in 4 volumes of 2% (vol/vol) paraformaldehyde (see table II.10-2) for 30 min at room temperature, centrifuged for 10 min at 16,000 x g, and washed once with 1 ml of 1x PBS and twice with 1 ml of 50% ethanol in PBS.

For CARD-FISH of archaeal *amoA* mRNA (see II.10.6.3 and II.10.6.5), samples from the RH soil SIP incubations after 12 weeks of fertilization with 15 µg NH_4^+-N g^{-1} *d.w.s.* were amended with 1.5 ml of 4% (vol/vol) paraformaldehyde and vortexed three times for 1 min (Eickhorst *et al.*, 2008). Larger soil particles were allowed to settle for 5 min. The supernatant was transferred to a new 1.5 ml tube and incubated for 5 h at 4°C. After incubation the sample was centrifuged for 5 min at 10,000 x g, washed once with 1ml 1x PBS, and centrifuged again.

For FISH of Marburg forest soil incubations, cells were separated from the soil using Histodenz (see II.10.3), then fixed following the protocol for clones and pure cultures.

For CARD-FISH of *hydB* mRNA (see II.10.6.4), samples of *Streptomyces* sp. PCB7 were fixed in 1.5 ml of 50% ethanol in 1x PBS for 24 h at 4°C, centrifuged for 10 min at 16,000 x g, and washed once with PBS.

Finally, all cells and soil samples were resuspended in 150 µl – 1 ml 50% ethanol in 1x PBS and stored at -20°C until further processing.

Table II.10-2: Solutions for fixation

1x PBS	Final conc.	Volume
NaCl	130 mM	7.6 g
K_2HPO_4	1.5 mM	3.26 g
Na_2HPO_4	8 mM	1.13 g
KCl	2.7 mM	0.2 g
A. bidest		1000 ml
→ Adjust pH to 7.4 and autoclave		

2% PFA	Final conc.	Volume
PFA (Roth)	2% (w/v)	1 g
1x PBS	1x	50 ml

4% PFA	Final conc.	Volume
PFA (Roth)	4% (w/v)	2 g
1x PBS	1x	50 ml

→ Dissolve in water bath (60°C), cool down, adjust pH to 7.2 and filter sterilize.

II.10.5 RING-FISH targeting *pmoA* of USCα

Recognition of individual genes-fluorescence in situ hybridization (RING-FISH) is a special variant of whole-cell hybridization. In RING-FISH a multiply labeled transcript polynucleotide probe is used to detect a single gene on the microbial chromosome during FISH. The folding of the single-stranded RNA probe molecules into secondary structures results in the formation of a network of probes around the cells during whole-cell hybridization, showing characteristical halo-shaped fluorescence signals in the periphery of the cells (see figure I.1-3). This network around the cell also offers the opportunity to combine RING-FISH with subsequent cell sorting. RING-FISH was performed with RNA probes targeting the *pmoA* gene of USCα to detect cells containing this gene in clones, pure cultures, and cells extracted from Marburg forest soil (see II.10.3). For each probe, formamide series (0-80% formamide in hybridization buffer) were used to determine optimal hybridization conditions.

II.10.5.1 RING-FISH with RNA polynucelotide probe

RING-FISH with A189f/Forest675r-T3 RNA polynucleotide probe (see II.10.1), targeting the *pmoA* gene of USCα, was used for clones, pure cultures and cells extracted from Marburg forest soil (see II.10.3) The following solutions were prepared for RING-FISH with RNA polynucleotide probes.

Table II.10-3: Solutions for RING-FISH with RNA polynucleotide probes

Hybridization buffer	Final conc.	Volume
NaCl, 5 M	75 mM	30 µl
Tris-HCl, 1 M pH 8	20 mM	40 µl
SDS, 10 % (w/v)	0.01%	2 µl
Formamide	variabel	variabel
Distilled water		ad 2 ml

Washing buffer	Final conc.	Volume
NaCl, 5 M	150 mM	30 ml
Tris-HCl, 1 M pH 8	100 mM	100 ml
SDS, 10 % (w/v)	0.01%	1 ml
Distilled water		ad 1 l
→ adjust to pH 7.4		

II.10.5.1.1 Hybridization on slides

Hybridization with RNA polynucleotide probe on slides, targeting the *pmoA* gene of USCα, was performed with clones, pure cultures, or cells extracted from Marburg forest soil (see II.10.3). Fixed sample (5-10 µl, see II.10.4) was applied to each well of a Teflon-coated glass slide (MAGV, Marburg, Germany) and dried for 10 min at 60°C. Cells were dehydrated in an ethanol series (50, 80, and 96%) for 3 min each and the slide was air dried. Fresh hybridization buffer (12 µl, see table II.10-3) was mixed with 2-4 µl (~2.5 µg) of RNA polynucleotide probe (see II.10.1) on a piece of Parafilm (Pechiney, Chicago, IL, USA) and applied to each well (except controls without probe). The slide was placed in a wet chamber with hybridization buffer (piece of tissue, soaked with 2 ml of hybridization buffer, in a 50 ml falcon tube) and denatured for 20 min at 80°C. Hybridization was performed in a hybridization oven (Hybaid, Heidelberg, Germany) for 22-24 h at 53°C. Afterwards, the slide was carefully rinsed with distilled water, air dried, and used for detection (see II.10.5.3).

II.10.5.1.2 Hybridization in solution

Hybridization with RNA polynucleotide probe in solution, targeting the *pmoA* gene of USCα, was performed with clones, pure cultures and cells extracted from Marburg forest soil (see II.10.3). Cell sorting using DNA-coated microplates was coupled to this protocol. Fixed sample (30 µl, see II.10.4) was mixed with 200 µl 1x PBS (see table II.10-2) in a 0.5 ml tube and centrifuged for 3 min at 11,000 x g to eliminate residual ethanol. The pellet was resuspended in 30 µl of fresh hybridization buffer (see table II.10-3) and mixed with 8 µl of

RNA polynucleotide probe (~5 µg). Samples were denatured for 20 min at 80°C and subsequently hybridized for 22-24 h at 53°C attached to the rotating device of the hybridization oven (Hybaid, Heidelberg, Germany). The tubes were centrifuged for 3 min at 8,000 x g and cells were resuspended in 100 µl of washing buffer (see table II.10-3). Care was taken to avoid the formation of cell aggregates, as this could disturb the efficiency of cell sorting afterwards. The samples were further incubated for 30 min at 53°C to wash off unbound probe. The cells were pelleted for 3 min at 8,000 x g and resuspended in 15-30 µl 1x PBS. The hybridized cells could be used for detection (see II.10.5.3) or cell sorting using DNA coated microplates (see II.5.4).

II.10.5.2 RING-FISH with synthetic oligo-oligonucleotide probes

A different approach to specifically target cells containing *pmoA* of USCα with RING-FISH consisted of a novel type of polynucleotide probes, so-called oligo-oligonucleotide probes. For the design of these oligo-oligonucleotide probes, at first specific oligonucleotide probes targeting *pmoA* of USCα were identified using the ARB Probe_Design tool (ARB probe match) with the sequences of the phylogenetic tree generated for *pmoA* (see II.9). Also oligonucelotide probe Ra14-598, previously used for microarray analysis of *pmoA* and also specific for USCα (Bodrossy *et al.*, 2003), was used to generate an oligo-oligonucleotide probe. The single stranded construct template consisted of an alternating sequence of the repetitive specific oligonucleotide sequence and poly-A or GC spacer regions. The T3 promoter was localized at the 5´end and the whole sequence was ordered at Biomers (Ulm, Germany). The sequences of these probes are listed in table II.10-4. The ability of the probes to form secondary structures was checked using the free program RNADraw (www.rnadraw.com) to calculate melting profiles.

Table II.10-4: Oligo-oligonucleotide probes for *pmoA* of USCα

Probe	DNA template 5'-3'	Length
MF08_25-175	ATAGGTATTAACCCTCACTAAAGTGCGGAAGCCGATGACATC GGCCAA*AAAAA*TGCGGAAGCCGATGACATCGGCCAA*AAAAA*T GCGGAAGCCGATGACATCGGCCAA*AAAAA*TGCGGAAGCCGA TGACATCGGCCAA*AAAAA*TGCGGAAGCCGATGACATCGGCCA A*AAAA*TGCGGAAGCCGATGACATCGGCCAA	175 bp without T3 promoter
RA14_GC121	ATAGGTATTAACCCTCACTAAAGGGCATCGAGGTACGAACGT T*CGCGC*GGCATCGAGGTACGAACGTT*CGCGC*GGCATCGAGG TACGAACGTT*CGCGC*GGCATCGAGGTACGAACGTT*CGCGC*G GCATCGAGGTACGAACGTT	121 bp without T3 promoter

Underlined: T3 promoter; italics: spacer region

For generation of single stranded RNA probes, *in vitro* transcription from single stranded template DNA was performed. The DNA-dependent RNA polymerase is able to synthesize in

3'-5' direction a single stranded RNA strand from different DNA templates. But at least the promoter region has to be double stranded. Therefore, it was necessary to built up a double stranded T3 promoter region. This has been performed by annealing of a single stranded nucleic acid primer constituting the T3 promoter sequence to a reverse complementary T3 promoter sequence that was part of the 3' prime end of the synthetic construct template. Therefore, 50 pmol of the single stranded synthetic probe DNA template were mixed with 50 pmol of a single stranded T3 promoter primer (sequence: ATAGGTATTAACCCTCACTAAAG), denatured for 5 min at 94°C and incubated for 20 min at 55°C using a PCR thermocycler. Afterwards, the samples were put on ice and used for *in vitro* transcription (see II.10.1).

II.10.5.2.1 Hybridization on slides

Hybridization with RNA oligo-oligonucleotide probes on slides, targeting the *pmoA* gene of USCα, was performed with clones, pure cultures, and cells extracted from Marburg forest soil (see II.10.3). Fixed sample (5-10 µl, see II.10.4) was applied to each well of a Teflon-coated glass slide (MAGV, Marburg, Germany) and dried for 10 min at 60°C. Cells were dehydrated in an ethanol series (50, 80, and 96%) for 3 min each and the slide was air dried. Fresh hybridization buffer (12 µl, 0-30% formamide, see table II.10-3) was mixed with 4 µl (~2.5 µg) of RNA oligo-oligonucleotide probe (see II.10.5.2) on a piece of Parafilm (Pechiney, Chicago, IL, USA) and applied to each well (except controls without probe). The slide was placed in a wet chamber with hybridization buffer (piece of tissue, soaked with 2 ml of hybridization buffer, in a 50 ml falcon tube) and denatured for 30 min at 80°C. Hybridization was performed in a hybridization oven (Hybaid, Heidelberg, Germany) for 4-22 h at 53°C. Afterwards, the slide was carefully rinsed with distilled water, air dried, and used for detection (see II.10.5.3).

II.10.5.2.2 Hybridization in solution

Hybridization with RNA oligo-oligonucleotide probes in solution, targeting the *pmoA* gene of USCα, was performed with clones and pure cultures. Cell sorting using DNA-coated microplates was coupled to this protocol. Fixed sample (30-100 µl, see II.10.4) was mixed with 3 volumes of 96% (v/v) ethanol, incubated for 3 min at 25°C and centrifuged for 3 min at 11,000 x g. The pellet was resuspended in 30 µl of fresh hybridization buffer (0-30% formamide, see table II.10-3) and mixed with 8 µl of RNA polynucleotide probe (~5 µg). Samples were denatured for 30 min at 80°C and subsequently hybridized for 22 h at 53°C attached to the rotating device of the hybridization oven (Hybaid, Heidelberg, Germany). The tubes were centrifuged for 3 min at 8,000 x g and cells were resuspended in 100 µl of washing buffer (see table II.10-3). Care was taken to avoid the building of cell clumps, as this could disturb the efficiency of cell sorting afterwards. The samples were further incubated for 30 min at 53°C to wash off unbound probe. The cells were pelleted for 3 min at 8,000 x g and

resuspended in 15-30 µl 1x PBS (see table II.10-2). The hybridized cells could be used for detection (see II.10.5.3) or cell sorting using DNA coated microplates (see II.5.4).

II.10.5.3 Detection

For detection of the digoxigenin labeled probes, anti-digoxigenin antibody coupled to the fluorescent dye fluorescein (Anti-digoxigenin-fluorescein, Fab fragments; Roche) was used. Probes labeled with biotin were detected using the antibody streptavidin coupled to the fluorescent dye Cy3 (Streptavidin-Cy3 Conjugate, Sigma Aldrich). For hybridizations on slides, 20 µl of Anti-DIG-fluorescein (diluted 1:2 in 1x PBS) or 20 µl of Streptavidin-Cy3 (diluted 1:100 in 1x PBS) were added to each well and the slide was incubated in a wet chamber (distilled water) in the dark for 1 h at 27°C (DIG) or 28°C (Biotin). To wash of unbound probe, slides were incubated for 10 min at 28°C in washing buffer (for DIG, see table II.10-3) or for 20 min at 29°C in DPBS (for Biotin). Finally, slides were washed carefully with distilled water and air dried in the dark. For hybridizations in solution, 30 µl of Anti-DIG-fluorescein (diluted 1:2 in 1x PBS) or Streptavidin-Cy3 (diluted 1:100 in 1x PBS) were added to the pellet resuspended in 15 µl 1x PBS. The solution was mixed and incubated in the dark for 1 h at 27°C (DIG) or 28°C (Biotin). Afterwards, the samples were transferred to 1.5 ml tubes, mixed with 1 ml of washing buffer (DIG) or DPBS (Biotin), and centrifuged for 15 min at 18,000 x g. The washing step was repeated and the pellets were resuspended in 25 µl 1x PBS.

II.10.5.4 Cell sorting using DNA-coated microplates

Cell sorting provides a method to “fish” labeled cells out of a cell mixture or environmental sample. The procedure consists of two different hybridization steps. The first step is the hybridization of the target cells with an unlabeled polynucleotide probe in solution, following the protocols described in II.10.5.1.2 and II.5.2.3. The second step is a hybridization step for separating the already hybridized cells from non-target cells via binding to microplate cavities coated with DNA complementary to the RNA probe. For this purpose, NucleoLink microplates (Nalge Nunc, Roskilde, Denmark) were used (Fichtl *et al.*, 2005). In these plates, 1-Ethyl-3-(3-Dimethylaminopropyl)-Carbodiimide (EDC) is a common agent to crosslink carboxyl- or amino groups via carbodiimide condensation, so DNA is bound covalently. The principle is depicted in figure II.10-1. The solutions used for cell sorting are listed in table II.10-5.

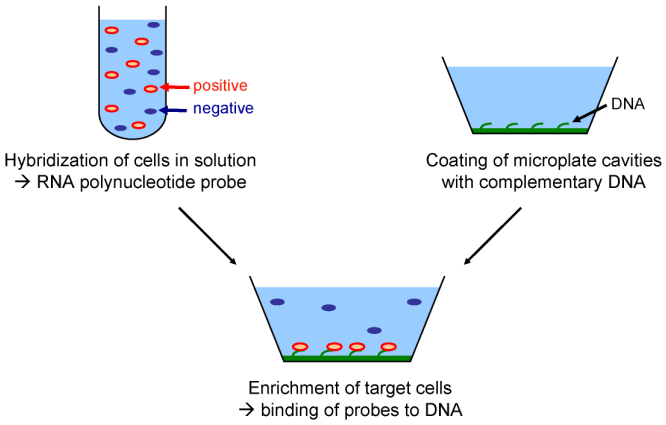


Figure II.10-1: Cell sorting using microplates

To generate the DNA to coat the microplates, a purified PCR product from USCα *pmoA* from Marburg forest soil was used as template for an asymmetric PCR of *pmoA*. The asymmetric PCR was performed as described in II.6.1, but without adding the reverse primer. Only the forward primer A189f was used. This way, a single stranded DNA sequence, complementary to the RNA polynucleotide probe, was generated. After the PCR, up to 10 PCR reactions were purified, pooled, and precipitated with 0.1 volume of NaAc (3 M, pH 5.2) and 2.5 volumes of 96% (v/v) ethanol overnight at -20°C. Samples were centrifuged (18,000 x g) for 20 min at 4°C, washed with 100 µl of ice cold 70% (v/v) ethanol, and centrifuged again. The pellet was shortly air dried, resuspended in 20-100 µl of nuclease-free water, and the concentration was determined using a NanoDrop instrument (Thermo Fisher Scientific, Schwerte, Germany) (see II.6.7). For coating, the DNA was denatured for 10 min at 94°C and directly put on ice to avoid renaturation. Fresh ice cold EDC-PBS/$MgCl_2$ was prepared (see table II.10-5) and 100 µl were mixed with 1 µg of DNA in each cavity of a microplate. For negative controls, only EDC-PBS/$MgCl_2$ was added. The microplate was closed with PCR film (Peqlab, Erlangen. Germany) and prehybridized overnight at 37°C. The supernatants were carefully discarded and cavities were dried for 1-2 h at 60°C. Coated plates sealed with PCR film can be stored at 4°C for several weeks. Before continuing with the enrichment, cavities were washed with 100 µl PBS to remove unbound DNA. After the RING-FISH hybridization in solution (see II.10.5.1.2 and II.10.5.2.3), the hybridization samples were centrifuged for 5 min at 13,000 x g. Pellets were washed with 100 µl 1x PBS and centrifuged again. Cells were resuspended in 100 µl of MP buffer (see table II.10-5). 50 µl of each sample were added into a microplate cavity coated with DNA and into one uncoated cavity (negative control). The microplate was covered with PCR film and incubated for 1 h at 37°C (for chromosomal DNA targeted probes, 53°C for rRNA targeted probes). After enrichment hybridization, the solution (containing the unbound cells) was carefully pipetted off the cavities. Corresponding supernatants were pooled, centrifuged and used for PCR. Microplate cavities were carefully

washed 1-2 times with 100 µl of 1x PBS. The immobilized target cells in the cavities were directly used for PCR. Therefore, the bacterial 16S rRNA gene was amplified as described in II.6.5, with minor modifications. The reaction mix (50 µl) was directly added to the microplate cavities and the initial denaturation phase was set to 12 min at 94°C. Also a hot-start DNA polymerase (Platinum® DNA Polymerase, Invitrogen) was used. The microplate was sealed with PCR film and after thermal cycling PCR products were checked on an agarose gel (see II.6.6).

Table II.10-5: Solutions for cell sorting using microplates

PBS/$MgCl_2$	Final conc.	Volume
$MgCl_2$	0.1 M	10.15 g
1x PBS (see table II.10-2)	1x	500 ml

EDC-PBS/$MgCl_2$	Final conc.	Volume
EDC (Thermo Scientific)	10 mM	19.2 mg
PBS/$MgCl_2$		10 ml
→ mix and cool for 5-10 min at -80°C		

20x SSC	Volume
NaCl	175.3 g
Sodium citrate	88.2 g
Distilled water	800 ml
→ adjust pH to 7.0, add 200 ml of distilled water and autoclave	

10% N-laurylsarcosin	Final conc.	Volume
N-laurylsarcosin	10%	10 g
Distilled water		100 ml
→ filter sterilize		

MP buffer	Initial conc.	Final conc.	Volume
SSC	20x	5x	262.5 µl
SDS	10%	0.02%	2.5 µl
Blocking reagent (Roche)	10%	2%	250 µl
N-laurylsarcosin	10%	0.1%	12.5 µl
Formamide		34%	412 µl
Distilled water			Ad 1.2 ml

II.10.6 CARD-FISH

Catalyzed reporter deposition (CARD) is another FISH variant that can be applied to increase signal intensities in various immunochemical and FISH applications. Through the use of horseradish peroxidase (HRP) labeled antibodies to detect the probes, many tyramide molecules, preconjugated with either haptens or fluorescent reporters, are deposited in close vicinity to the HRP binding site, resulting in superior spatial resolution (see figure I.1-2). In combination with HRP-labeled antibodies, the CARD-FISH method has the potential to detect low-abundance mRNAs or 16S rRNAs. CARD-FISH was performed with RNA probes targeting mRNA of *pmoA* USCα (see II.6.2), archaeal *amoA* (see II.6.3), and *hydB Streptomyces* sp. PCB7 (see II.6.4). CARD-FISH of archaeal 16S rRNA was also carried out, coupled to detection of *amoA* of ammonia oxidizing archaea (see II.6.5). For each probe, formamide series (0-80% formamide in hybridization buffer) were used to determine optimal hybridization conditions. CARD-FISH for mRNA was performed based on a protocol by Pernthaler and Amann (2004).

II.10.6.1 Solutions for CARD-FISH

The following solutions were prepared for CARD-FISH.

Table II.10-6: Solutions for CARD-FISH

0.2 M HCl	Volume
1 M HCl	5 ml
Distilled water	20 ml

20% SDS	Volume
Sodium dodecyl sulphate	10 g
Distilled water	40 ml
→ Stir, then fill up to 50 ml with water	

20x PBS	Volume
NaCl	160 g
KCl	4 g
Na_2HPO_4	28.8 g
KH_2PO_4	4.8 g
Distilled water	800 ml
→ Adjust pH to 7.6 with HCl, fill up to 1 l with water, and autoclave	

1x PBS	Volume
20x PBS	15 ml
Distilled water	285 ml

2x saline-sodium citrate buffer (SSC)	Volume
NaCl	17.53 g
Sodium citrate	8.82 g
Distilled water	800 ml
→ Adjust pH to 7.0 with HCl, fill up to 1 l with water, and autoclave	

1x Maleic acid buffer	Volume
Maleic acid	5.8 g
NaCl	4.4 g
Distilled water	500 ml
→ Adjust pH to 7.5 with NaOH pellets	

10% Blocking reagent	Volume
Blocking reagent (Roche)	50 g
1x Maleic acid buffer	500 ml
→ Stir and heat up, until powder is completely dissolved. Aliquot, autoclave and store at -20°C (or at 4°C when in use).	

Hybridization buffer - 50% formamide	Volume
5 M NaCl	3.6 ml
1 M Tris-HCl pH 8.0	0.4 ml
20% SDS	20 µl
10% Blocking reagent	2 ml
Dextran sulphate	2 g
Formamide	10 ml
Nuclease-free water	3.98 ml
→ Dissolve at 40-60°C in water bath, aliquot, and store at -20°C	

Amplification buffer	Volume
5 M NaCl	16 ml
20x PBS	2 ml
10% Blocking reagent	0.4 ml
Dextran sulphate	4 g
Nuclease-free water	21.5 ml
→ Dissolve at 40-60°C in water bath and store at 4°C	

Hybridization buffer - 10% formamide	Volume
5 M NaCl	0.9 ml
1 M Tris-HCl pH 8.0	0.1 ml
20% SDS	5 µl
Dextran sulphate	0.5 g
Formamide	500 µl
Nuclease-free water	3.495 ml
→ For CARD-FISH of archaeal 16S rRNA. Dissolve at 40-60°C in water bath, aliquot, and store at -20°C	

Washing buffer for 16S rRNA CARD	Volume
5 M NaCl	4.4 ml
0.5 M Na-EDTA	0.5 ml
1 M Tris-HCl pH 8.0	1 ml
20% SDS	25 µl
Distilled water	44.075 ml
→ equivalent to 10% formamide in hybridization buffer	

1 mg ml^{-1} DAPI solution	Volume
4',6-diamidino-2-phenylindole (Sigma)	2 mg
Nuclease free water	2 ml

II.10.6.1.1 Synthesis of tyramide conjugates

CARD is based on the deposition of a large number of labeled tyramine molecules by peroxidase activity. HRP reacts with hydrogen peroxide and the phenolic part of labeled tyramide to produce a quinine-like structure bearing a radical on the C2 group. This "activated" tyramide then covalently binds to tyrosine residues in the target cell. This results in

greatly enhanced FISH sensitivity as compared to probes with a single fluorochrome. Synthesis of tyramide conjugates was performed based on a protocol by Pernthaler *et al.* (2004) and is described below.

Synthesis of tyramide labeled with 5- (and 6-) carboxyfluorescein:

a) Active dye stock:

100 mg 5- (and 6-) carboxyfluorescein succinimidyl ester (Invitrogen)
+ 10 ml dimethylformamide

→ Don't expose to light!

b) Tyramine HCl stock:

33 µl triethylamine
+ 3.3 ml dimethylformamide
+ 33 mg tyramine-HCl (Sigma)

Mix 10 ml of active dye stock with 3.3 ml of tyramine HCl stock. Incubate for 6-12 h in the dark at room temperature. Add 86.7 ml 96% (v/v) ethanol to achieve 1 mg active dye ml^{-1}. Aliquot (20 µl - 1 ml) and dry overnight in SpeedVac. Store dried tyramide conjugates at -20°C. Dissolve tyramides in dimethylformamide.

Synthesis of tyramide labeled with $Alexa_{546}$:

a) Active dye stock:

1 mg succinimidyl ester AlexaFluor546 (Invitrogen)
+ 100 µl dimethylformamide
→ Don't expose to light!

b) Tyramine HCl stock:

10 µl triethylamine
+ 1 ml dimethylformamide
+ 10 mg tyramine-HCl (Sigma)

Add 14.7 µl of tyramine HCl stock to active dye stock. Incubate for 6-12 h in the dark at room temperature. Add 875.3 ml 96% (v/v) ethanol to achieve 1 mg active dye ml^{-1}. Aliquot (50 µl) and dry overnight in SpeedVac. Store dried tyramide conjugates at -20°C. Dissolve tyramides in 50 µl of dimethylformamide.

II.10.6.2 CARD-FISH mRNA USCα *pmoA*

CARD-FISH was performed with RNA polynucleotide probe A189f/Forest675r-T3 (see II.10.1) targeting the *pmoA* transcripts of USCα to detect cells expressing this gene in expression clones (induced and uninduced, see II.10.2), pure cultures, cells extracted from Marburg forest soil (see II.10.3), and in incubations of this soil on filters of soil substrate membrane systems (see II.11.1). The protocol is described below.

mRNA CARD-FISH of USCα pmoA:

1. Pretreatment

1. Add 5-10 µl of sample or a small filter piece to each well of a Teflon-coated glass slide (MAGV)
2. Dry slide for 5-10 min at 46°C
3. Boil up 0.1% low gelling point agarose (0.02 g in 20 ml distilled water), pour in Petri dish, let cool down to 35-40°C, and cover slide with agarose
4. Dry slide for 15-20 min at 46°C
5. Dehydrate slide in 50 ml 96% (v/v) ethanol for 1 min at room temperature
6. Air dry slide
7. Cover slide with 1 ml of 0.2 M HCl (see table II.10-6) and incubate for 10 min at room temperature
8. Wash slide in 50 ml 1x PBS for 1 min at room temperature
9. Incubate slide in 50 ml of 0.1% DEPC (50 µl DEPC + 50 ml 1x PBS, for carboxyethylation) for 12 min at room temperature
10. Wash slide in 50 ml 1x PBS for 1 min at room temperature
11. Wash slide in 50 ml distilled water for 1 min at room temperature

2. Permeabilization

1. Cover slide with 1 ml of lysozyme solution (5 mg ml^{-1} in 1x TE buffer) and incubate for 30 min at room temperature
2. Wash slide in 50 ml distilled water for 1 min at room temperature
3. Cover slide with 1 ml of proteinase K solution (1 µg ml^{-1} in 1x TE buffer) and incubate for 15 min at room temperature → not for clones!
4. Wash slide 3 times in 50 ml distilled water for 1 min at room temperature
5. Dehydrate slide in 50 ml 96% (v/v) ethanol for 1 min at room temperature

6. Air dry slide

3. CARD-FISH

1. Cover slide with 100 µl of hybridization buffer (50% formamide, see table II.10-6)
2. Prepare wet chamber soaked with 2 ml of 50% formamide-1x SSC (1 ml formamide + 1 ml 2x SSC)
3. Prehybridize slide in wet chamber for 1h at 58°C in hybridization oven
4. Mix 100 µl of hybridization buffer with 50 ng of RNA polynucleotide probe (~ 2-4 µl), denature mixture for 5 min at 80°C
5. Add probe mixture to wells (final probe concentration: 250 ng ml^{-1})
6. Prepare wet chamber soaked with 1 ml of hybridization buffer
7. Hybridize slide in wet chamber overnight at 58°C in hybridization oven
8. Wash slide in 50 ml of 50% formamide-1x SSC (25 ml formamide + 25 ml 2x SSC) for 1 h at 58°C
9. Wash slide in 50 ml of 0.2xSSC-0.01% (w/v) SDS (5 ml 2x SSC + 25 µl 20% SDS + 44.975 ml distilled water) for 30 min at 58°C
10. Incubate slide in 50 ml of 1x PBS-0.5% blocking reagent (2.5 ml 20x PBS + 2.5 ml 10% blocking reagent + 45 ml distilled water) for 30 min at room temperature
11. Mix 975 µl 1x PBS with 10 µl 10% blocking reagent and 15 µl of anti-DIG-HRP antibody (= 0.75 U ml^{-1}, Roche), add mixture on slide, and incubate for 1 h at 37°C
12. Wash slide 3 times in 50 ml 1x PBS for 10 min at room temperature
13. Mix 1 ml of amplification buffer (see table II.10-6) with 10 µl 100x H_2O_2 stock (1 ml 1x PBS + 5 µl 30% H_2O_2, Sigma Aldrich) and 5 µl of fluorescein labeled tyramide (see II.10.6.1.1)
14. Add mixture on slide and incubate for 5 min at room temperature in the dark
15. Wash slide in 50 ml 1x PBS for 3 min at room temperature
16. Wash slide 3 times in 50 ml distilled water for 1 min at room temperature
17. Dehydrate slide in 50 ml 50% (v/v) ethanol for 1 min at room temperature
18. Dehydrate slide in 50 ml 96% (v/v) ethanol for 1 min at room temperature
19. Air dry slide in the dark

4. DAPI staining

1. Add 20 µl of DAPI solution (see table II.10-6) per well and incubate for 10 min at room temperature
2. Wash slide in 50 ml distilled water for 1 min at room temperature
3. Dehydrate slide in 50 ml 96% (v/v) ethanol for 1 min at room temperature
4. Air dry slide in the dark
5. Continue with epifluorescence microscopy (see II.10.7)

II.10.6.3 CARD-FISH mRNA archaeal *amoA*

CARD-FISH was performed with RNA polynucleotide probe amo111f/amo643r-T3 (see II.10.1) targeting the *amoA* transcripts of ammonia-oxidizing archaea to detect cells expressing this gene in expression clones (induced and uninduced, see II.10.2) and in incubations of Rauischholzhausen agricultural soil incubated for stable isotope probing (see II.10.2). The protocol is described in II.10.6.2 with the following modifications:

2. Permeabilization

3. Cover slide with 1 ml of proteinase K solution (**15 µg ml^{-1}** in 1x TE buffer) and incubate for **10** min at room temperature → for soil samples

3. CARD-FISH → hybridization and washing temperature was **60.5°** instead of 58°C!

II.10.6.4 CARD-FISH mRNA *hydB*

CARD-FISH was performed with RNA polynucleotide probe NiFe-1129f/NiFe-1640r-T3 (see II.10.1) targeting the *hydB* transcripts of *Streptomyces* sp. PCB7 to detect cells expressing this gene in expression clones (induced and uninduced, see II.10.2) and pure cultures. The protocol is described in II.10.6.2 with the following modifications:

2. Permeabilization → for *Streptomyces* sp. PCB7

1. Cover slide with 1 ml of lysozyme solution (**10 mg ml^{-1}** in 1x TE buffer) and incubate for **1 h at 37°C**

3. Cover slide with 1 ml of **achromopeptidase** solution (**60 U ml^{-1}** in 0.01 M NaCl and 0.01 M Tris-HCl; Sigma) and incubate for **30 min at 37°C**

II.10.6.5 CARD-FISH 16S rRNA archaea

CARD-FISH of archaeal 16S rRNA was attached to CARD-FISH of archaeal *amoA* transcripts. HRP-labeled probe Arch915 (Biomers, Ulm, Germany) was used for detection of *Archaea* in soil samples with Alexa546-labeled tyramide. The protocol was inserted after step 3.17 of the mRNA CARD-FISH protocol (see II.10.6.3) and is described below.

CARD-FISH of archaeal 16S rRNA attached to mRNA CARD-FISH:

1. Incubate slide in 0.01 M HCl for 10 min at room temperature (to inactivate anti-DIG-HRP antibody)
2. Wash slide in 50 ml 1x PBS for 1 min at room temperature

3. Wash slide in 50 ml distilled water for 1 min at room temperature
4. Dehydrate slide in 50 ml 50% (v/v) ethanol for 1 min at room temperature
5. Dehydrate slide in 50 ml 96% (v/v) ethanol for 1 min at room temperature
6. Air dry slide in the dark
7. Mix 200 µl of hybridization buffer (10% formamide, see table II.10-6) with 2 µl of probe working solution of HRP-labeled probe Arch915 (50 ng μl^{-1}) and add 20 ml of solution to each well
8. Prepare wet chamber with 1 ml of hybridization buffer (10% formamide)
9. Hybridize slide in wet chamber for 2.5 h at 46°C in hybridization oven
10. Wash slide in prewarmed washing buffer (see table II.10-6) for 15 min at 48°C
11. Dip slide shortly in cold water (4°C)
12. Wash slide in 50 ml 1x PBS for 15 min at room temperature
13. Mix 1 ml of amplification buffer (see table II.10-6) with 5 µl of $Alexa_{546}$ tyramide (1 mg ml^{-1}, see II.10.6.1.1) and add mixture on slide.
14. Incubate slide for 10 min at room temperature
15. Wash slide in 50 ml 1x PBS for 10 min at room temperature
16. Wash slide in 50 ml distilled water for 1 min at room temperature
17. Dehydrate slide in 50 ml 96% (v/v) ethanol for 1 min at room temperature
18. Air dry slide in the dark
19. Continue with DAPI-staining (mRNA CARD-FISH protocol, step 4.1)

II.10.7 Fluorescence microscopy

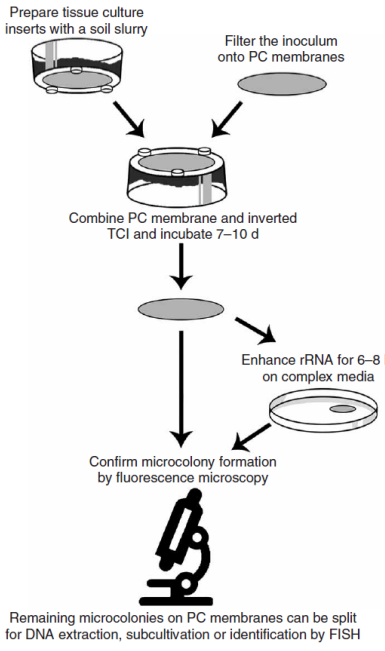
After hybridization and detection, all slides were mounted with the antifading agent Citifluor AF1 (Citifluor, London, UK), covered with a cover slide, and hybridization preparations were visualized by fluorescence microscopy (Axiophot; Carl Zeiss Microimaging GmbH, Jena, Germany). The different emission peaks of the fluorescent dyes (fluorescein: 523 nm, Cy-3: 570 nm, Alexa546: 573 nm, DAPI: 461 nm) allowed for visual discrimination of the signals (fluorescein: green signal, Cy-3 and Alexa546: orange-red signal, DAPI: blue signal). Results of the hybridizations were documented using a camera (INTAS, Goettingen, Germany).

II.11 Enrichment of Upland Soil Cluster α

The Marburg forest soil (MF) was also used for approaches to enrich Upland Soil Cluster α. The first approach was based on a soil substrate membrane system incubated at methane concentrations close to atmospheric concentration (see II.11.1). The second approach was based on incubating the soil with alternating incubation periods of acetate and methane (see II.11.2). Abundance of USCα was investigated in both enrichment approaches using a variety of molecular analyses (qPCR, T-RFLP, CARD-FISH, etc.).

II.11.1 Soil substrate membrane system (SSMS)

The soil substrate membrane system (SSMS) provides a technique to mimic the natural terrestrial environment of soil bacteria (Ferrari *et al.*, 2008). The substrate used for cultivation consists of natural non-sterilized wet soil as the sole growth component. The soluble soil carbon components released after soil wetting is sufficient for microcolony-forming (mCFU) growth of microorganisms. This is supposed to facilitate the isolation and continued cultivation slow growing microorganisms in the soil. In the SSMS approach, microcolony formation occurs on a polycarbonate membrane (PCM) in immediate contact with the slurry of soil substrate. The principle of SSMS is depicted in figure II.11-1.



(Ferrari *et al.*, 2008)

Figure II.11-1: Principle of soil substrate membrane systems

The intention of using this method was to enrich Upland Soil Cluster α from Marburg forest soil. Therefore, 20 g of fresh MF soil were mixed with water and vortexed, until a homogenous slurry formed. 3 g of soil were each weighed carefully in 25 mm tissue culture inserts (TCI, 0.02 µm Anopore membrane; Nunc, Roskilde, Denmark). To evenly distribute the soil in the TCI and to remove air bubbles, the benchtop mixer was turned on to full speed and touched carefully with the TCI at the edge of the vortexer plate. The vibrations cause the soil to evenly distribute over the whole membrane. Into the middle of a well within a sterile 6-well plate (Greiner Bio One), 500 µl of distilled water were pipetted to prevent the TCI from drying out during the incubation period. The TCI was inverted so that the membrane was facing upwards and placed in the middle of the well over the sterile water. For inoculation of the filters, 3 g of the previously homogenized soil were diluted 1:10 with 30 ml of sterile water in a 50 ml falcon tube, and vortexed vigorously. Large soil particles were allowed to sediment by letting the sample stand for 30 s. From the supernatant, 100 µl were diluted with 1 ml of sterile water, resulting in a 1:100 dilution of the soil. This dilution was used to inoculate the filters. Therefore, sample filtration manifold was set up and a vacuum pump was attached to its exhaust. For every TCI (except negative controls), a sterile 0.2 µm isopore polycarbonate membrane filter (PCM, 25 mm; Millipore) was placed on top of a prewet glass fiber filter. Then, a sterile cylinder was placed on top of the PCM on the manifold. 10 ml of sterile water was added into each cylinder followed by 50 µl of the 1:100 soil dilution. The solution was gently mixed by pipetting to ensure an even distribution of cells on the PCM during filtration. The vacuum pump was turned on to draw the diluted inoculum through the PCM. Valves were closed as soon as the inoculum had passed through the PCM. The PCM was carefully remove from the filtration manifold using sterile tweezers and placed on top of the inverted, prepared TCI, prewetted with ~10 µl of sterile water. To further prevent the SSMS from drying out during the incubation period 1 ml of distilled water was pipetted between the wells. The 6-well plate was closed with a lid and sealed with Parafilm. The SSMS was transferred to a gas tight jar (2 l). The jar was closed and methane was added to a final concentration of 20 ppmv. The set-up is shown in figure II.1-2. The SSMS was incubated at 25°C in the dark. A filter without cell-inoculation was used as negative control. Methane concentration in the jar was monitored by GC (see II.4.6) and maintained at 20 ppmv CH_4. Every week, the water level between the wells was readjusted. Every 3-5 days, the jar was flushed with fresh air. After 3 and 6 weeks of incubation, single filters were taken off and quartered using a sterile and sharp scalpel. The quarters were used for CARD-FISH of *pmoA* USCα transcripts (see II.10.6.2) and DNA was extracted for qPCR (see II.8).

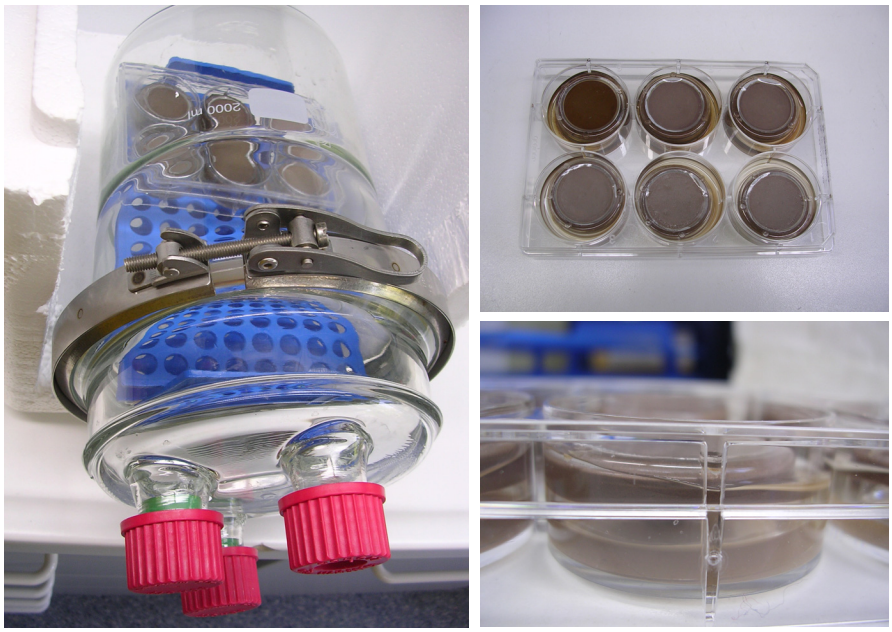


Figure II.11-2: Set-up of SSMS

II.11.2 Incubation of Marburg forest soil with alternating acetate and CH_4 treatments

Because of recent results, showing that methanotrophs are capable of using acetate as carbon source, an enrichment approach was set up using alternating incubation periods of acetate and methane. Therefore, Marburg forest soil incubations were set up either as normal soil (20 g) or as slurry (20 g soil + 40 ml of medium DSM 922, see table II.2-1) in 120 ml serum bottles capped with butyl rubber stoppers. The soil was incubated with 2 ppmv, 20 ppmv, and 100 ppmv of methane, respectively. Additionally, three different concentrations of acetate were added (100, 400, or 800 µg g^{-1} *d.w.s.*). Incubations were performed in duplicates for each treatment and at 25°C in the dark. Slurries were shaken at ~170 rpm. Methane concentration in the incubations was monitored by GC (see II.4.6). Every week, bottles were flushed with synthetic air (20% O_2, 80% N_2), and methane treatments were renewed. Acetate was added every 3rd week of incubation, to allow enough time for acetate to be depleted and to starve microorganisms depending on the acetate. Consumption of acetate was checked 1-2 days after a new acetate treatment using HPLC (II.4.5). During the incubation period, aliquots of the soil (0.5 g) were extracted (see II.5.1). The extracted DNA was used for qPCR to investigate the abundance of *pmoA* USCα and for T-RFLP to monitor potential changes in bacterial population (see II.6.8).

III. USCα in upland forest soil

III.1 Assimilation of acetate by the putative atmospheric methane oxidizing clade USCα

Jennifer Pratscher, Marc G. Dumont and Ralf Conrad

Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany

Submitted to *Nature Geoscience* in October 2010

III.1.1 Abstract

Forest soils are the major biological sink for atmospheric methane, yet the identity and function of the microorganisms responsible for this process remain unclear. Although members of the upland soil cluster α (USCα) are assumed to represent methanotrophic bacteria adapted to the trace level of atmospheric methane and to play an essential part in the removal of this greenhouse gas from the atmosphere, so far they resisted isolation. Especially the question, whether USCα are able to obtain all their energy and carbon solely from methane or use additional carbon compounds, still awaits to be answered. In this study, we performed stable-isotope probing (SIP) of RNA and DNA to investigate assimilation of $^{13}CH_4$ and ^{13}C-acetate by USCα in an acidic forest soil. RNA-SIP using $^{13}CH_4$ showed no incorporation of labeled carbon into nucleic acids of USCα, although CARD-FISH targeting *pmoA* mRNA of USCα still detected expression in the incubated soil. However, stable isotope probing of RNA using labeled acetate revealed assimilation by USCα, but DNA-SIP showed no growth, probably due to prolonged generation times. Our results demonstrate that the contribution of alternative carbon sources, such as acetate, to the metabolism of potential atmospheric methane oxidizers like USCα in upland forest soils might be substantial.

III.1.2 Introduction

Methane (CH_4) is an important greenhouse gas with a global warming potential 20 times more effective than CO_2 and a current atmospheric concentration of 1.7 ppmv. One major sink for atmospheric methane are upland soils, where aerobic methanotrophic communities consume approximately 30 Tg y^{-1} (1, 2). The key step in methane oxidation, the initial oxidation of CH_4 to methanol, is catalyzed via the methane monooxygenase enzyme which occurs as a particulate, membrane bound form (pMMO), and as a soluble, cytosolic form (sMMO) (3). The *pmoA* gene, which encodes the α-subunit of pMMO, has been generally used as a biomarker

to investigate methanotrophic communities in various environments. Phylogenetic analyses of soils showing uptake of atmospheric methane revealed the presence of predominant, possible "high affinity" methanotrophic bacteria, which represent novel sequence lineages of *pmoA* and were therefore named upland soil cluster (USC) α and γ (4-6). Further studies showed that *pmoA* of USCα is most closely related to *Methylocapsa acidiphila* (5, 7) while USCγ exhibits next relation to the *Methylococcaceae* (4). These findings all indicate the potential role of those upland soil clusters in the removal of methane from the atmosphere (8). Unfortunately, only little is known about the molecular phylogeny and function of these microorganisms. So far no successful isolation approach has been reported. Several *Methylocystis* and *Methylosinus* species have been shown to contain two types of pMMO, the conventional enzyme pMMO1 and pMMO2, a second monooxygenase, which catalyses oxidation of CH_4 at atmospheric levels (9-11). These strains, however, are also not able to grow at atmospheric concentrations of methane and might require additional carbon sources (10, 12, 13).

A promising method to link function of USCα to phylogeny is provided by stable isotope probing (SIP). This technique allows the specific identification of microorganisms assimilating labeled substances, most commonly carbon from a particular ^{13}C-labeled substrate (14). Previous studies investigated the assimilation of CH_4 by USCα in soils showing uptake of atmospheric CH_4 by using stable isotope probing of PLFAs or DNA, and detected labeling of lipids proposed to be characteristic for USCα but no incorporation of labeled carbon into DNA (4, 15-19).

The aim of this study was to investigate potential oxidation of methane and assimilation of acetate by upland soil cluster α in an acidic forest soil using the more sensitive RNA-SIP and DNA-SIP in comparison. We further wanted to monitor the expression of USCα *pmoA* by mRNA catalyzed reporter deposition – fluorescence *in situ* hybridization (CARD-FISH). Our findings provide novel evidence that these potential atmospheric methane oxidizers in upland soils might not depend on methane as sole carbon and energy source.

III.1.3 Results

Methane oxidation activity in Marburg Forest soil

A high-affinity CH_4 oxidation potential of 41 pmol *g.d.w.* $soil^{-1}$ h^{-1} at atmospheric methane concentration (~2 ppmv) was observed for the Marburg forest soil collected in July 2008. Addition of acetate (100, 400, 800 µg g^{-1} *d.w.s*) to "normal" soil incubations did not effect methane oxidation; however, methane oxidation was strongly inhibited when soil was incubated in slurry and shaken (see supplementary figure 1). USCα showed to be the predominant methanotroph in this soil and made up 94.6% of the methanotrophic bacterial community (1.42±0.11 x10^6 *pmoA* gene copies g^{-1} *d.w.s.*), while the general methanotrophs (all cultured methanotrophs) accounted only for 5.4% (8.12±0.15 x10^4 *pmoA* gene copies g^{-1} *d.w.s.*), as detected by qPCR.

RNA-SIP using $^{13}CH_4$ labeling

After 6 and 10 weeks of incubation, the 200 ppmv CH_4 microcosms had consumed a total of 82.3 and 137.1 µmol ^{13}C *g.d.w.* soil^{-1}, respectively. Because incorporation of $^{13}CH_4$ in the other incubations (2 ppmv and 20 ppmv) was not sufficient for stable isotope probing, only these microcosms were used for RNA-SIP of methane oxidizing bacteria. Therefore, buoyant density centrifugation was conducted with the respective RNA extracts from ^{12}C and ^{13}C microcosms after 6 and 10 weeks of incubation with 200 ppmv CH_4. The quantitative distribution of *pmoA* transcripts in these gradients was analyzed by qPCR of cDNA (Figure 1). After 6 weeks of incubation the copy number of USCα *pmoA* transcripts peaked only in the 'light' RNA fraction (1.77 - 1.79 g ml^{-1}) and showed no detectable labeling in the 'heavy' fraction (1.81 – 1.83 g ml^{-1}). After 10 weeks of incubation still no enrichment of labeled RNA could be observed. *pmoA* transcripts of general methanotrophs (see supplementary figure 2) and controls with $^{12}CH_4$ also did not show any labeling. These results suggest that the ^{13}C was not incorporated into nucleic acids by the USCα, comprising 94.6% of the methanotrophic bacterial community.

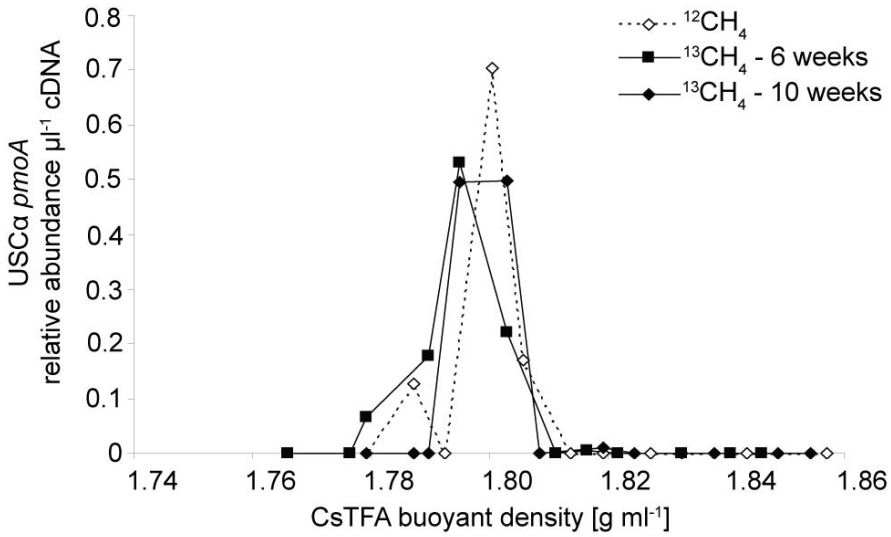


Fig. 1. Distribution of *pmoA* transcripts of USCα in RNA-SIP after 6 and 10 weeks of incubation with 200 ppmv CH_4 (^{12}C or ^{13}C). Distribution of *pmoA* transcripts was measured by real-time PCR of cDNA from gradient fractions.

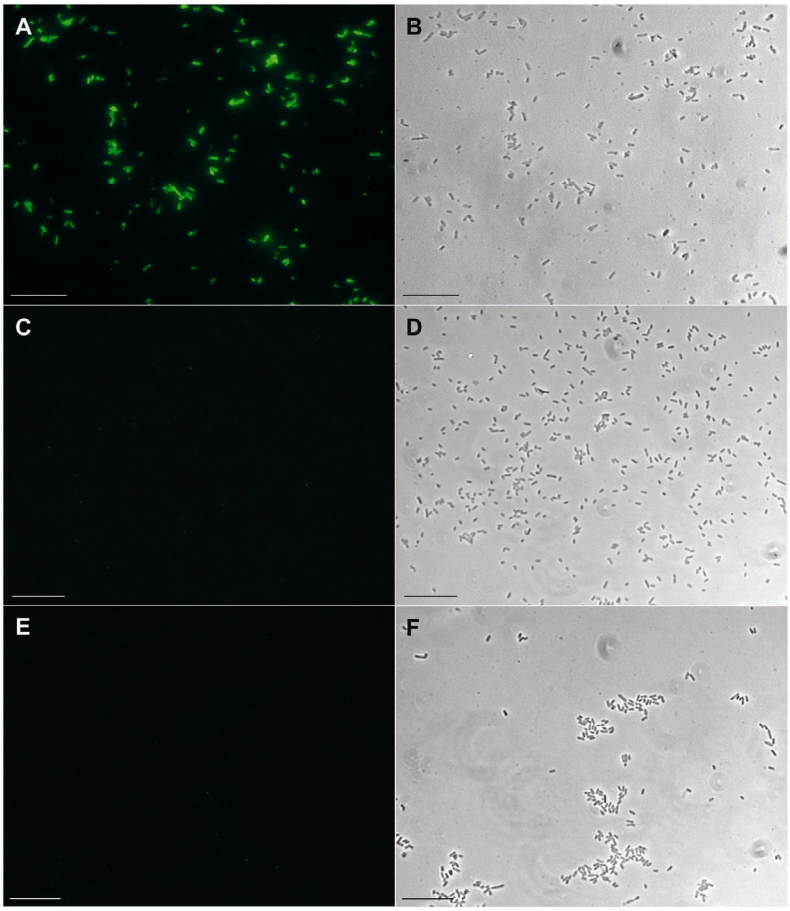


Fig. 2. Detection of *pmoA* mRNA transcripts of USCα by application of CARD-FISH with *pmoA* antisense probe in clones expressing *pmoA* of upland soil cluster α (A and B) or *Methylocapsa acidiphila* (E and F), and in an uninduced clone harboring *pmoA* of USCα (C and D). Fluorescence images for *pmoA* CARD-FISH (A, C, and E) and respective phase contrast (B, D, and F). Bars = 10 µm.

mRNA CARD-FISH of USCα *pmoA* in soil

To detect the expression of USCα *pmoA* mRNA in the Marburg forest soil, CARD-FISH of *pmoA* transcripts was conducted based on the protocol by Pernthaler and Amann (20). Clones and soil samples were hybridized with an USCα *pmoA* mRNA antisense probe, followed by detection with an anti-DIG-antibody labeled with horseradish peroxidase and signal amplification by catalyzed reporter deposition with fluorescein-labeled tyramide. The specificity of the *pmoA* mRNA antisense probe for USCα in the Marburg forest soil was tested with expression clones (Figure 2). Induced cells showed strong hybridization signals (Figure 2A and B), whereas no signal was observed in uninduced cells (Figure 2C and D) or clones expressing the partial *pmoA* gene of *Methylocapsa acidiphila* (Figure 2E and F), indicating a high specificity of the *pmoA* mRNA CARD-FISH for USCα. The hybridizations with the soil, incubated with 200 ppmv, resulted in few but strong signals for the *pmoA* CARD-FISH (Figure 3), demonstrating that *pmoA* was expressed in the incubations. USCα showed to be small, rod shaped cells with a length of approximately 1 µm.

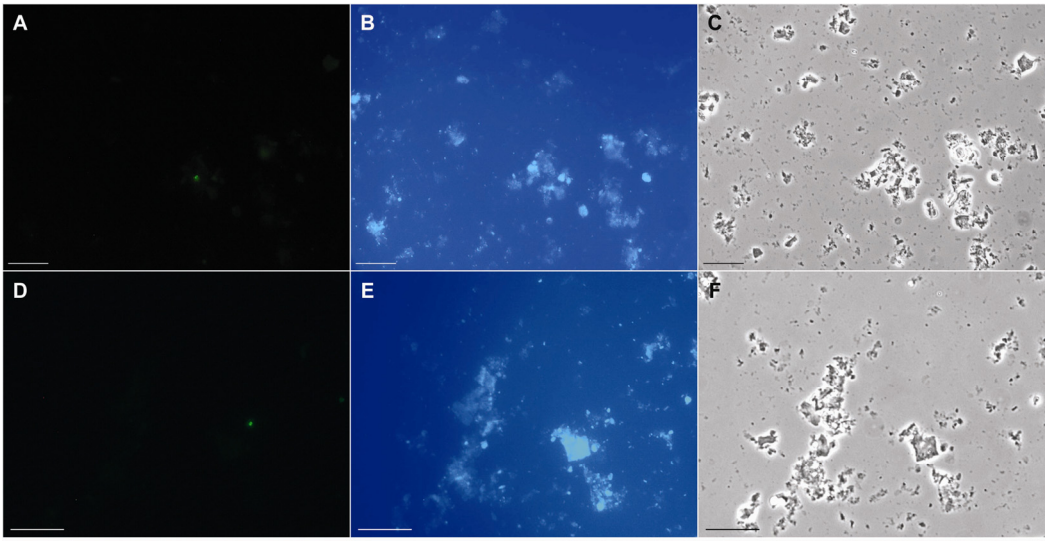


Fig. 3. Detection of *pmoA* mRNA transcripts by application of CARD-FISH with USCα *pmoA* antisense probe in forest soil after 10 weeks of incubation with 200 ppmv CH_4. Fluorescence images for *pmoA* CARD-FISH (A and D), DAPI DNA staining (B and E), and respective phase contrast (C and F). Bars = 10 µm.

Stable isotope probing using ^{13}C-acetate

After 3 weeks of incubation with ^{12}C- and ^{13}C-acetate, the microcosms and slurries had each consumed a total of about 400 µmol C *g.d.w.* soil^{-1}. Gradient centrifugation of RNA was performed with all respective RNA extracts from ^{12}C- and ^{13}C-acetate exposed microcosms after 3 weeks of incubation. The quantitative distribution of *pmoA* transcripts in these gradients was analyzed by qPCR of cDNA (Figure 4). After 3 weeks of incubation with 10 mM acetate, the copy number of *pmoA* transcripts of USCα and general methanotrophs from the wet soil incubations was highest in the 'light' RNA fraction (1.77 - 1.79 g ml^{-1}), and could not be detected in the 'heavy' fraction (Figure 4a and d). The *pmoA* transcripts of USCα in the ^{13}C slurry treatments, however, showed a strong labeling and were nearly completely shifted into the 'heavy' (1.81 – 1.83 g ml^{-1}) RNA fraction (Figure 4b and c). This could not be seen for the transcripts of general *pmoA* in these incubations, which still exhibited no labeling (Figure 4e and f). Also, no labeling was observed in the controls with ^{12}C-acetate. This indicates that acetate was actively consumed by USCα in the slurry incubations but not by the general methanotrophic community in this forest soil. Differences in RNA-SIP results compared to the wet soil microcosms might be due to a better distribution and accessibility of the acetate in the slurry incubations.

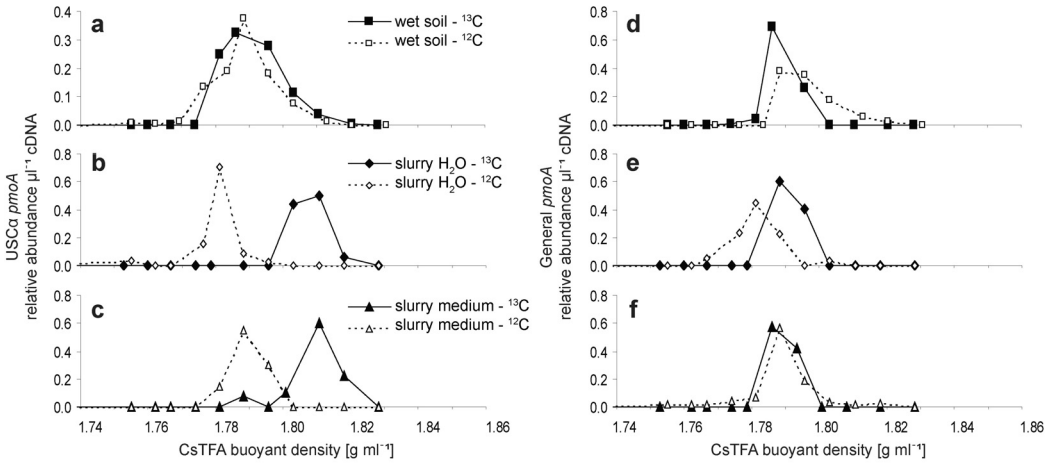


Fig. 4. Distribution of *pmoA* transcripts of USCα (a-c) and general methanotrophs (d-f) in RNA-SIP after 3 weeks of incubation with 10 mM acetate (^{12}C or ^{13}C). Acetate was added to the soil dissolved in 6 ml H_2O (wet soil), 20 ml H_2O (slurry H_2O), or 40 ml of medium DSMZ 922 (slurry medium), respectively. Distribution of *pmoA* transcripts was measured by real-time PCR of cDNA from gradient fractions.

For DNA stable isotope probing of methane oxidizing bacteria, buoyant density centrifugation was conducted with all respective DNA extracts from ^{12}C and ^{13}C microcosms after 3 weeks of incubation. The quantitative distribution of *pmoA* genes in these gradients was analyzed by qPCR of DNA (Figure 5). The copy numbers of the *pmoA* genes for both USCα (Figure 5a-c) and general methanotrophs (Figure 5d-f) peaked only in the 'light' fractions (1.69 – 1.72 g ml^{-1}), suggesting that although USCα were actively involved in acetate oxidation, as shown in the RNA-SIP approach, replication did not occur.

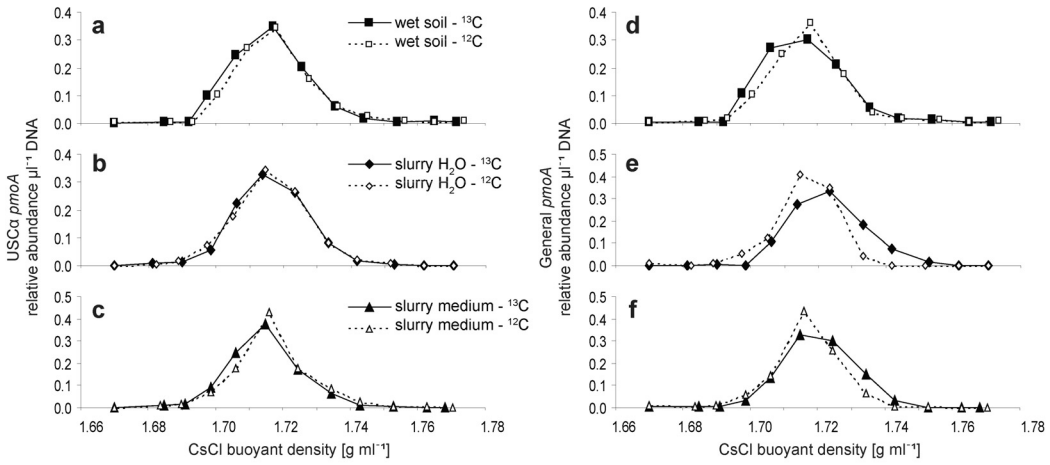


Fig. 5. Distribution of *pmoA* genes of USCα (a-c) and general methanotrophs (d-f) in DNA-SIP after 3 weeks of incubation with 10 mM acetate (^{12}C or ^{13}C). Acetate was added to the soil dissolved in 6 ml H_2O (wet soil), 20 ml H_2O (slurry H_2O), or 40 ml of medium DSMZ 922 (slurry medium), respectively. Distribution of *pmoA* genes was measured by real-time PCR of DNA from gradient fractions.

Furthermore, strong labeling of the bacterial 16S rRNA genes and transcripts in all ^{13}C-acetate incubations indicates that acetate was used significantly by the bacterial community in this soil (see supplementary figure 3). However, no labeling of *pmoA* or bacterial 16S rRNA was observed in the controls with 5% $^{13}CO_2$ (see supplementary figure 4), demonstrating that the 'heavy' RNA and DNA from the gradients indeed resulted directly from acetate incorporation into selected microbes and were not due to secondary labeling by CO_2 (21, 22).

III.1.4 Discussion

Upland soils are a major sink for atmospheric methane, but to date it remains elusive whether the upland soil cluster α (USCα), proposed to exhibit a crucial role in atmospheric methane oxidation, rely on methane as sole carbon and energy source. Previous studies using stable isotope probing of DNA to detect assimilation of CH_4 by USCα did not detect incorporation of labeled carbon, although tested soils consistently showed consumption of CH_4 (18). PLFA-SIP with labeled CH_4, on the other hand, detected labeling of lipids proposed to be characteristic for USCα (4, 15-19).

In our study, we tested the more sensitive RNA stable isotope probing approach to investigate potential incorporation of labeled ^{13}C-CH_4 into mRNA of USCα in an upland forest soil. RNA-SIP, however, also detected no labeling of USCα and general *pmoA* transcripts after 6 and 10 weeks of incubation with 200 ppmv CH_4, although microcosms showed consistent uptake of methane. According to Neufeld *et al.* (23), 5-500 µmol ^{13}C *g.d.w.* soil^{-1} are required for efficient DNA-SIP. For the more sensitive RNA-SIP, these values might even be lower (24, 25). This suggests that enough labeled carbon was taken up by soil incubations to generally allow for detection of labeling in SIP, but USCα did not incorporate carbon from CH_4 into nucleic acids and hence were not labeled. It is unlikely that the remaining general methanotrophic community in the Marburg forest soil, which besides the predominant USCα solely consists of type I methanotrophs (6), was responsible for the methane uptake at 200 ppmv, since they do not possess a high affinity pMMO required for oxidation of methane <600 ppmv (10).

Nonetheless, cells expressing USCα *pmoA* could still specifically be detected by mRNA CARD-FISH in the soil incubated with CH_4. This observation could indicate that expression of *pmoA* might not necessarily be coupled to enzyme activity in USCα, as previously reported for the type II methanotroph *Methylocystis* strain H2s, where transcripts of *pmoA* were detected although cells were grown on acetate without addition of methane (12). Additionally, Baani and Liesack (10) observed constitutive expression of the *pmoCAB2* genes encoding for pMMO2, a particulate methane monooxygenase also able to oxidize methane at concentrations close to atmospheric level, in *Methylocystis* sp. strain SC2. As methane consumption, however, was observed during the whole incubation period, a more plausible explanation could be the use of CH_4 by USCα, but solely for energy conservation (or restricted as carbon source for e.g. PLFAs) and not as general carbon source, as already

suggested for oxidation of atmospheric methane in forest soils (26). In this case, USCα might require and assimilate alternative carbon compounds, such as acetate. Furthermore, using CARD-FISH we were able to visualize USCα *in situ* for the first time, showing that cells exhibited a small, rod shaped appearance.

Previous studies already described the ability of several type II methanotrophs to assimilate acetate (12, 13) as well as stimulation of atmospheric methane oxidation by acetate in an alpine tundra soil (27). Moreover, several *Methylocella* species were even found to be facultative methanotrophs, showing higher growth rates and carbon conversion efficiency on acetate than on methane, and inhibition of methane oxidation when acetate was present (28). Our results now reveal active assimilation of acetate by upland soil cluster α in a forest soil using an RNA-SIP approach. USCα *pmoA* transcripts were consistently labeled during incubation of soil slurries with 10 mM ^{13}C-acetate for 3 weeks. Incubation of wet soil did not show any labeling, probably due to insufficient dispersal of the acetate in comparison to the slurry incubations. Controls with ^{12}C-acetate and $^{13}CO_2$ also did not show any labeling, confirming that labeling resulted from true label incorporation by assimilation of acetate. These findings support that USCα indeed are able to use other carbon sources and are not restricted to methane.

Acetate, however, seems to be rather unsuitable for enrichment or isolation of upland soil cluster α from forest soil, since it also served as a carbon source for other microorganisms in this soil, which outgrew USCα as observed in stable isotope probing of bacterial 16S rRNA genes and transcripts. Addition of acetate also showed no effect on oxidation of methane in soil microcosms. But since methane oxidation was inhibited in slurries, where distribution of acetate is much better than in the microcosms, we can not exclude that this inhibition was due to acetate and could only not be observed in the “normal” soil because the major population of USCα did not get access to the acetate. Nevertheless, this supports the hypothesis that USCα might only utilize atmospheric CH_4 as an additional energy source or survival strategy.

In contrast to the RNA-SIP approach, we were not able to detect labeling of USCα *pmoA* when using DNA-SIP. While RNA-SIP targeting *pmoA* of upland soil cluster α in the slurry incubations clearly demonstrated that acetate was assimilated, DNA-SIP revealed no growth of these organisms. Since detection of label incorporation in DNA-SIP depends on active replication of cells, we assume that the USCα might have actively assimilated the ^{13}C-acetate, as seen in RNA-SIP, but probably grew insufficiently for detection in DNA-SIP. This is supported by previous findings where growth on acetate in *Methylocystis* species, initially described as obligate methanotrophs, was detected but found to be 3-10 times slower than growth on methane (12, 13). This suggests that USCα probably grew too slowly within the 3 weeks of incubation to be detected in DNA-SIP or that replication did not occur due to still unfavourable growth conditions.

Unlike for USCα, the *pmoA* transcripts and copies of the general methanotrophic community did not show any labeling in RNA- and DNA-SIP after 3 weeks of incubation with

^{13}C-acetate. This was not surprising, since the only general methanotrophic bacteria in the Marburg forest soil have been found to be type I methanotrophs (6), which so far are not reported to possess the ability to utilize acetate. The observation, that *pmoA* transcripts could still be detected in both SIP incubations, although the general methanotrophic community in this soil showed neither incorporation of CH_4 (at 200 ppmv) nor of acetate, also probably indicates a general high stability or large turnover without biosynthesis of new biomolecules of *pmoA* mRNA and other transcripts presumed to be involved in 'high-affinity' activities in soils and other environments. This has also been observed for *pmoA* transcripts in anoxic lake sediments (Dumont *et al.*, unpublished) and for transcripts of a high-affinity naphthalene dioxygenase (NDO) of *Acidovorax* in a groundwater system (29).

Considering all these observations, our results provide first insight into the function, morphology, and phylogeny of the upland soil cluster α, proposed to be substantially involved in the oxidation of atmospheric methane in many upland soils. Our data further strengthens the hypothesis that these potential atmospheric methane oxidizers might utilize additional carbon compounds for growth, such as acetate, and could use CH_4 mainly as energy replenishment and survival strategy under limited conditions, suggesting the USCα represents rather facultative than obligate methanotrophs. These findings could promote the knowledge and understanding of upland soils as a sink for atmospheric methane and the microorganisms proposed to be responsible for this process.

III.1.5 Materials and methods

Soil incubation

Soil was sampled using 10 cm long soil cores in October 2008 (for CH_4 labeling) and in August 2009 (for acetate labeling) from a forest soil in Marburg, Germany. The field site and soil properties were described previously (4, 6). The soil was homogenized, sieved through 3 mm mesh and stored at 4°C until further use. The rate of oxidation of ambient (~2 ppmv) CH_4 was measured over a 24 h period.

Incubation for stable isotope probing with CH_4 was performed in duplicates for each treatment. Soil (5 g *d.w.s*) was amended with 1 ml H_2O and incubated at 25°C and darkness in 120 ml serum bottles capped with butyl stoppers. The methane concentration in the headspace was adjusted to and maintained at 2 ppmv, 20 ppmv, and 200 ppmv of CH_4 ($^{12}CH_4$ or $^{13}CH_4$), respectively. Every week, bottles were flushed with fresh air and CH_4 treatments were renewed. An additional incubation with 1000 ppmv $^{13}CO_2$ was set up as a control for secondary labeling.

Incubation for stable isotope probing with acetate was performed in duplicates for each treatment. Soil (20 g *d.w.s.*) was amended with a final concentration of 10 mM acetate (^{12}C or ^{13}C), dissolved in 6 ml H_2O, 20 ml H_2O, or 40 ml of medium DSMZ 922 (designed for *Methylocapsa acidiphila*; 7), respectively. Fully labeled $^{13}C_2$ sodium acetate (99 atom%) was purchased from Campro Scientific GmbH. Incubations were performed on a shaker (200 rpm)

at 25°C and darkness in 250 ml Schott bottles capped loosely to allow incubations to stay oxic. An additional incubation with 5% $^{13}CO_2$ was set up as a control for secondary labeling. Acetate-free controls received an equal amount of distilled water. Every week, acetate treatments were renewed.

Chemical analyses

For chemical analysis of pH and acetate, aliquots of the soil were removed weekly from each treatment. Concentrations of acetate in the microcosms incubated with either labeled or unlabeled acetate were analyzed by high pressure liquid chromatography (HPLC; Thermo Fisher Scientific, San Jose, CA, USA). For SIP incubations with methane, consumption of CH_4 was monitored by gas chromatography (SRI 8610C gas chromatograph equipped with a flame ionization detector; SRI Instruments, Torrance, CA, USA). The $^{13}CH_4$ (99 atom%) was purchased from Sigma-Aldrich Co.

Nucleic acid extraction and SIP fractionation

For both SIP incubations, every week 0.5 g of soil of each bottle were sampled, frozen immediately in liquid nitrogen and stored at -80°C until further processing. Nucleic acids were extracted from soil using a hexadecyltrimethylammonium bromide (CTAB) -based protocol (30) with minor modifications. Soil (0.5 g) was mixed with 200 µl of zirconia-silica beads (0.1 mm; Roth, Karlsruhe, Germany), 0.5 ml of 6% CTAB extraction buffer, and 0.5 ml phenol chloroform isoamyl alcohol (25:24:1) in 2.0 ml screw-cap tubes. Cells were lysed in a FastPrep instrument (MP Biomedicals, Eschwege, Germany) for 30 s at 5.5 m s^{-1} and supernatants were extracted using twice phenol chloroform isoamyl alcohol (25:24:1) and chloroform isoamyl alcohol (24:1). Nucleic acids were precipitated with polyethylene glycol (PEG) 6000 solution (30%) and dissolved in 100 µl of nuclease free water. In case of the incubations with acetate, the samples showed a high release of fulvic and humic acids and had to be further purified using Illustra MicroSpin S-400 HR spin columns (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions (31).

For stable isotope probing of RNA, 50 µl of extract were treated with RNase-free DNase I for digestion of DNA. RNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and stored at -80°C until further usage. Integrity of nucleic acids was checked on agarose gels and concentration was determined using a NanoDrop instrument (Thermo Fisher Scientific, Schwerte, Germany).

Stable isotope probing fractionation of total DNA extract (5.0 µg) was performed with an initial cesium chloride (CsCl) buoyant density of 1.72 g ml^{-1} subjected to centrifugation at 177 000 x *g* for 36 h at 20°C (32). Gradient centrifugation of RNA was carried out in cesium trifluoroacetate (CsTFA) as described previously (32) with an initial buoyant density of 1.79 g ml^{-1} and centrifugation at 130 000 x *g* for 65 h at 20°C. DNA- and RNA-gradients were fractionated from bottom to top by displacing the gradient medium with nuclease free water at the top of the tube using a syringe pump (Kent Scientific, Torrington, CT, USA) at a flow rate

of 0.45 ml min^{-1}, generating twelve fractions per density gradient. The density of each fraction was determined by refractometry (Reichert, Depew, NY, USA). DNA was recovered by PEG 6000 precipitation with glycogen (23) and dissolved in 30 µl of nuclease free water. RNA was precipitated from CsTFA with two volumes of ethanol and 20 µg glycogen and resuspended in 10 µl of nuclease free water. RNA samples from density gradient fractions were reverse transcribed with random hexamer primers and SuperScript III reverse transcriptase (Invitrogen, Darmstadt, Germany).

Quantitative PCR of *pmoA* and 16S rRNA genes

The abundance of USCα *pmoA* genes and transcripts in the different SIP fractions was quantified by quantitative PCR (qPCR) using primers A189f and Forest675r as previously described (33). The 25 µl reaction mixture contained 12.5 µl of SYBRGreen Jump-Start™ Taq ReadyMix™, 1 µM of each primer, 500 ng BSA ml^{-1}, 4.0 mM $MgCl_2$, 1.0 µl template DNA or cDNA (33).

The abundance of general *pmoA* genes and transcripts in the different SIP fractions was quantified by qPCR using primers A189f and mb661 as previously described (33). The 25 µl reaction mixture contained 12.5 µl of SYBRGreen Jump-Start™ Taq ReadyMix™, 0.667 µM of each primer, 4 mM $MgCl_2$, 1.0 µl template DNA or cDNA.

qPCR of bacterial 16S rRNA genes and transcripts was performed using primers Ba519f and Ba907r as previously described (34). The 25 µl reaction mixture contained 12.5 µl of SYBRGreen Jump-Start™ Taq ReadyMix™, 0.25 µM of each primer, 200 ng BSA ml^{-1}, 4 mM $MgCl_2$, 1.0 µl template DNA or cDNA.

All assays were performed in an iCycler™ (Applied Biosystems, Darmstadt, Germany), respective qPCR standards were used and controls were always run with water instead of DNA or cDNA extract.

CARD-FISH of USCα *pmoA* mRNA

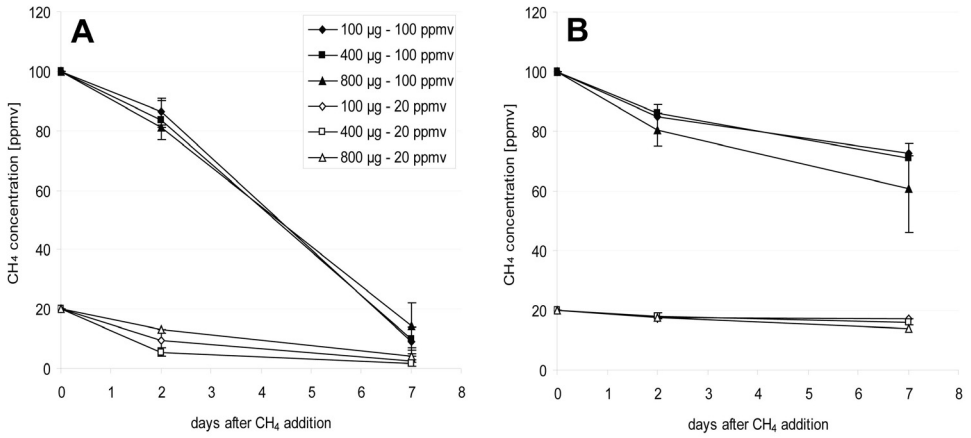
To generate controls for mRNA CARD-FISH, partial *pmoA* genes of USCα from soil (positive control) and of *Methylocapsa acidiphila* DSM13967 and *Methylocystis sp.* SC2 (negative controls) were cloned into *E. coli* Top10 competent cells using primers A189f/Forest675r (33) for soil and A189f/A682r (35) for pure cultures, respectively, and expressed by using vector pBAD as previously described by Pernthaler and Amann (20). Clones were fixed in 2% (vol/vol) formaldehyde for 30 min at room temperature, centrifuged, and washed once with PBS and twice with 50% ethanol in PBS. Cells from the soil incubations after 10 weeks of incubation with 200 ppmv CH_4 were extracted using a Nycodenz density centrifugation method (36), fixed in 4% (vol/vol) formaldehyde for 12-16 h at 4°C, centrifuged, and washed once with PBS. Cells were then resuspended in 50% ethanol in PBS and stored at -20°C until further processing. Polynucleotide antisense RNA probes of USCα *pmoA* were generated by *in vitro* transcription and were simultaneously labeled by incorporating digoxigenin-11-UTP (Roche Molecular Diagnostics, Basel, Switzerland) (37). The hybridization and detection

procedures were based on the protocol described by Pernthaler and Amann (20), with some minor modifications. Five microlitres of cell suspensions were applied to each well of a Teflon-coated glass slide (MAGV, Marburg, Germany) and dried at 46°C. Expression clones of *pmoA* were permeabilized with lysozyme (5 mg ml^{-1} in Tris-EDTA buffer (TE); Sigma, Taufkirchen, Germany) for 30 min at room temperature. Hybridization was performed overnight at 58°C using a formamide concentration of 50% in the hybridization buffer. Finally, slides were stained with 4',6-diamidino-2-phenylindole (DAPI), mounted with the antifading agent Citifluor AF1 (Citifluor, London, UK), and hybridization preparations were visualized by fluorescence microscopy (Axiophot; Carl Zeiss Microimaging GmbH, Jena, Germany).

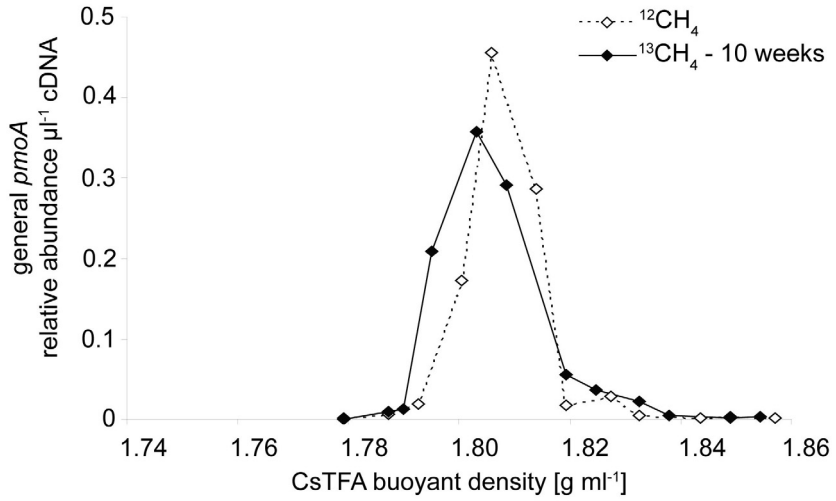
Acknowledgements

We thank Peter Claus, Melanie Klose, and Bianca Pommerenke for skillful technical assistance.

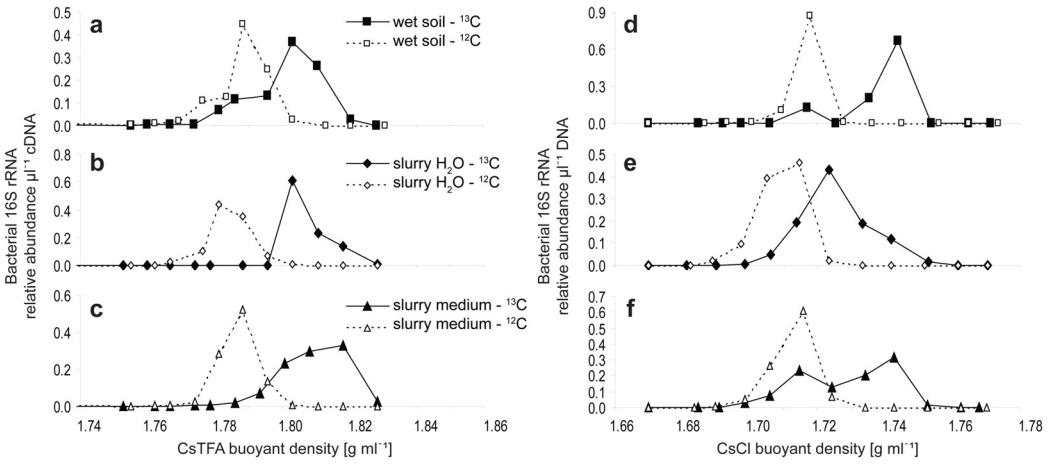
III.1.6 Supplementary material



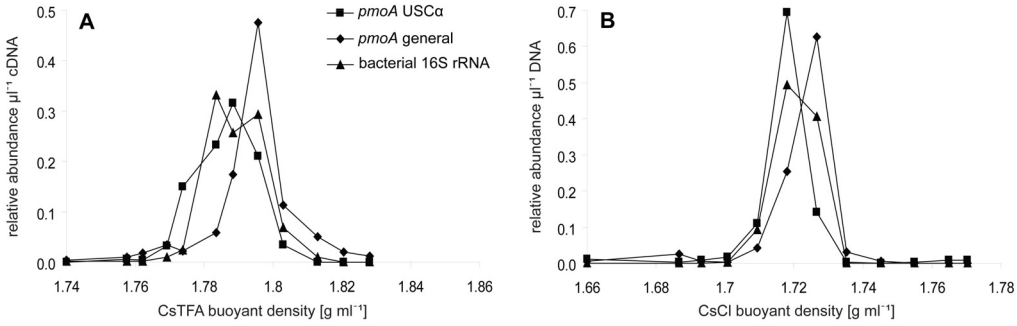
SI figure 1. Uptake of methane in soil microcosms (A; 20 g of Marburg forest soil) and slurries (B; 20 g of Marburg forest soil mixed with 40 ml medium DSM 922) incubated under 20 ppmv and 100 ppmv of methane and amended with different concentrations of acetate (100, 400, or 800 µg g^{-1} *d.w.s.*). Methane concentrations were measured by gas chromatography.



SI figure 2. Distribution of *pmoA* transcripts of general methanotrophs in RNA-SIP after 10 weeks of incubation with 200 ppmv CH_4 (^{12}C or ^{13}C). Distribution of *pmoA* transcripts was measured by real-time PCR of cDNA from gradient fractions.



SI figure 3. Distribution of bacterial 16S rRNA transcripts (a-c) and genes (d-f) in RNA-SIP and DNA-SIP gradients, respectively, after 3 weeks of incubation with 10 mM acetate (^{12}C or ^{13}C). Acetate was added to the soil dissolved in 6 ml H_2O (wet soil), 20 ml H_2O (slurry H_2O), or 40 ml of medium DSMZ 922 (slurry medium), respectively. Distribution of bacterial 16S rRNA transcripts and genes was measured by real-time PCR of cDNA and DNA from gradient fractions.



SI figure 4. Distribution of *pmoA* and bacterial 16S rRNA transcripts (A) and genes (B) in RNA-SIP and DNA-SIP gradients, respectively, after 3 weeks of incubation with 5% $^{13}CO_2$. Distribution of transcripts and genes was measured by real-time PCR of cDNA and DNA from gradient fractions.

III.1.7 References

1. Denman, K. L. *et al.* Couplings between changes in the climate system and biogeochemistry. In *Climate Change 2007: The Physical Science Basis*. Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, *et al*. (eds). Cambridge, UK: Cambridge University Press, pp. 499–587 (2007).

2. Dalal, R. C. & Allen, D. E. Greenhouse gas fluxes from natural ecosystems. *Aust. J. Bot.* **56**, 369–407 (2008).

3. Hanson, R. S. & Hanson, T. E. Methanotrophic bacteria. *Microbiol. Rev.* **60**, 439–471 (1996).

4. Knief, C., Lipski, A. & Dunfield, P. F. Diversity and activity of methanotrophic bacteria in different upland soils. *Appl. Environ. Microbiol.* **69**, 6703–6714 (2003).

5. Ricke, P. *et al.* First genome data from uncultured upland soil cluster alpha methanotrophs provide further evidence for a close phylogenetic relationship to *Methylocapsa acidiphila* B2 and for high-affinity methanotrophy involving particulate methane monooxygenase. *Appl. Environ. Microbiol.* **71**, 7472–7482 (2005).

6. Kolb, S., Knief, C., Dunfield, P. F. & Conrad, R. Abundance and activity of uncultured methanotrophic bacteria involved in the consumption of atmospheric methane in two forest soils. *Environ. Microbiol.* **7**, 1150–1161 (2005).

7. Dedysh, S. N. *et al. Methylocapsa acidiphila* gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from *Sphagnum* bog. *Int. J. Syst. Evol. Microbiol.* **52**, 251–261 (2002).

8. Kolb, S. The quest for atmospheric methane oxidizers in forest soils. *Environ. Microbiol. Rep.* **1**, 336–346 (2009).

9. Dunfield, P.F. *et al.* Isolation of a *Methylocystis* strain containing a novel *pmoA*-like gene. *FEMS Microbiol. Ecol.* **41**, 17–26 (2002).

10. Baani, M. & Liesack, W. Two isozymes of particulate methane monooxygenase with different methane oxidation kinetics are found in *Methylocystis* sp. strain SC2. *Proc. Natl. Acad. Sci. USA* **105**, 10203–10208 (2008).

11. Kravchenko, I. K., Kizilova, A. K., Bykova, S. A., Men'ko, E. V. & Gal'chenko, V. F. Molecular analysis of high-affinity methane-oxidizing enrichment cultures isolated from a forest biocenosis and agrocenoses. *Microbiology* **79**, 106-114 (2010).

12. Belova, S. E. *et al.* Acetate utilization as a survival strategy of peat-inhabiting *Methylocystis* spp.. *Environ. Microbiol. Rep.* (in press): doi:10.1111/j.1758-2229.2010.00180.x (2010).

13. Dunfield, P. F., Belova, S. E., Vorob'ev, A. V., Cornish, S. L. & Dedysh, S. N. *Methylocapsa aurea* sp. nov., a facultatively methanotrophic bacterium possessing a particulate methane monooxygenase. *Int. J. Syst. Evol. Microbiol.* (in press): doi: 10.1099/ijs.0.020149-0. (2010).

14. Dumont, M. G. & Murrell, J. C. Stable isotope probing – linking microbial identity to function. *Nat. Rev. Microbiol.* **3**, 499-504 (2005).

15. Holmes, A. J. *et al.* Characterization of methanotrophic bacterial populations in soils showing atmospheric methane uptake. *Appl. Environ. Microbiol.* **65**, 3312–3318 (1999).

16. Bull, I. D., Parekh, N. R., Hall, G. H., Ineson, P. & Evershed, R. P. Detection and classification of atmospheric methane oxidizing bacteria in soil. *Nature* **405**, 175-178 (2000).

17. Chen, Y. *et al.* Diversity of the active methanotrophic community in acidic peatlands as assessed by mRNA and SIP-PLFA analyses. *Environ. Microbiol.* **10**, 446–459 (2008).

18. Bengtson, P. *et al.* Links between methanotroph community composition and CH_4 oxidation in a pine forest soil. *FEMS Microbiol. Ecol.* **70**, 356–366 (2009).

19. Menyailo, O. V., Abraham, W.-R. & Conrad, R. Tree species affect atmospheric CH_4 oxidation without altering community composition of soil methanotrophs. *Soil Biol. Biochem.* **42**, 101–107 (2010).

20. Pernthaler, A. & Amann, R. Simultaneous fluorescence in situ hybridization of mRNA and rRNA in environmental bacteria. *Appl. Environ. Microbiol.* **70**, 5426–5433 (2004).

21. Neufeld, J., Dumont, M. G., Vohra, J. & Murrell, J. Methodological considerations for the use of stable isotope probing in microbial ecology. *Microb. Ecol.* **53**, 435–442 (2007).

22. Lueders, T. Stable isotope probing of hydrocarbon-degraders. In: Timmis KN (ed). *Handbook of Hydrocarbon and Lipid Microbiology*. Springer: Berlin, Heidelberg, pp 4011–4026 (2009).

23. Neufeld JD, *et al.* DNA stable-isotope probing. *Nat. Protoc.* **2**, 860-866 (2007).

24. Manefield, M., Whiteley, A. S., Griffiths, R. I. & Bailey, M. J. RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Appl. Environ. Microbiol.* **68**, 5367–5373 (2002).

25. Whiteley, A. S., Manefield, M. & Lueders, T. Unlocking the 'microbial black box' using RNA-based stable isotope probing technologies. *Curr. Opin. Microbiol.* **17**, 67–71 (2006).

26. Degelmann, D. M., Borken, W., Drake, H. L. & Kolb, S. Different atmospheric methane-oxidizing communities in European beech and Norway spruce soils. *Appl. Environ. Microbiol.* **76**, 3228–3235 (2010).

27. West, A. E. & Schmidt, S. K. Acetate stimulates atmospheric CH_4 oxidation by an alpine tundra soil. *Soil Biol. Biochem.* **31**, 1649-1655 (1999).

28. Dedysh, S. H., Knief, C. & Dunfield, P. F. *Methylocella* species are facultatively methanotrophic. *J. Bacteriol.* **187**, 4665–4670 (2005).

29. Huang, W. E. *et al.* Resolving genetic functions within microbial populations: in situ analyses using rRNA and mRNA stable isotope probing coupled with single-cell Raman-fluorescence in situ hybridization. *Appl. Environ. Microbiol.* **75**, 234-241 (2009).

30. Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G. & Bailey, M. J. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. *Appl. Environ. Microbiol.* **66**, 5488–5491 (2000).

31. Wang, Y., Morimoto, S., Ogawa, N., Oomori, T. & Fujii, T. An improved method to extract RNA from soil with efficient removal of humic acids. *J. Appl. Microbiol.* **107**, 1168–1177 (2009).

32. Lueders, T., Manefield, M. & Friedrich, M. W. Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ. Microbiol.* **6**, 73–78 (2004).

33. Kolb, S., Knief, C., Stubner, S. & Conrad, R. Quantitative detection of methanotrophs in soil by novel *pmoA* targeted real-time PCR assays. *Appl. Environ. Microbiol.* **69**, 2423–2429 (2003).

34. Stubner, S. Enumeration of 16S rDNA of *Desulfotomaculum* lineage 1 in rice field soil by real-time PCR with SybrGreen detection. *J. Microbiol. Methods* **50**, 155–164 (2002).

35. Holmes, A. J., Costello, A., Lidstrom, M. E. & Murrell, J. C. Evidence that particulate methane monooxygenase and ammonium monooxygenase may be evolutionarily related. *FEMS Microbiol. Lett.* **132**, 203–208 (1995).

36. Caracciolo, A. B., Grenni, P., Cupo, C. & Rossetti, S. In situ analysis of native microbial communities in complex samples with high particulate loads. *FEMS Microbiol. Lett.* **253**, 55-58 (2005).

37. Zwirglmaier, K., Ludwig, W. & Schleifer, K.-H. Improved fluorescence in situ hybridization of individual microbial cells using polynucleotide probes: the network hypothesis. *Syst. Appl. Microbiol.* **26**, 327–337 (2003).

III.2 RING-FISH and enrichment of Upland Soil Cluster α in Marburg forest soil

III.2.1 RING-FISH and cell sorting of Upland Soil Cluster α

The probes that were designed for application of RING-FISH were first tested with *E. coli* clones harboring the partial gene of the particulate methane monooxygenase (*pmoA*) of Upland Soil cluster α from Marburg forest soil as positive control and partial *pmoA* genes of *Methylocapsa acidiphila* and *Methylocystis* sp. strain SC2 as negative controls. RING-FISH for all probes was evaluated under formamide concentrations of 5-55%. The potential of the probes to form secondary structures was analysed *in silico* (RNAdraw V1.1, www.rnadraw.com, Matzura et *al.*, 1996). It was suggested (Zwirglmaier *et al.*, 2003) that the feature of RNA polynucleotide probes to form a network within and around the cell envelope is decisive for the appearance of halo signals of target bacteria.

III.2.1.1 RING-FISH with long RNA polynucelotide probe MF02

The long RNA polynucleotide probe MF02 (~500 bp), derived from the PCR product of an USCα soil clone (see II.10.1), was analysed *in silico* using RNAdraw and showed a high potential to form secondary structures at hybridization conditions of 53°C and 15% of formamide (see figure III.2-1).

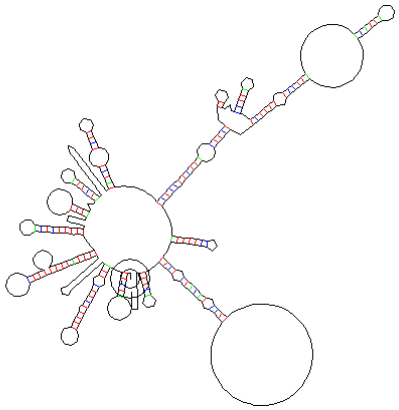


Figure III.2-1: Secondary structure model (RNAdraw V1.1) of USCα *pmoA* RNA polynucleotide probe MF02 (~500 bp) with 15% formamide in the hybridization buffer and hybridization temperature of 53°C.

Because USCα has not been isolated so far, no “real” cells of this cluster could be used as positive control for RING-FISH. Therefore, clones harboring the partial gene of the particulate methane monooxygenase of USCα from Marburg forest soil were used as positive control. These cells showed bright whole cell fluorescence after hybridization. Occasionally, halo signals could also be observed, but only in small, single areas on the well, never in larger areas or as dominating signal type after hybridization (see figure III.2-2). This was

independent from formamide concentration and incubation time. Above a formamide concentration of 35%, all signals completely disappeared.

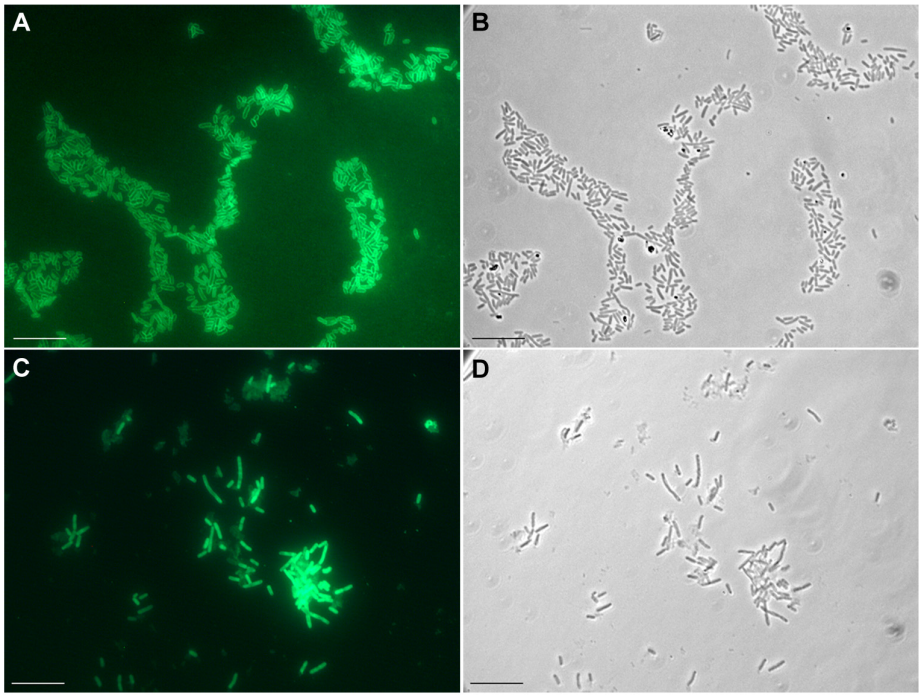


Figure III.2-2: Halo signals (A) and whole cell fluorescence (C) observed after RING-FISH of particulate methane monooxygenase (*pmoA*) genes of USCα in clones harboring *pmoA* of Upland Soil cluster α (positive control) using polynucleotide probe MF02. Fluorescence images (A and C) and respective phase contrast (B and D). Bars = 10 µm.

The negative controls, *E. coli* clones harboring *pmoA* of *Methylocapsa acidiphila* and *Methylocystis* sp. strain SC2, also continuously showed whole cell fluorescence after RING-FISH, independent from formamide concentration and incubation time (see figure III.2-3). Above a formamide concentration of 35%, signals completely disappeared. This indicates that hybridizations were not specific enough to target only *pmoA* of USCα, since successfull discrimination between the control clones could not be achieved with polynucelotide probe MF02.

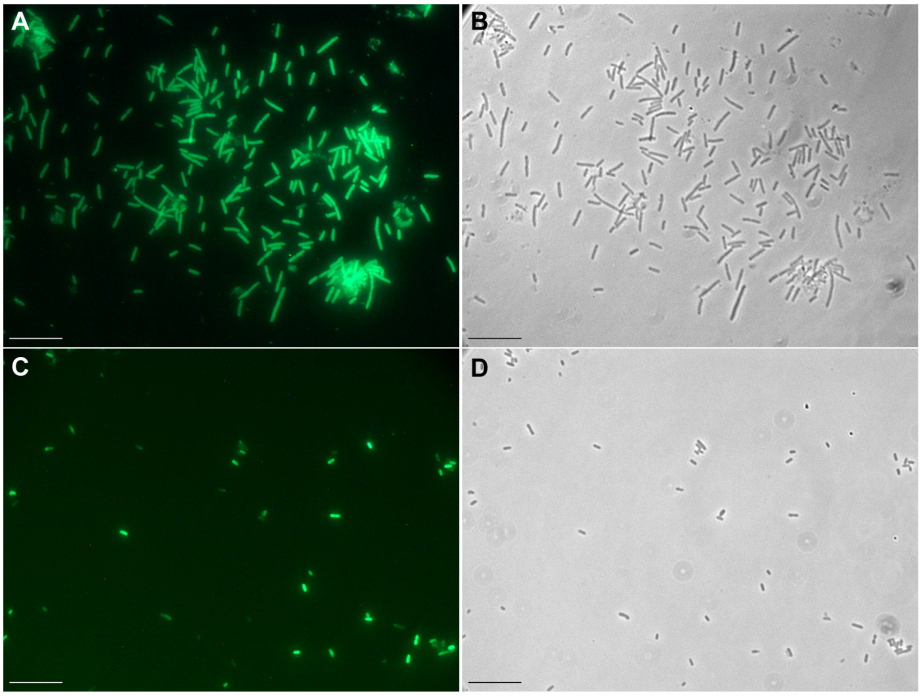


Figure III.2-3: Whole cell fluorescence observed after RING-FISH of particulate methane monooxygenase (*pmoA*) genes of USCα in clones harboring *pmoA* of *Methylocapsa acidiphila* (A and B) and *Methylocystis* sp. strain SC2 (C and D) (negative controls) using polynucleotide probe MF02. Fluorescence images (A and C) and respective phase contrast (B and D). Bars = 10 µm.

To exclude the possibility that these observations resulted from cells damaged by wrong fixation, thawing, or other circumstances, clones were fixed new every month and only fresh aliquots were used for hybridization. Also clones fixed with 2% paraformaldehyde instead of 4% were tested. But all hybridizations resulted in either whole cell fluorescence or no signal of the positive and negative controls.

Another negative control for RING-FISH was represented by *Methylocapsa acidiphila*. This was done to test whether actual methanotroph cells might behave differently in the hybridization procedure compared to clones harboring the same target gene. *Methylocapsa acidiphila* cells showed no signal after RING-FISH of *pmoA* gene of USCα in contrast to the *E. coli* harboring the same *pmoA* gene. This observation was independent from formamide concentration and incubation time, suggesting that the different cell morphologies of clones and the methanotroph might have had a significant impact on the hybridization results and therefore clones might not be universally applicable as controls in RING-FISH. Above a formamide concentration of 80%, cells suddenly showed a bright halo signal (see figure III.2-4). To test the specificity of this halo signal, cells of *Methylocapsa acidiphila* were also hybridized with a probe targeting archaeal *amoA* as a negative control, also leading to bright halo signals. This indicates that formamide concentrations of 80% and above might have damaged the cell wall and allowed for unspecific formation of the probe network.

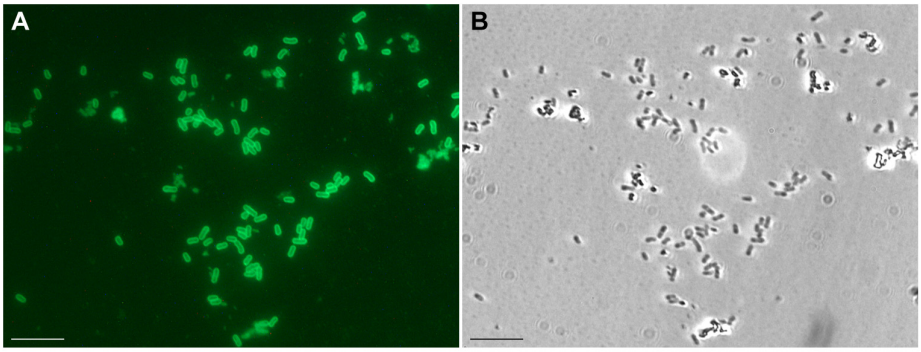


Figure III.2-4: Unspecific halos signals observed after RING-FISH of particulate methane monooxygenase (*pmoA*) genes of USCα in *Methylocapsa acidiphila* (negative control) at formamide concentration of 80% using polynucleotide probe MF02. Fluorescence image (A) and respective phase contrast (B). Bars = 10 µm.

Hybridizations using RING-FISH probe MF02 with cells extracted from Marburg forest soil using Histodenz density gradient centrifugation (see II.10.3) showed sporadic halo signals when hybridized at 53°C with 15% formamide in the hybridization buffer (see figure III.2-5). Due to the unspecific results obtained with the clones, however, no statement can be made about the specificity of these signals in soil.

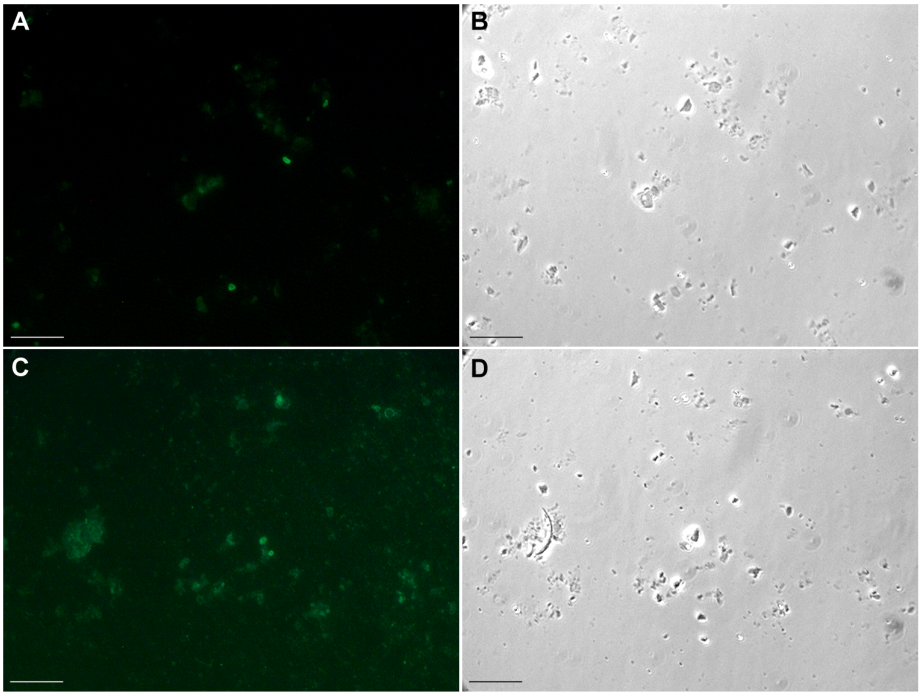


Figure III.2-5: Halo signals observed after RING-FISH of particulate methane monooxygenase (*pmoA*) genes of USCα in cells extracted from Marburg forest soil using polynucleotide probe MF02. Fluorescence images (A and C) and respective phase contrast (B and D). Bars = 10 µm.

III.2.1.2 RING-FISH with monospecific oligo-oligonucleotide probes

The monospecific RNA oligo-oligonucleotide probe MF08_25-175 (175 bp) targeting *pmoA* of USCα (see II.10.5.2), was analysed *in silico* using RNAdraw and showed a high potential to form secondary structures at hybridization conditions of 53°C and 15% of formamide (see figure III.2-6).

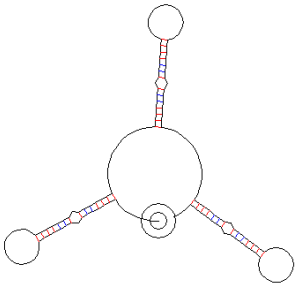


Figure III.2-6: Secondary structure model (RNAdraw V1.1) of monospecific USCα *pmoA* RNA oligo-oligonucleotide probe MF08_25-175 (175 bp) with 15% formamide in the hybridization buffer and hybridization temperature of 53°C.

Also using this probe, cells from clones harboring *pmoA* of Upland Soil cluster α (positive control) showed bright whole cell fluorescence and only single partial halos, independent from formamide concentration and incubation time (see figure III.2-7). Above a formamide concentration of 30% and below an incubation time of 12 h, signals completely disappeared.

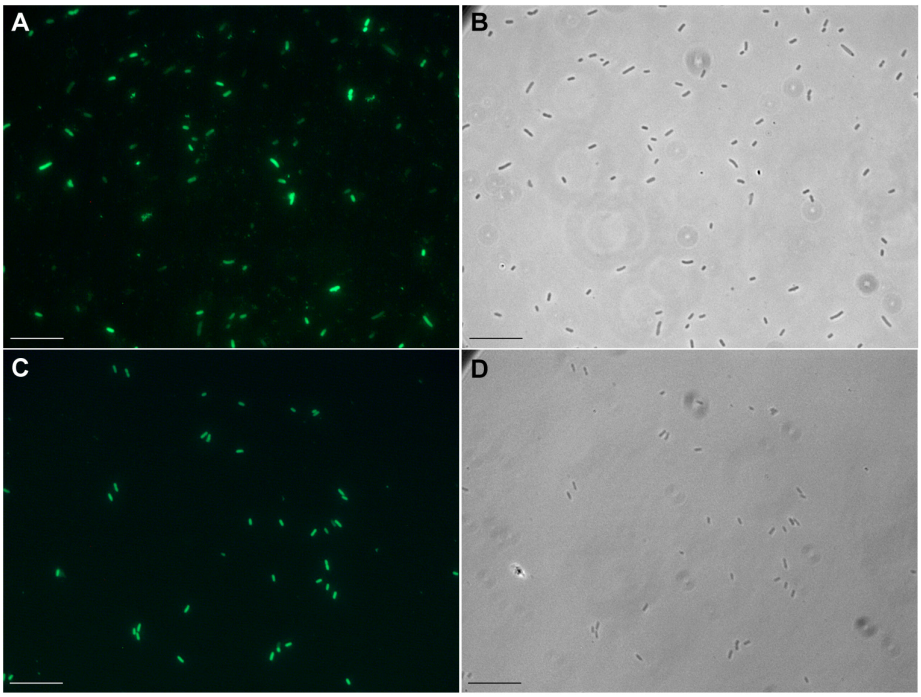


Figure III.2-7: Partial halo signals (A) and whole cell fluorescence (C) observed after RING-FISH of particulate methane monooxygenase (*pmoA*) genes of USCα in clones harboring *pmoA* of Upland Soil cluster α (positive control) using the monospecific oligo-oligonucleotide probe MF08_25-175. Fluorescence images (A and C) and respective phase contrast (B and D). Bars = 10 µm.

The same was observed with the negative controls, clones harboring *pmoA* of *Methylocapsa acidiphila* and *Methylocystis* sp. strain SC2. Cells consistently showed whole cell fluorescence, independent from formamide concentration and incubation time (see figure III.2-8). Above a formamide concentration of 30%, signals completely disappeared. This shows that also with this probe, no successful discrimination between the control clones to target *pmoA* of USCα could be achieved.

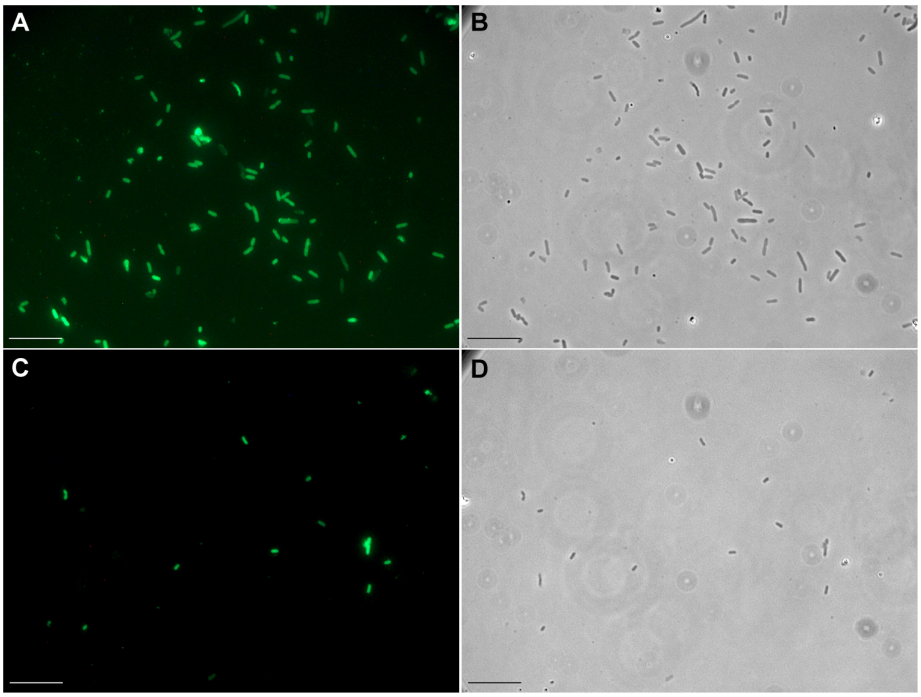


Figure III.2-8: Whole cell fluorescence observed after RING-FISH of particulate methane monooxygenase (*pmoA*) genes of USCα in clones harboring *pmoA* of *Methylocapsa acidiphila* (A and B) and *Methylocystis* sp. strain SC2 (C and D) (negative controls) using the monospecific oligo-oligonucleotide probe MF08_25-175. Fluorescence images (A and C) and respective phase contrast (B and D). Bars = 10 µm.

Also with probe MF08_25-175, hybridizations using RING-FISH with cells extracted from Marburg forest soil using Histodenz density gradient centrifugation (see II.10.3) showed sporadic halo signals when hybridized at 53°C with 15% formamide in the hybridization buffer (see figure III.2-9). Due to the unspecific results obtained with the clones, however, no statement can be made about the specificity of these signals in soil.

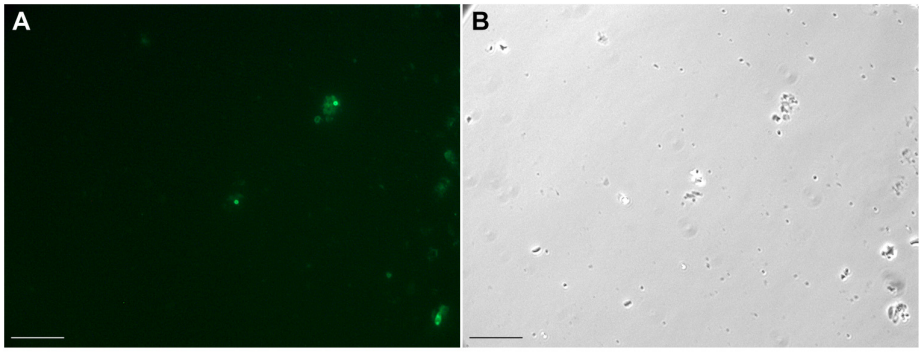


Figure III.2-9: Halo signals observed after RING-FISH of particulate methane monooxygenase (*pmoA*) genes of USCα in cells extracted from Marburg forest soil using the monospecific oligo-oligonucleotide probe MF08_25-175. Fluorescence image (A) and respective phase contrast (B). Bars = 10 µm.

The monospecific RNA oligo-oligonucleotide probe RA14_GC121 (121 bp) targeting *pmoA* of USCα (see II.10.5.2), was analysed *in silico* using RNAdraw and showed a high potential to form secondary structures at hybridization conditions of 53°C and 10% of formamide (see figure III.2-10).

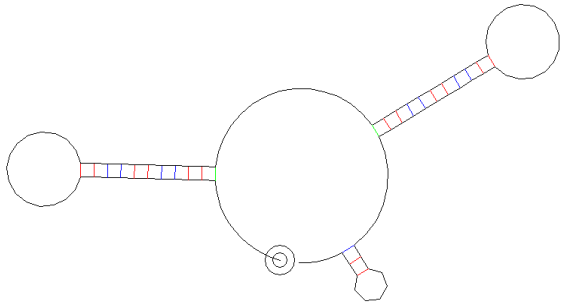


Figure III.2-10: Secondary structure model (RNAdraw V1.1) of monospecific USCα *pmoA* RNA oligo-oligonucleotide probe RA14_GC121 (121 bp) with 10% formamide in the hybridization buffer and hybridization temperature of 53°C.

After RING-FISH and detection, cells from clones harboring *pmoA* of Upland Soil cluster α (positive control) showed bright whole cell fluorescence and also only single partial halos, independent from formamide concentration and incubation time (see figure III.2-11). Above a formamide concentration of 30% and below an incubation time of 12 h, signals completely disappeared.

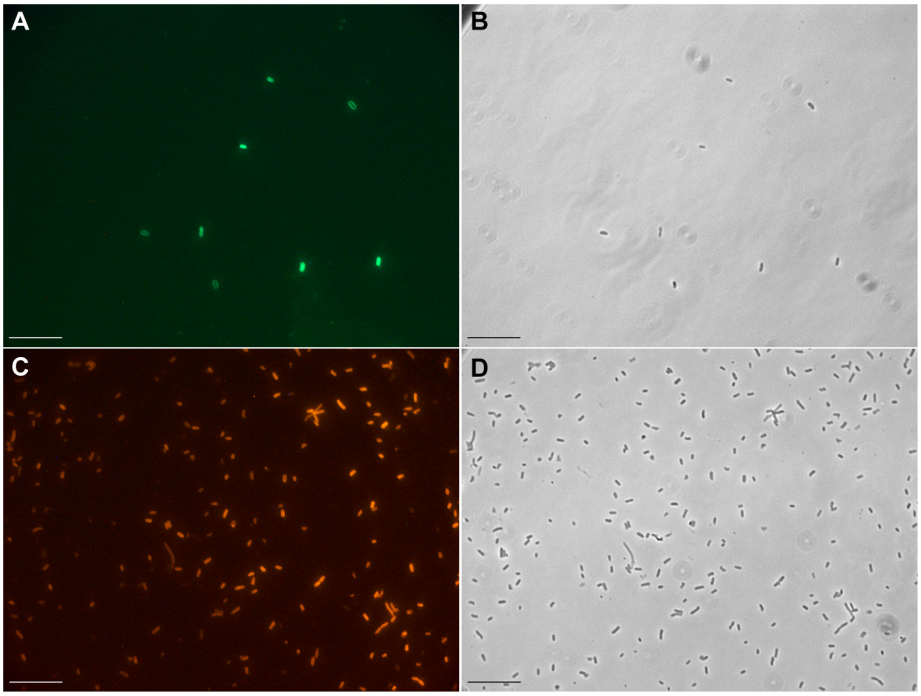


Figure III.2-11: Partial halo signals and whole cell fluorescence observed after RING-FISH of particulate methane monooxygenase (*pmoA*) genes of USCα in clones harboring *pmoA* of Upland Soil cluster α (positive control) using the monospecific oligo-oligonucleotide probe RA14_GC121. Fluorescence images (A and C) and respective phase contrast (B and D). Bars = 10 µm.

Like for the monospecific oligo-oligonucleotide probe RA14_GC121, negative controls, clones harboring *pmoA* of *Methylocapsa acidiphila* and *Methylocystis* sp. strain SC2, continuously showed whole cell fluorescence after RING-FISH, independent from formamide concentration and incubation time (see figure III.2-12). Above a formamide concentration of 30%, signals completely disappeared. These results indicate that there was no difference in the hybridization efficiency and specificity between probe MF08_25-175 with poly-A spacer regions and probe RA14_GC121 containing GC spacers. Also with probe RA14_GC121, specific hybridization of only USCα *pmoA* was not possible.

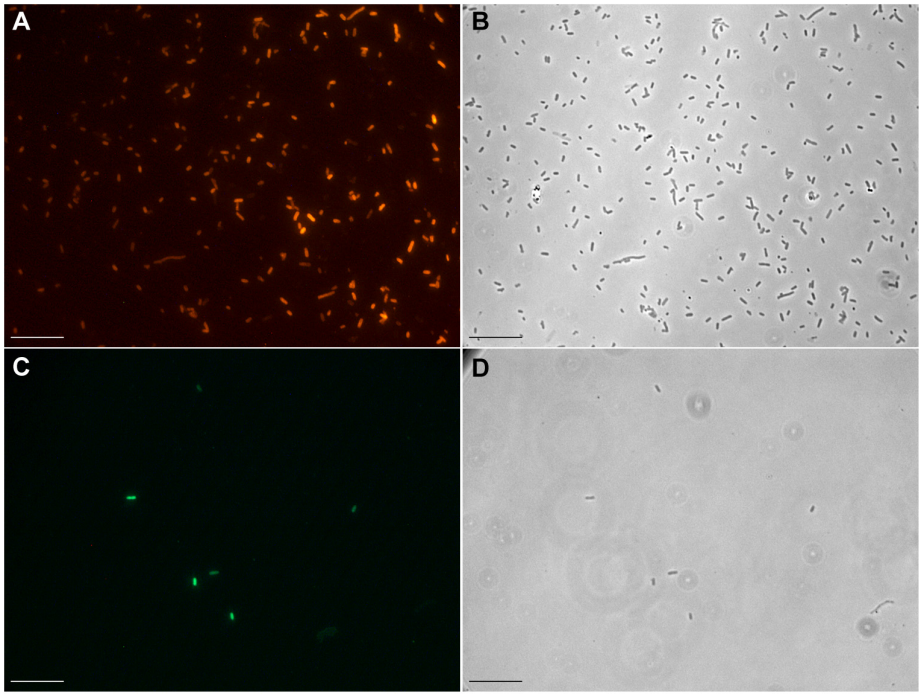


Figure III.2-12: Whole cell fluorescence observed after RING-FISH of particulate methane monooxygenase (*pmoA*) genes of USCα in clones harboring *pmoA* of *Methylocapsa acidiphila* (A and B) and *Methylocystis* sp. strain SC2 (C and D) (negative controls) using the monospecific oligo-oligonucleotide probe RA14_GC121. Fluorescence images (A and C) and respective phase contrast (B and D). Bars = 10 µm.

As for the other probes, hybridizations using RING-FISH with probe RA14_GC121 cells extracted from Marburg forest soil using Histodenz density gradient centrifugation (see II.10.3) resulted in sporadic halo signals when hybridized at 53°C with 15% formamide in the hybridization buffer (see figure III.2-9). But again, due to the unspecific results obtained with the clones, no statement can be made about the specificity of these signals in soil.

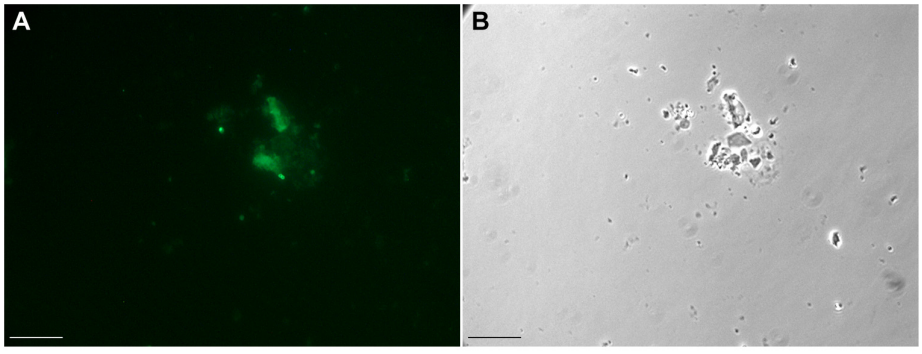


Figure III.2-13: Halo signals observed after RING-FISH of particulate methane monooxygenase (*pmoA*) genes of USCα in cells extracted from Marburg forest soil using the monospecific oligo-oligonucleotide probe RA14_GC121. Fluorescence image (A) and respective phase contrast (B). Bars = 10 µm.

III.2.3 Cell sorting

Cell sorting was applied despite the unspecific RING-FISH results to test whether the probe networks around the target cells, essential for efficient cell sorting, after RING-FISH might be present but just not visible due to additional whole cell fluorescence. Cell sorting was carried out with *E. coli* clones harboring *pmoA* of USCα (positive control) or of *Methylocapsa acidiphila* (negative control), respectively, using RING-FISH RNA polynucleotide probe MF02 and monospecific oligo-oligonucelotide probe RA14_GC121 targeting the *pmoA* gene of Upland Soil cluster α methanotrophs for hybridization in solution (see II.10.5.1.2 and II.10.5.2.2) prior to immobilization (see II.10.5.4). The microplate was directly used for PCR of the bacterial 16S rRNA gene. PCR directly targeting *pmoA* of USCα could not be used because this would also have amplified the *pmoA* DNA coating present in the wells.
In most of the cell sorting approaches, no 16S rRNA gene PCR products of both clones were detectable. If the washing steps were decreased, PCR products could be detected, but were random and unspecific (see figure III.2-14), comparable to the RING-FISH results.

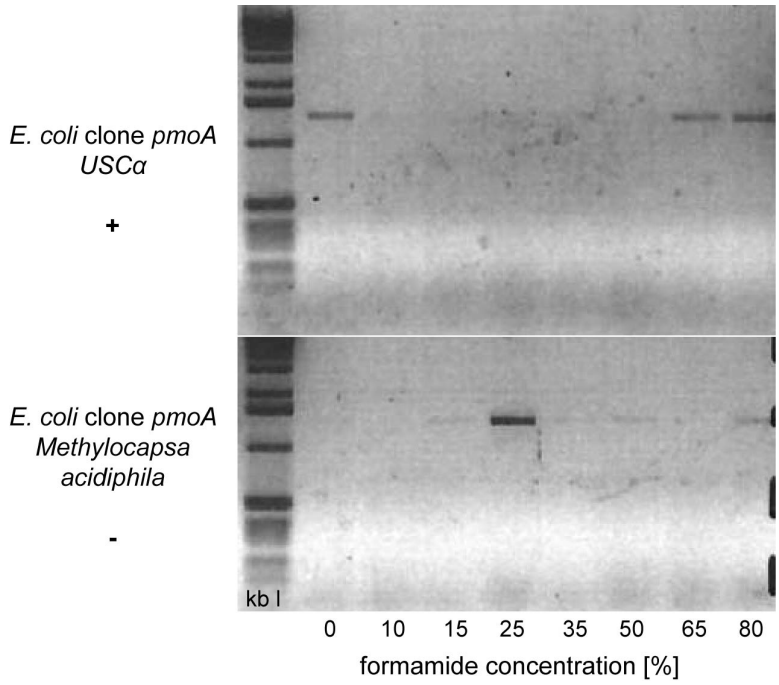


Figure III.2-14: Unspecific PCR detection of *E. coli* clones harboring *pmoA* of USCα (positive control) or of *Methylocapsa acidiphila* (negative control) obtained after immobilization with reduced washing steps. For RING-FISH in solution, USCα *pmoA* gene targeted RING-FISH probe MF02 was used.

This suggests that for the clones, no network formation of probe during RING-FISH in solution took place, hence cells could not hybridize to the DNA-coating of the wells, were finally washed out and not detected by PCR. Fewer washing steps led to unhybridized cells remaining in the wells, which were then detected by PCR. Specific and successful cell sorting could not be observed. Cells extracted from Marburg forest soil were also used as positive

control for cell sorting but detected bands were also very random and not linked to formamide concentration in the hybridization buffer. This might have resulted from unhybridized cell aggregates remaining in the wells, leading to different results in every immobilization.

III.2.2 Enrichment approaches of Upland Soil Cluster α

III.2.2.1 Enrichment approach 1: Soil substrate membrane system (SSMS)

Enrichment or isolation of Upland Soil cluster α using conventional isolation strategies and media so far remained unsuccessful. Therefore, the soil substrate membrane system (SSMS) was tested in this study as an approach to enrich USCα because this technique closely mimics the natural terrestrial environment of soil bacteria, providing natural non-sterilized wet soil as the sole growth component in immediate contact with a polycarbonate membrane as a surface for microcolony formation. The system was set up using Marburg forest soil, incubated with 20 ppmv CH_4 and potential growth of USCα was monitored using mRNA CARD-FISH (see II.10.6.2) and qPCR (see II.8).

The SSMS consumed about 403 nmol CH_4 per week, throughout the whole incubation period. After 3 weeks of incubation in the SSMS, mRNA CARD-FISH of USCα *pmoA* detected single cells, but no microcolonies on the filter pieces (see figure III.2-14).

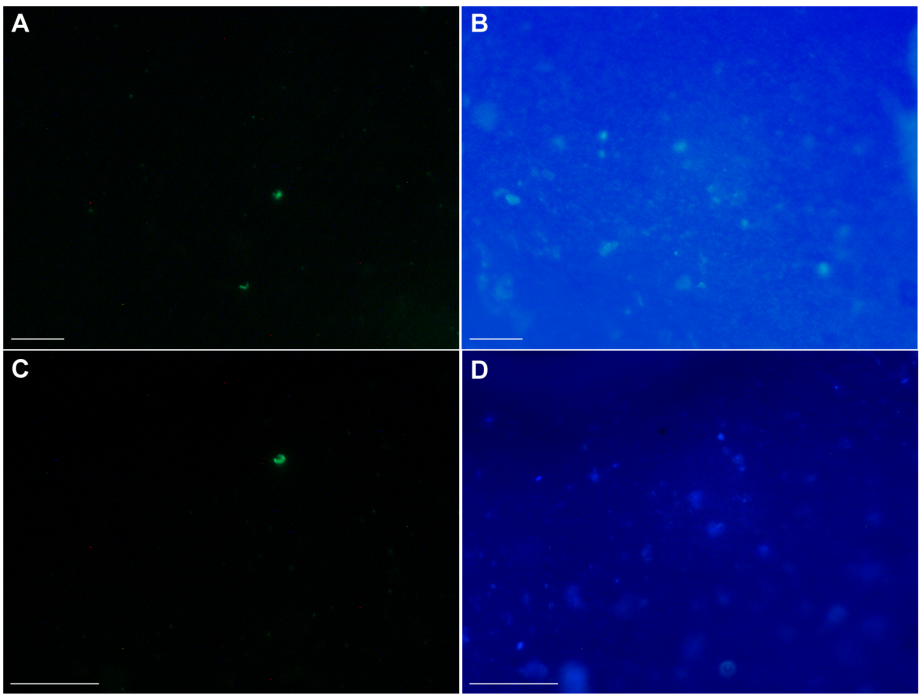


Figure III.2-14: Detection of USCα *pmoA* mRNA transcripts by application of CARD-FISH with USCα *pmoA* antisense probe on filters of soil substrate membrane system after 3 weeks of incubation with 20 ppmv CH_4. Fluorescence images for *pmoA* CARD-FISH (A and C) and respective phase contrast (B and D). Bars = 10 µm.

Also after 6 weeks of incubation, still no microcolonies could be observed by CARD-FISH, only single cells (see figure III.2-15). This indicates that USCα cells were present on the filters and actively transcribed *pmoA* but did not grow during the 6 weeks of incubation under 20 ppmv CH_4. Quantitative PCR with DNA extracts from filter pieces detected *pmoA* genes of USCα throughout the whole incubation period. Copy numbers, however, were not comparable, because quantities varied too much between duplicates and different incubation time points, probably due to an uneven distribution of the cells on the filters.

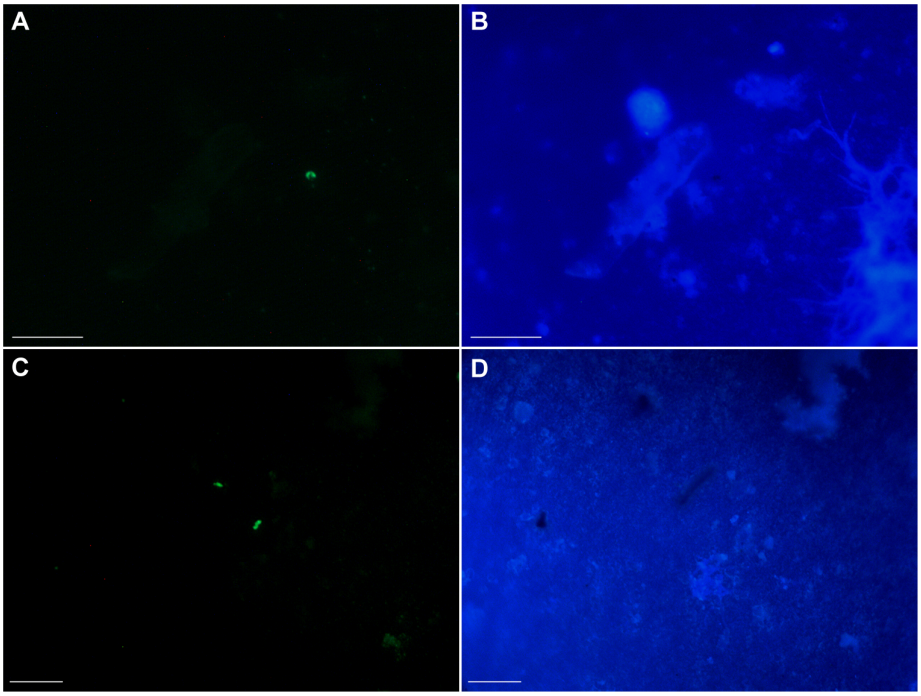


Figure III.2-15: Detection of USCα *pmoA* mRNA transcripts by application of CARD-FISH with USCα *pmoA* antisense probe on filters of soil substrate membrane system after 6 weeks of incubation with 20 ppmv CH_4. Fluorescence images for *pmoA* CARD-FISH (A and C) and respective phase contrast (B and D). Bars = 10 µm.

III.2.2.2 Enrichment approach 2: Incubation of Marburg forest soil with alternating acetate and CH_4 treatments

Because of recent results showing that methanotrophs are capable of using acetate as carbon source (West & Schmidt, 1999; Dunfield *et al.*, 2010), an enrichment approach was set up using alternating incubation periods of acetate and methane. The intention was to provide acetate for growth to USCα and methane for survival in between the acetate incubation when acetate was completely depleted. In increasing cycles, this should lead to the starvation of the microbial community during absence of acetate and to an enrichment of methanotrophs like USCα, which can survive on methane but might not necessarily grow on it. Normal soil (20 g) and slurry (20 g soil + 40 ml of medium DSM 922, see table II.2-1) microcosms were incubated under 2 ppmv, 20 ppmv, and 100 ppmv of methane, respectively, with three different concentrations of acetate (100, 400, or 800 µg g^{-1} *d.w.s.*). HPLC analysis (see II.4.5) showed that acetate was completely consumed in all incubations within 2 days after addition. The soil microcosms consistently oxidized methane during the incubation time (see figure III.2-16). Methane oxidation was higher using 100 ppmv CH_4 compared to 20 ppmv. The different acetate treatments did not affect methane uptake. Consumption of methane in the 2 ppmv incubations could not be measured, because bottles were constantly exposed to fresh air to avoid limitation of methane in the microcosms.

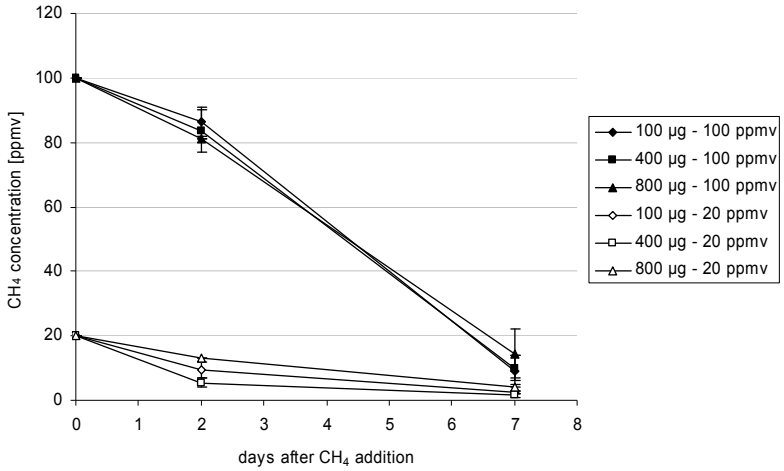


Figure III.2-16: Uptake of methane in soil microcosms incubated under 20 ppmv and 100 ppmv of methane and amended with different concentrations of acetate (100, 400, or 800 µg g^{-1} *d.w.s.*). Methane concentrations were measured by gas chromatography.

The slurry microcosms consumed much less methane in comparison to the “normal” soil incubations (see figure III.2-17), possibly due to disturbance of the USCα during shaking. But also in this case, the different acetate treatments had no effect on the methane consumption.

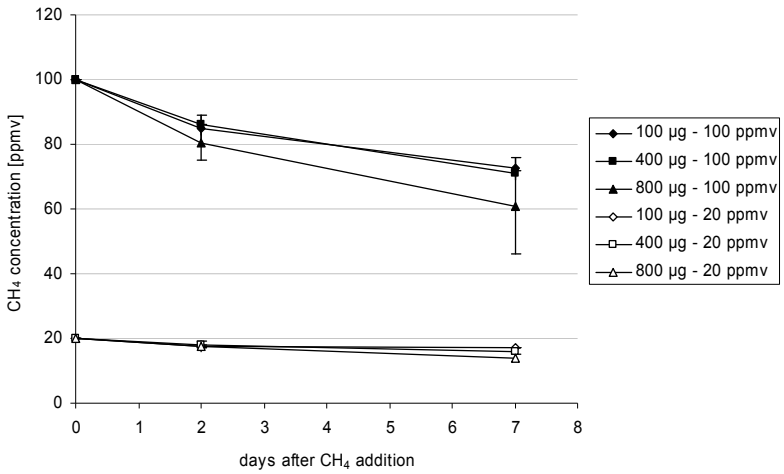


Figure III.2-17: Uptake of methane in slurry microcosms (with medium DSM 922) incubated under 20 ppmv and 100 ppmv of methane and amended with different concentrations of acetate (100, 400, or 800 µg g^{-1} *d.w.s.*). Methane concentrations were measured by gas chromatography.

After 4 cycles of acetate addition (~12 weeks), T-RFLP of the bacterial 16S rRNA genes in the microcosms incubated under 100 ppmv CH_4 and treated with 400 µg acetate g^{-1} *d.w.s.* showed a strong enrichment of a 154 bp fragment in comparison to the untreated Marburg forest soil (see figure III.2-18).

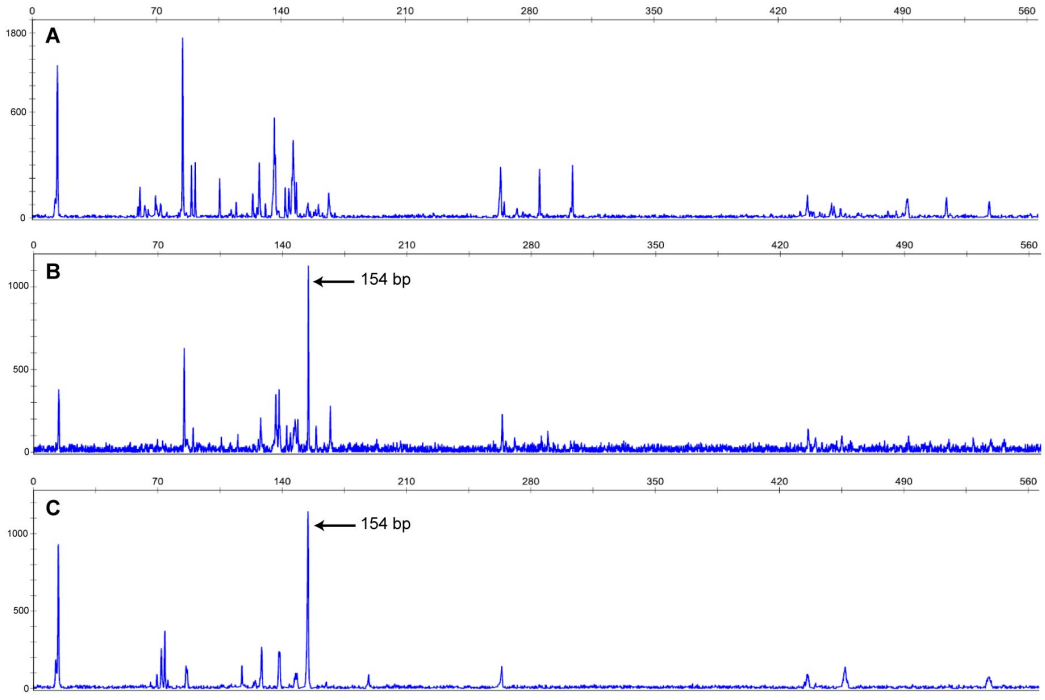


Figure III.2-18: T-RFLP analysis of bacterial 16S rRNA genes from soil microcosms. Untreated Marburg forest soil (A) and soil microcosms incubated under 100 ppmv CH_4 and treated with 400 µg acetate g^{-1} *d.w.s.* for 12 weeks (B and C) .

Soil from these incubations was inoculated into 20 ml of medium DSM 922 with 100 µg acetate ml^{-1} and 100 ppmv CH_4, and shaken at ~170 rpm and 25°C in the dark. After 1 week, the enrichments turned slightly turbid and were further inoculated into fresh medium. After 2 further inoculations, turbid growth could be observed within 2 days. The bacterial composition of these enrichments was checked by T-RFLP, showing the enrichments were nearly pure cultures with one very dominant peak at 154 bp fragment length (see figure III.2-19), as observed in the initial incubations.

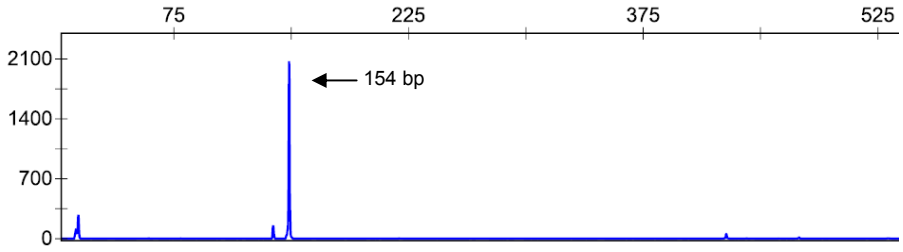


Figure III.2-19: T-RFLP analysis of bacterial 16S rRNA genes in enrichment culture. Restriction analysis was performed using *Msp*I restriction enzyme.

pmoA, however, could not be detected by PCR, which was supported by the fact that the enrichments did not consume methane and were not able to grow without addition of acetate. Quantitative PCR of *pmoA* (see II.8) also detected no growth of USCα. Copy numbers remained stable at around 14.2±0.11 $x10^5$ *pmoA* copies per gram dry weight of soil. Furthermore, the bacterial 16S rRNA gene contributing to the 154 bp peak observed in the T-RFLP, was cloned and sequenced. Sequence analysis using NCBI nucleotide BLAST (www.ncbi.nlm.nih.gov/BLAST/) revealed that the sequence showed highest similarity to *Burkholderia* species (see figure III.2-20). This strongly indicates that USCα was not enriched in the soil microcosms, instead were other microorganisms, which were able to survive between the acetate treatments and finally overgrew the bacterial population.

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
FJ434110.1	Burkholderia sp. IMER-A1-8 16S ribosomal RNA gene, partial sequence	1195	1195	99%	0.0	96%	
AB201286.1	Burkholderia ginsengisoli gene for 16S rRNA, partial sequence, strain: K	1195	1195	99%	0.0	96%	
CP001044.1	Burkholderia phymatum STM815 chromosome 2, complete sequence	1173	2341	100%	0.0	96%	
CP001043.1	Burkholderia phymatum STM815 chromosome 1, complete sequence	1173	4678	100%	0.0	96%	
HM019521.1	Burkholderia phymatum strain GR05 16S ribosomal RNA gene, partial s	1168	1168	100%	0.0	96%	
FJ560957.2	Burkholderia phymatum strain GR01 16S ribosomal RNA gene, partial s	1168	1168	100%	0.0	96%	
EU680455.1	Uncultured bacterium clone S7-78 16S ribosomal RNA gene, partial seq	1168	1168	100%	0.0	96%	
GU472974.1	Uncultured Burkholderia sp. clone bulk69 16S ribosomal RNA gene, part	1162	1162	99%	0.0	95%	
GU472961.1	Uncultured Burkholderia sp. clone bulk56 16S ribosomal RNA gene, part	1162	1162	99%	0.0	95%	
AF508806.1	Burkholderia sp. TNFYE-5 16S ribosomal RNA gene, partial sequence	1158	1158	100%	0.0	95%	
GU731260.1	Bacterium enrichment culture clone heteroA7_4W 16S ribosomal RNA g	1157	1157	100%	0.0	95%	
GU472976.1	Uncultured Burkholderia sp. clone bulk71 16S ribosomal RNA gene, part	1157	1157	99%	0.0	95%	
HM019519.1	Burkholderia phymatum strain GR03 16S ribosomal RNA gene, partial s	1157	1157	100%	0.0	95%	
AY904779.1	Burkholderia cepacia strain SEMIA 6390 16S ribosomal RNA gene, parti	1153	1153	100%	0.0	95%	
GU473151.1	Uncultured Burkholderia sp. clone BfP74 16S ribosomal RNA gene, parti	1151	1151	100%	0.0	95%	
GU473146.1	Uncultured Burkholderia sp. clone BfP69 16S ribosomal RNA gene, parti	1151	1151	100%	0.0	95%	
HM019522.1	Burkholderia phymatum strain GR06 16S ribosomal RNA gene, partial s	1151	1151	100%	0.0	95%	
EF635856.1	Burkholderia sp. LMG 22948 16S ribosomal RNA gene, partial sequence	1151	1151	100%	0.0	95%	
DQ451490.1	Uncultured bacterium clone FAC51 16S ribosomal RNA gene, partial seq	1147	1147	99%	0.0	95%	
GQ401700.1	Uncultured bacterium clone CafTC09 16S ribosomal RNA gene, partial s	1146	1146	100%	0.0	95%	
EF467847.1	Burkholderia xenovorans strain B2-5 16S ribosomal RNA gene, partial s	1146	1146	100%	0.0	95%	
CP000271.1	Burkholderia xenovorans LB400 chromosome 2, complete sequence	1146	3438	100%	0.0	95%	
CP000270.1	Burkholderia xenovorans LB400 chromosome 1, complete sequence	1146	3438	100%	0.0	95%	
DQ451458.1	Uncultured beta proteobacterium clone FAC19 16S ribosomal RNA gene,	1144	1144	99%	0.0	95%	
AY904781.1	Burkholderia cepacia strain SEMIA 6394 16S ribosomal RNA gene, parti	1144	1144	100%	0.0	95%	
FJ025134.1	Burkholderia cepacia strain SEMIA 6417 16S ribosomal RNA gene, parti	1142	1142	98%	0.0	95%	
AB438046.1	Burkholderia sp. MED-7 gene for 16S rRNA, partial sequence	1140	1140	99%	0.0	95%	

Figure III.2-20: 16S rRNA gene sequence comparison of enrichment culture using nucleotide BLAST.

IV. Ammonia oxidation coupled to CO_2 fixation by Archaea and Bacteria in an agricultural soil

Jennifer Pratscher, Marc G. Dumont and Ralf Conrad

Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany

Submitted to *Proceedings of the National Academy of Sciences of the United States of America* on 30th July 2010

IV.1 Abstract

Ammonia oxidation is an essential part of the global nitrogen cycling and was long thought to be driven only by *Bacteria*. Recent findings, however, expanded this pathway also to the *Archaea*. But most questions concerning the metabolism of ammonia oxidizing archaea, like ammonia oxidation and potential CO_2 fixation, yet remain open, especially for terrestrial environments. Here, we investigated the activity of ammonia oxidizing archaea and bacteria in an agricultural soil by comparison of RNA- and DNA-stable isotope probing. RNA-SIP demonstrated CO_2 assimilation coupled to ammonia oxidation by *Archaea* and *Bacteria* in an agricultural soil, while DNA-SIP showed no growth of ammonia oxidizing archaea, in contrast to their bacterial counterparts. Furthermore, the analysis of labeled RNA found transcripts of the archaeal acetyl-CoA/propionyl-CoA carboxylase (*accA*/*pccB*) to be expressed and labeled. This strongly suggests that ammonia oxidizing archaea in soil autotrophically fix CO_2 using the 3-hydroxypropionate-4-hydroxybutyrate cycle, one of the two new pathways recently discovered for CO_2 fixation in *Crenarchaeota*. CARD-FISH targeting *amoA* mRNA and 16S rRNA of *Archaea* also revealed ammonia oxidizing archaea to be numerically relevant among the *Archaea* in this soil. Our results demonstrate that the contribution of ammonia oxidizing archaea in soil to nitrification and CO_2 assimilation and their importance to the overall archaeal community might be larger than previously thought.

IV.2 Introduction

Ammonia oxidation, the first step in nitrification, is crucial for the global nitrogen cycle. While for a long time bacteria were believed to be solely responsible for this process and to exclusively possess the genes for the ammonia monooxygenase (AMO), the key enzyme of nitrification (1), there is now increasing evidence that also *Archaea* are involved. *amoA* genes of *Archaea* encoding subunit A of ammonia monooxygenase have been found to occur in a wide variety of environments including marine systems, hot springs, and soils (2-5).

Furthermore, molecular studies revealed that ammonia oxidizing archaea often outnumber the nitrifying *Bacteria* in most environments by orders of magnitude (3, 5, 6). These findings all demonstrate the potentially significant role of *Archaea* in the process of nitrification. New evidence also suggests the assignment of the ammonia oxidizing archaea to the new archaeal phylum *Thaumarchaeota* instead of to the *Crenarchaeota* (7, 8). In addition, these *Archaea* may be of importance for the global carbon cycle. The ammonia oxidizing archaea isolated from aquatic environments were all shown to be autotrophs (9-11), like their bacterial counterparts, and analysis of ^{13}C-bicarbonate labeled lipid biomarkers of natural *Crenarchaeota* in the North Sea indicated an autotrophic metabolism (12). However, it is still unclear whether this also applies to ammonia oxidizing archaea in soil and to what extent they are functionally active. An answer to this question and a link of phylogeny to function could be provided by stable isotope probing (SIP) of nucleic acids. This technique allows the specific identification of microorganisms assimilating labeled substances, most commonly carbon from a particular ^{13}C-labeled substrate (13). Direct demonstration of ammonia oxidation by this method is not possible, as nitrite, the product of ammonia oxidation, is not assimilated. But assuming that ammonia oxidation is coupled to autotrophic CO_2 fixation, one should be able to identify the active autotrophic ammonia oxidizing prokaryotes using SIP. So far, DNA-SIP analyses successfully showed autotrophy of ammonia oxidizing bacteria in sediments of a lake (14) and an estuary (15), but failed to detect CO_2 fixation of ammonia oxidizing archaea in agricultural soil (16), although potential activity of these *Archaea* in soil was reported before (26, 53). Efficiency of DNA-SIP, however, depends solely on replication of cells, thus excluding microorganisms that might be active but not growing. In this case, stable isotope probing of RNA (17) is assumed to yield more detailed information regarding activity.

The aim of this study was to investigate CO_2 assimilation linked to nitrification of ammonia oxidizing prokaryotes in an agricultural soil using RNA-SIP and DNA-SIP in comparison. We also wanted to detect expression of archaeal *amoA* by mRNA catalyzed reporter deposition – fluorescence *in situ* hybridization (CARD-FISH). Our findings provide novel evidence that the contribution of nitrifying *Archaea* to ammonia oxidation and CO_2 fixation in terrestrial environments might be substantial.

IV.3 Results

Nitrification activity in SIP incubations

For stable isotope probing, agricultural soil microcosms were incubated with 5% ^{13}C-labeled or unlabeled ^{12}C-CO_2 for 12 weeks. Concentrations of 1–5% CO_2 are considered typical in soil (18). Weekly fertilization of the soil with either 15 µg or 100 µg $(NH_4)_2SO_4$-N g^{-1} *d.w.s.* resulted in stepwise production and increase of nitrate (SI Fig. 1), while concentration in the unfertilized control did not increase. As expected, the largest nitrate production was observed in the microcosms fertilized with the higher concentration of ammonia (100 µg N g^{-1} *d.w.s.*). Ammonium and nitrite did not accumulate over time, indicating that nitrate production indeed resulted from ammonia oxidation. Ammonia and nitrate concentrations were not balanced because net nitrification generally underestimates gross nitrification in soils due to additional nitrogen cycling (54).

RNA stable isotope probing

For RNA stable isotope probing of ammonia-oxidizing prokaryotes buoyant density centrifugation was conducted with all respective RNA extracts from ^{12}C and ^{13}C microcosms after 8 and 12 weeks of incubation. The quantitative distribution of archaeal and bacterial *amoA* transcripts in these gradients was analyzed by qPCR of cDNA (Fig. 1). The obtained copy numbers represent mean results from the triplicate microcosms and repeated qPCR analyses. After 8 weeks of incubation with 5% $^{13}CO_2$ and fertilization with 15 µg N g^{-1} *d.w.s.* the copy number of archaeal *amoA* transcripts was highest in the 'light' RNA fraction (1.77 - 1.79 g ml^{-1}), but also showed detectable labeling in the 'heavy' fraction (1.81 – 1.83 g ml^{-1}) already (Fig. 1a). This could not be seen in the fertilization treatment with 100 µg N g^{-1} *d.w.s.* (Fig. 1c). Instead, here a shift of the archaeal *amoA* mRNA towards the partially labeled, 'intermediate' gradient fraction (1.79 – 1.80 g ml^{-1}) of ^{13}C gradients was observed. This indicates that *Archaea* might have been inhibited by the elevated ammonia concentration in the 100 µg N g^{-1} *d.w.s.* treatment, resulting in a slower activation and lower activity of ammonia oxidation. This is supported by the 12-week incubations. While the archaeal *amoA* transcripts in the 15 µg N g^{-1} *d.w.s.* treatment were nearly completely shifted into the 'heavy' fraction (Fig. 1b), the transcripts of the 100 µg N g^{-1} *d.w.s.* fertilization were still only partially labeled (Fig. 1d). The copy number of bacterial *amoA* transcripts, on the other hand, already peaked after 8 weeks of incubation in the fractions with 'heavy' RNA from the $^{13}CO_2$ treatment (Fig. 1e and g), suggesting that the ammonia oxidizing bacteria were probably activated more rapidly and synthesised new mRNA faster than their archaeal counterparts. Here, the labeling was stronger in the 100 µg N g^{-1} *d.w.s.* treatment (Fig. 1e) than in the microcosms fertilized with 15 µg N g^{-1} *d.w.s.* (Fig. 1g). After 12 weeks, bacterial *amoA* mRNA of both treatments was completely shifted to the 'heavy' fraction (Fig. 1f and 1h). No labeling was observed in the unfertilized treatment and the controls with $^{12}CO_2$ (SI Fig. 2), demonstrating that the 'heavy' RNA from the other gradients indeed resulted from true label incorporation into

selected microbes in SIP (19, 20). The fact that no labeling was observed in the unfertilized treatment also shows that CO_2 fixation was coupled to ammonia oxidation.

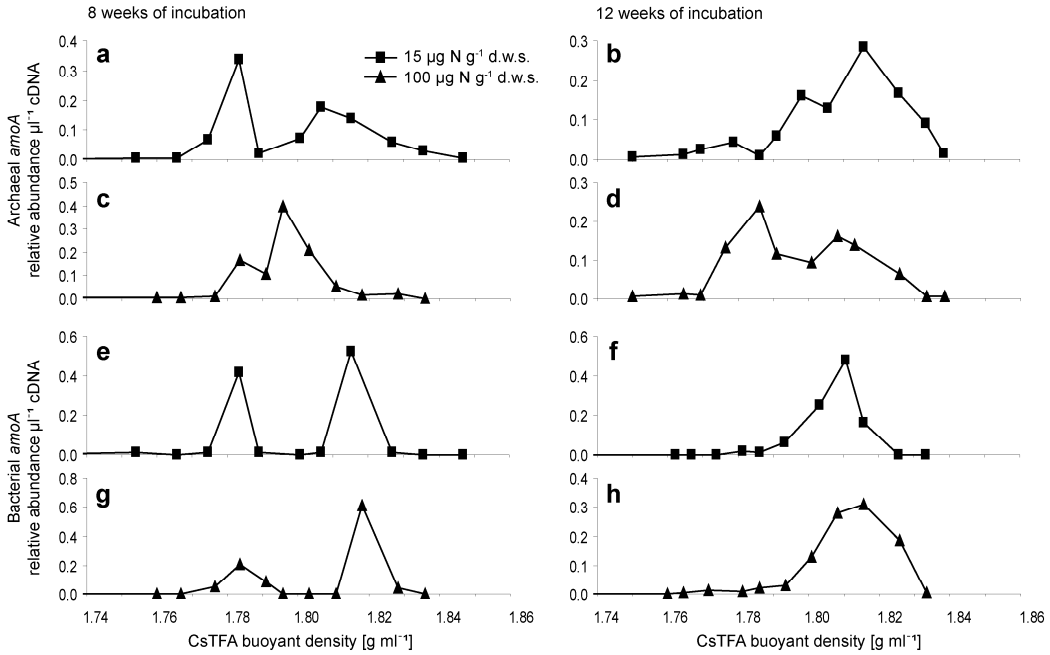


Fig. 1. Distribution of *amoA* transcripts from *Archaea* (a-d) and *Bacteria* (e-h) in RNA-SIP gradients after 8 (a, c, e, and g) and 12 (b, d, f, and h) weeks of incubation with $^{13}CO_2$ and fertilization with either 15 µg (a-b, e-f) or 100 µg (c-d, g-h) $(NH_4)_2SO_4$-N g^{-1} *d.w.s.*. Distribution of *amoA* transcripts was measured by qPCR of cDNA from gradient fractions.

DNA stable isotope probing

Gradient centrifugation of DNA was performed with all respective DNA extracts from $^{12}CO_2$- and $^{13}CO_2$-exposed microcosms after 8 and 12 weeks of incubation. The quantitative distribution of archaeal and bacterial *amoA* genes in these gradients was analyzed by qPCR (Fig. 2). The obtained copy numbers represent mean results from the triplicate microcosms and repeated qPCR analyses. After 8 weeks of incubation, the copy numbers of archaeal and bacterial *amoA* genes for both fertilization treatments peaked only in the 'light' fractions (1.69 – 1.72 g ml^{-1}) (Fig. 2a, c, e and g). Note that the small shift in buoyant density is due to different GC contents of *Archaea* and *Bacteria* (21, 22). While after 12 weeks of incubation still no labeling was detected for *Archaea* (Fig. 2b and d), the bacterial *amoA* genes were completely shifted to the 'heavy' fraction (1.73 – 1.76 g ml^{-1}) (Fig. 2f and h). A difference regarding the N-fertilization treatments as seen for the RNA-SIP could not be observed. Also here, controls with $^{12}CO_2$ and the unfertilized samples did not show any labeling (SI Fig. 3). These results suggest that although *Archaea* were actively involved in ammonia oxidation and CO_2 assimilation, as shown in the RNA-SIP approach, they did not replicate. By contrast, the ammonia oxidizing bacteria acquired 'heavy' DNA to such an extent that those not

proliferating could no longer be detected in the 'light' DNA fractions. This was supported by comparison of the copy numbers of archaeal and bacterial *amoA* genes in the initial soil versus copy numbers in the incubated soil, determined by qPCR. While ammonia oxidizing bacteria showed strong growth within the 12 weeks of incubation (initial soil: 5.47±0.75 x10^6; 12 week incubation: 7.80±0.67 x10^7 [copy number g^{-1} *d.w.s.*]), this could not be observed for the archaea containing *amoA* (initial soil: 4.77±0.51 x10^7; 12 week incubation: 5.35±0.39 x10^7 [copy number g^{-1} *d.w.s.*]).

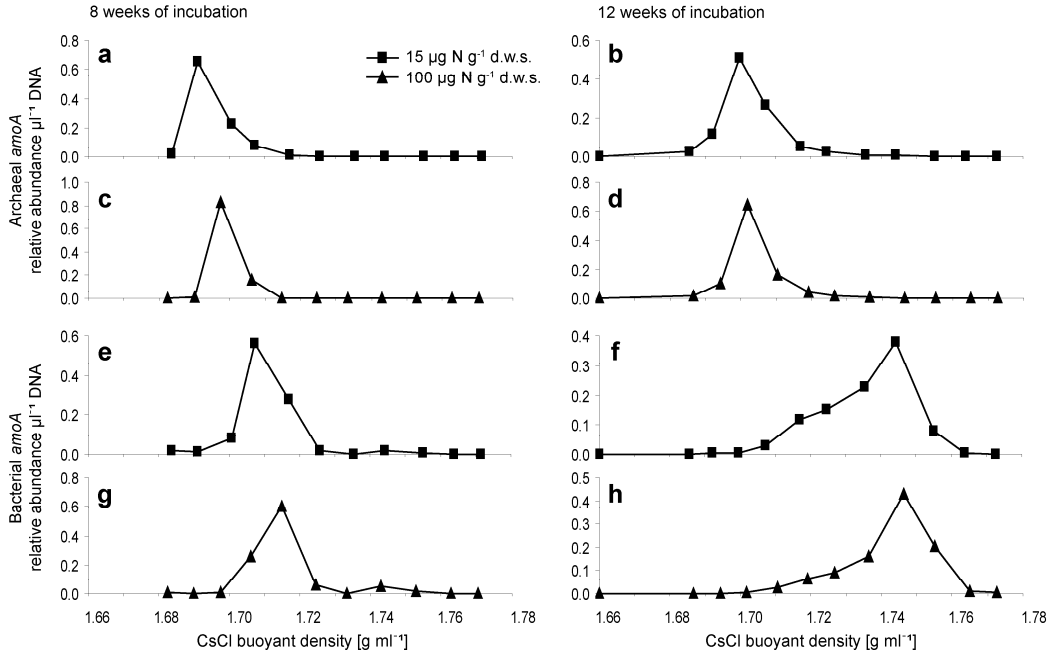


Fig. 2. Distribution of *amoA* genes from *Archaea* (a-d) and *Bacteria* (e-h) in DNA-SIP gradients after 8 (a, c, e, and g) and 12 (b, d, f, and h) weeks of incubation with $^{13}CO_2$ and fertilization with either 15 µg (a-b, e-f) or 100 µg (c-d, g-h) $(NH_4)_2SO_4$-N g^{-1} *d.w.s.*. Distribution of *amoA* gene abundance was measured by qPCR of DNA from gradient fractions.

Phylogenetic analyses of archaeal 16S rRNA transcripts in RNA-SIP

The 'light' and 'heavy' RNA fractions (1.815 and 1.783 g ml^{-1}, respectively) from the $^{13}CO_2$ incubated and low fertilizer (15 µg N g^{-1} *d.w.s.*) treated microcosms were used after 8 weeks of incubation to analyze the sequences of archaeal 16S rRNA transcripts. Sequence analysis revealed that all sequences (40 clones), from both 'light' (20 clones) and 'heavy' fraction (20 clones), showed highest similarity (95% maximum identity) to the ammonia oxidizer *Nitrososphaera gargensis* (SI Fig. 6). This homogeneity among the 'light' and 'heavy' fractions might be explained by an incomplete labeling and/or activation of the archaeal ammonia oxidizers after 8 weeks of incubation, as also observed in the RNA-SIP and qPCR of *amoA* transcripts (Fig. 1a). This indicates a high homogeneity of the community of ammonia oxidizing archaea in this soil regarding phylogeny and function.

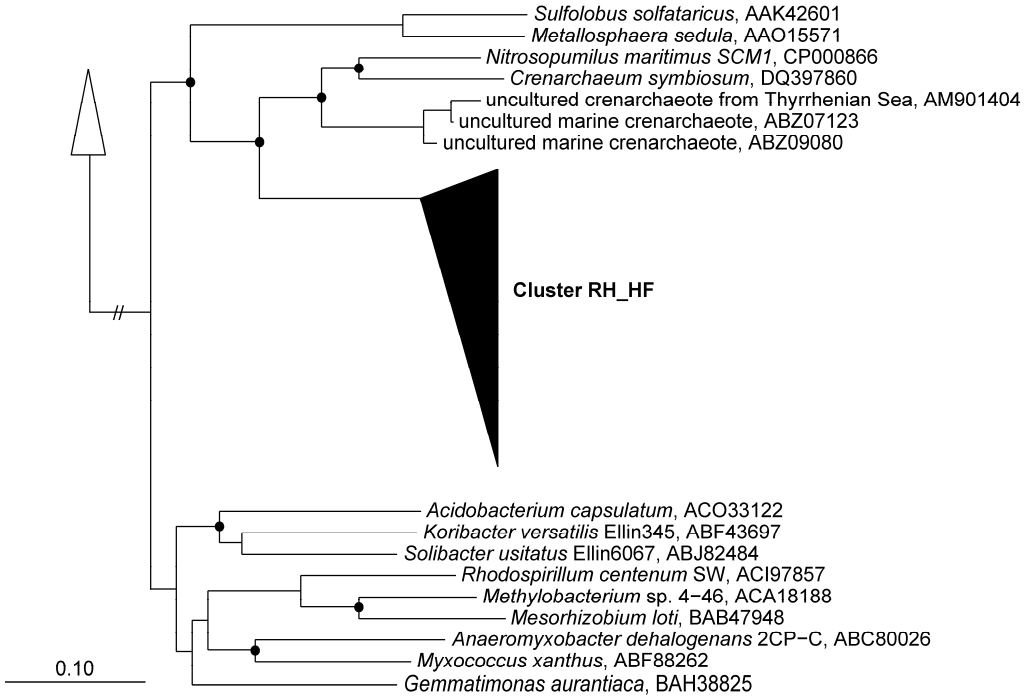


Fig. 3. Phylogenetic affiliation of putative acetyl- and propionyl- CoA-carboxylase (AccA/PccB) transcript clones derived from $^{13}CO_2$ RNA-SIP gradient fractions of soil after 12 weeks of incubation and fertilization with 15 µg N g^{-1} *d.w.s.*. Neighbor-joining analysis using 1000 bootstrap replicates was used to infer tree topology and the nodes with the percentage of bootstrap re-sampling above 90% is indicated by filled circles. The clones from ^{13}C-labeled 'heavy' RNA are shown as Cluster RH_HF (GenBank accession numbers HM996921-HM996934). The tree is rooted with *accA* gene of *Haloquadratum walsbyi* DSM 16790 (YP_658717). The scale bar represents 10% amino acid sequence divergence.

Archaeal *accA* transcripts detected in 'heavy' RNA fractions

The labeled and unlabeled RNA fractions (1.815 and 1.783 g ml^{-1}, respectively) from the $^{13}CO_2$ incubated and low fertilizer (15 µg N g^{-1} *d.w.s.*) treated microcosms were used after 12 weeks of incubation to generate clone libraries targeting bacterial and archaeal acetyl-CoA carboxylase (*accA*) transcripts (Fig. 3). *AccA* sequences could only be detected in the library of the 'heavy' fraction and exclusively consisted of a cluster of archaeal *accA* sequences (Cluster RH_HF), most closely related to *Nitrosopumilus maritimus*, *Crenarchaeum symbiosum* and *accA* genes of marine *Crenarchaeota* from deep Thyrrhenian Sea (23). These results strongly indicate an involvement of the acetyl-CoA/propionyl-CoA carboxylase in the CO_2-fixation process of ammonia oxidizing archaea in soil.

mRNA CARD-FISH of archaeal *amoA* and abundance of ammonia oxidizing archaea and bacteria in soil

To visually investigate the expression of *amoA* mRNA in ammonia oxidizing archaea, CARD-FISH of *amoA* transcripts and archaeal 16S rRNA was conducted, based on the protocol by Pernthaler and Amann (24). Clones and soil samples were hybridized with an archaeal *amoA* mRNA antisense probe, followed by detection with an anti-DIG-antibody labeled with horseradish peroxidase and signal amplification by catalyzed reporter deposition with fluorescein-labeled tyramide. For soil samples, detection of mRNA was also coupled to CARD-FISH of archaeal 16S rRNA using probe Arch915. The specificity of the *amoA* mRNA antisense probe for ammonia oxidizing archaea in this soil was tested with expression clones (SI Fig. 4). Induced cells showed strong hybridization signals (SI Fig. 4A and B), whereas no signal was observed in uninduced cells (SI Fig. 5) or clones expressing the partial *amoA* gene of ammonia oxidizing bacteria (SI Fig. 4C and D), indicating a high specificity of the *amoA* mRNA CARD-FISH. The hybridizations with the soil, incubated for 12 weeks with 5% CO_2 and fertilized weekly with 15 µg N g^{-1} *d.w.s.*, resulted in good signals, both for the *amoA* and 16S rRNA CARD-FISH (Fig. 4).

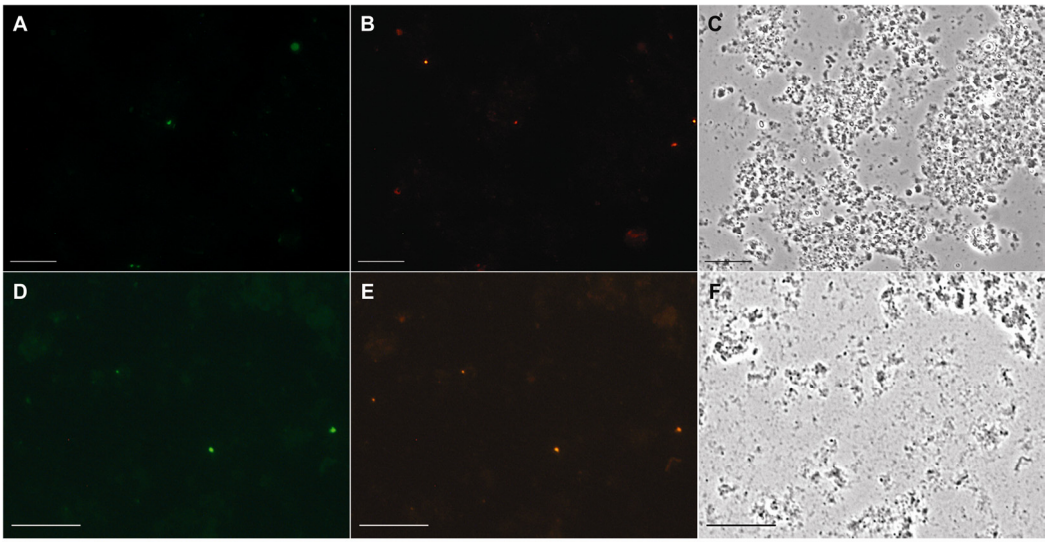


Fig. 4. Detection of *amoA* mRNA transcripts by application of CARD-FISH with archaeal *amoA* antisense probe in agricultural soil after 12 weeks of incubation with 5% CO_2 and fertilization with 15 µg N g^{-1} *d.w.s.*. Fluorescence images for *amoA* CARD-FISH (A and D) and respective phase contrast (C and F). Archaeal cells in the soil incubation were additionally detected by 16S rRNA CARD-FISH using HRP-labeled probe Arch915 (B and E). Bars = 10 µm.

Only cells that exhibited a signal with the 16S rRNA probe Arch915 (Fig. 4B and E) also showed detection by *amoA* mRNA FISH (Fig. 4A and D). Out of the detected archaeal cells in this incubated soil, half of the cells also showed a signal for *amoA* expression. To further test this observation, a comparison of copy numbers of *amoA* genes versus 16S rRNA genes in the incubated soil, determined by qPCR, was performed. While ammonia oxidizing bacteria

slightly outnumbered their archaeal counterparts, most likely due to the proliferation also observed in DNA-SIP, and made up 4% of the bacterial communitiy (*amoA*: 7.80±0.67 x10^7; 16S rRNA: 2.02±0.23 x10^9 [copy number g^{-1} *d.w.s.*]), archaea containing *amoA* accounted for 54% of all archaea (*amoA*: 5.35±0.39 x10^7; 16S rRNA: 9.83±0.91 x10^7 [copy number g^{-1} *d.w.s.*]), supporting the CARD-FISH observation. This indicates that ammonia oxidizing archaea might play an important role in the archaeal community in soil.

IV.4 Discussion

To date it remains elusive whether ammonia oxidizing archaea in soil can assimilate CO_2. A previous study using this soil by Jia and Conrad (16) based on DNA-SIP only detected labeling of *Bacteria* by CO_2 fixation and concluded that AOA in soil might be heterotrophic or mixotrophic rather than autotrophic. Here we were now able to demonstrate active CO_2 fixation coupled to ammonia oxidation by *Archaea* in an agricultural soil using an RNA-SIP approach. Archaeal *amoA* transcripts were consistently labeled during incubation of soil microcosms with $^{13}CO_2$ and fertilization with either 15 µg or 100 µg $(NH_4)_2SO_4$-N g^{-1} *d.w.s.*. Controls without fertilization and with $^{12}CO_2$ did not show any labeling, confirming that CO_2 fixation was coupled to ammonia oxidation and that labeling resulted from true label incorporation. Labeling of ammonia oxidizing archaea due to cross-feeding on labeled organic carbon derived from autotrophic ammonia oxidizing bacteria was unlikely, since labeling of archaeal *amoA* mRNA was weaker and less pronounced with higher concentration of ammonia (100 µg N g^{-1} *d.w.s.*) although ammonia oxidizing bacteria showed better and faster labeling at the elevated concentration. If labeling of archaeal ammonia oxidizers had been coupled to labeling of bacterial nitrifiers due to cross-feeding, the same response in SIP of both groups to the different ammonia concentrations should have been observed. Furthermore, stable isotope probing of DNA showed assimilation of CO_2 into biomass of ammonia oxidizing bacteria not until the time course from 8-week to 12-week incubation. This is supported by the fact, that also the main activity of ammonia oxidation was recorded during these four weeks. Hence, ammonia oxidizing bacteria grew very slowly and thus are unlikely to have provided organic carbon for cross-feeding of the *Archaea*. Our results show that NH_4^+ fertilization stimulated CO_2 assimilation by archaeal ammonia oxidizers, though CO_2 fixation and ammonia oxidation were stimulated to a greater extent by lower concentration of ammonia (15 µg N g^{-1} *d.w.s.*). The 100 µg N g^{-1} *d.w.s.* treatment led to incomplete labeling of archaeal *amoA* mRNA even after 12 weeks of incubation. This strengthens the hypothesis that ammonia oxidizing archaea are rather adapted to low nutrient environments and inhibited by high fertilizations (25-28). Our results agree with previous findings in *Nitrososphaera gargensis* isolated from hot spring which was also inhibited by relatively high concentrations of ammonium (11) and a recent publication demonstrating growth of ammonia oxidizing archaea in soil only at acetylene-sensitive nitrification under low ammonia concentrations (53). Like Jia and Conrad (16), however, we also were not able to detect labeling of archaeal

amoA when using DNA-SIP. While RNA-SIP targeting *amoA* of ammonia oxidizing archaea clearly demonstrated that CO_2 was assimilated, DNA-SIP revealed no growth of these organisms because detection of label incorporation in DNA-SIP can only take place when cells are actively replicating. We assume that the ammonia concentrations in the soil for both fertilization treatments might have allowed ammonia oxidation activity of *Archaea*, as seen in RNA-SIP, but still did not provide favourable conditions for them to grow. This uncoupling should generally be considered, when investigating the activity of microorganisms in natural environments. Because methods depending on cell growth to detect incorporation of label might not or only insufficiently detect cells that are active but grow more slowly. For these microorganisms, RNA-SIP might be the more appropriate approach.

Unlike the *Archaea*, the *amoA* copies of ammonia oxidizing bacteria were completely labeled in RNA- and DNA-SIP for both fertilization treatments after 12 weeks of incubation. This was not surprising, since it has been known for a long time that ammonia oxidizing bacteria fix CO_2 using the Calvin Cycle and its key enzyme, the ribulose bisphosphate carboxylase (RubisCO) (29, 30). The results of the stable isotope probing demonstrated activity and growth to such an extent that the whole community of ammonia oxidizing bacteria could only be detected in the 'heavy' fractions. The labeling of *amoA* mRNA took place more rapidly in the fertilization treatments with 100 µg N g^{-1} *d.w.s.*, showing that nitrifying *Bacteria*, as expected, responded even better to higher concentrations of ammonia. In addition, we were able to specifically and quantitatively detect archaeal cells expressing *amoA* in incubated soil directly by mRNA CARD-FISH using expression clones as controls. The high abundance of ammonia oxidizing archaea (~ 50%) within the *Archaea* in this soil, as visualized by fluorescence microscopy, was also confirmed by qPCR of *amoA* and 16S rRNA genes. Since ammonia oxidizing archaea are thought to harbor only one copy of both the 16S rRNA gene and the *amoA* gene, like *Nitrosopumilus maritimus*, gene copy numbers derived by qPCR should be equivalent to cell numbers (31, 32). We also analyzed the archaeal 16S rRNA transcripts from light and heavy fractions of RNA-SIP and observed a high homogeneity within the archaeal ammonia oxidizers regarding phylogeny and function, and a close relation to *Nitrososphaera gargensis*. Taken together, these results demonstrate the high relative abundance of ammonia oxidizing archaea among the overall archaeal community in soil. Furthermore, we wanted to determine which pathway enables ammonia oxidizing archaea in soil to fix CO_2. Two new autotrophic carbon fixation cycles have been recently described in *Crenarchaeota*, the dicarboxylate-4-hydroxybutyrate cycle and the 3-hydroxypropionate-4-hydroxybutyrate cycle, and all *Crenarchaeota* studied so far use either the former or the latter cycle (33). Because of the oxygen sensitivity of some of its enzymes, the dicarboxylate-hydroxybutyrate cycle is restricted to anaerobic or microaerobic *Crenarchaeota* of the orders Thermoproteales and Desulfurococcales (34-36). The enzymes of the hydroxypropionate-butyrate cycle, on the other hand, are oxygen tolerant. Therefore, this cycle fits well with the lifestyle of aerobic *Crenarchaeota* (37). The hydroxypropionate-butyrate cycle occurs in the autotrophical crenarchaeal order Sulfolobales, e.g. *Metallosphaera sedula* (38-40). The CO_2-

fixing enzyme of this process is the bifunctional biotin-dependent acetyl-CoA/propionyl-CoA carboxylase (Acc/Pcc). Recent studies already detected sequences of the *accA*/*pccB* by genome analysis in members of the mesophilic marine group I *Crenarchaeota*, including ammonia oxidizers *Crenarchaeum symbiosum* and *Nitrosopumilus* spp. (33, 40, 41), and in metagenomic libraries of uncultured ammonia oxidizing marine Crenarchaeotes (23, 42-44), but existence of these sequences alone could not be linked to functionality so far. Our results now show that transcripts of this acetyl-CoA/propionyl-CoA carboxylase (*accA*/*pccB*), closely related to the sequences of the ammonia oxidizing marine *Crenarchaeota*, were not only expressed but furthermore also labeled by assimilation of $^{13}CO_2$. This strongly suggests that ammonia oxidizing archaea in upland soils are able to autotrophically fix CO_2 using the hydroxypropionate-hydroxybutyrate cycle, hence providing an additional sink for CO_2 in terrestrial environments.

In summary, we were able to show that ammonia oxidizing archaea were actively involved in microbial ammonia oxidation in an agricultural soil and did fix CO_2 autotrophically, presumably via the hydroxypropionate-hydroxybutyrate cycle. These results and the observed numerical importance of the archaeal ammonia oxidizers to the overall archaeal community in this environment give new insights into the function and characteristics of ammonia oxidizing archaea in soil.

IV.5 Materials and methods

Soil incubation

Soil was sampled using 40 cm long soil cores in April 2009 from maize plots at the long-term experiment field site of the University of Giessen, Germany. Maize, wheat, and barley are annually rotated at the field site. The field site and soil properties were described previously (16). The soil was air-dried, sieved through 1 mm mesh, homogenized, and stored at 4°C until further use. Incubation for stable isotope probing with 5% CO_2 was performed in triplicates for each treatment. Soil (10 g *d.w.s*) was incubated at 60% maximum water holding capacity (WHC), 25°C and darkness in 120 ml serum bottles capped with butyl stoppers. Five percent of CO_2 ($^{12}CO_2$ or $^{13}CO_2$) were added to the headspace and the soil was fertilized with either 100 µg or 15 µg $(NH_4)_2SO_4$-N g^{-1} *d.w.s.*, respectively, dissolved in distilled water. Nitrogen-free controls received an equal amount of distilled water. Every week, bottles were flushed with synthetic air (20% O_2, 80% N_2), 5% of CO_2 was added, and fertilization treatments were renewed. For chemical analysis of pH, ammonium, nitrite, and nitrate, aliquots of the soil were removed from each treatment every week (see supplementary information).

Nucleic acid extraction and SIP fractionation

After 8 and 12 weeks of incubation with $^{13}CO_2$ and $^{12}CO_2$, respectively, 0.5 g of soil of each bottle were sampled, frozen immediately in liquid nitrogen and stored at -80°C until further processing. Nucleic acids were extracted from soil using a sodium dodecyl sulfate (SDS) -

based protocol (45) with minor modifications. Soil (0.5 g) was mixed with 200 µl of zirconia-silica beads (0.1 mm; Roth, Karlsruhe, Germany) and 1 ml of SDS extraction buffer in 2.0 ml screw-cap tubes. Cells were lysed in a FastPrep beat beating system for 45 s at 6 m s^{-1} and supernatants were extracted using phenol chloroform isoamyl alcohol (25:24:1) and chloroform isoamyl alcohol (24:1). Nucleic acids were precipitated with polyethylene glycol (PEG) 6000 solution (20%) and dissolved in 100 µl of nuclease free water. For stable isotope probing of RNA, 50 µl of extract were treated with RNase-free DNase I for digestion of DNA. RNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and stored at -80°C until further usage. Integrity of nucleic acids was checked on agarose gels and concentration was determined using a NanoDrop instrument (Thermo Fisher Scientific, Schwerte, Germany). Stable isotope probing fractionation of total DNA extract (5.0 µg) was performed with an initial cesium chloride (CsCl) buoyant density of 1.72 g ml^{-1} subjected to centrifugation at 177 000 *g* for 36 h at 20°C (21). Gradient centrifugation of RNA was carried out in cesium trifluoroacetate (CsTFA) as described previously (21) with an initial buoyant density of 1.79 g ml^{-1} and centrifugation at 130 000 *g* for 65 h at 20°C. DNA- and RNA-gradients were fractionated from bottom to top by displacing the gradient medium with nuclease free water at the top of the tube using a syringe pump (Kent Scientific, Torrington, CT, USA) at a flow rate of 0.45 ml min^{-1}, generating twelve fractions per density gradient. The density of each fraction was determined by refractometry (Reichert, Depew, NY, USA). DNA was recovered by PEG 6000 precipitation with glycogen (46) and dissolved in 30 µl of nuclease free water. RNA was precipitated from CsTFA with two volumes of ethanol and 20 µg glycogen and resuspended in 10 µl of nuclease free water. RNA samples from density gradient fractions were reverse transcribed with random hexamer primers (Invitrogen, Darmstadt, Germany) and M-MLV reverse transcriptase (Promega, Mannheim, Germany).

Quantitative PCR of *amoA* genes

The abundance of archaeal *amoA* genes and transcripts in the different SIP fractions was quantified by quantitative PCR (qPCR) using primers amo196F and amo277R as previously described (5, 16). The 25 µl reaction mixture contained 12.5 µl of SYBRGreen Jump-Start™ Taq ReadyMix™, 0.5 µM of each primer, 200 ng BSA ml^{-1}, 4.0 mM $MgCl_2$, 1.0 µl template DNA or cDNA (47). The abundance of bacterial *amoA* genes and transcripts in the different SIP fractions was quantified by qPCR using primers amoA-1F and amoA-2R as previously described (16, 48). The 25 µl reaction mixture contained 12.5 µl of SYBRGreen Jump-Start™ Taq ReadyMix™, 0.5 µM of each primer, 200 ng BSA ml^{-1}, 3.0 mM $MgCl_2$, 1.0 µl template DNA or cDNA. All assays were performed in an iCycler™ (Applied Biosystems, Darmstadt, Germany), respective qPCR standards were used and controls were always run with water instead of DNA or cDNA extract. PCR efficiencies for all qPCR assays were between 90-104% with r^2 values between 0.976-0.997.

Analysis of archaeal 16S rRNA and *accA* transcripts from light and heavy fractions of RNA-SIP

Complementary DNA (cDNA) of the heavy and light fractions of the $^{13}CO_2$ treatments with 15 µg $(NH_4)_2SO_4$-N g^{-1} *d.w.s.* was used for PCR amplification of archaeal 16S rRNA cDNA fragments using primers Arch109F and Arch934R as previously described (49). Bacterial and archaeal acetyl-CoA carboxylase alpha subunit (*accA*) cDNA fragments were amplified by primers PcB_388F and PcB_1271R (23). PCR products were cloned using pGEM-T Easy vector and *E. coli* JM109 competent cells (Promega, Mannheim, Germany). Sequencing was carried out on an ABI 3130 genetic analyzer (Applied Biosystems, Darmstadt, Germany), and analyzed by DNAStar software package. Phylogenetic trees were reconstructed from sequence data using the ARB software package (50). *accA* tree topology was checked by neighbor-joining algorithm using 1000 bootstrap replicates and was verified with a tree calculated using maximum likelihood. Archaeal 16S rRNA tree topology was checked by neighbor-joining algorithm using 500 bootstrap replicates and Jukes-Cantor correction of distances. Data deposition: The sequences reported in this paper have been deposited in the GenBank database (*accA*: accession nos. HM996921-HM996934; archaeal 16S rRNA: accession nos. HQ293120-HQ293148).

CARD-FISH of *amoA* mRNA and archaeal 16S rRNA

To generate controls for mRNA CARD-FISH, partial archaeal (positive control) and bacterial (negative control) *amoA* genes from soil were cloned into *E. coli* Top10 competent cells using primers amo111F/amo643R (51) and amoA-1F/amoA-2R (52), respectively, and expressed by using vector pBAD as previously described by Pernthaler and Amann (24). Clones and soil samples after 12 weeks of fertilization with 15 µg NH_4^+-N g^{-1} *d.w.s.* were fixed and CARD-FISH was performed as described in supplementary information.

Acknowledgements

We are grateful to Dr. Lothar Behle-Schalk for the access to the long-term experimental trial of the University of Giessen. We thank Peter Claus, Melanie Klose, and Bianca Pommerenke for skilful technical assistance.

IV.6 Supplementary material

Chemical analyses

Soil NO_3^--N and NO_2^--N were measured by ion chromatography (Sykam, Fuerstenfeldbruck, Germany). Ammonium was extracted from 0.15 g of soil after mixing with 1.5 ml of 2 N KCl and measured fluorometrically at an emission wavelength of 470 nm on a SAFIRE microplate reader (TECAN, Crailsheim, Germany) (1). Additionally, CO_2 concentration in the headspace was measured by a gas chromatograph-combustion-isotope ratio mass spectrometry system (2). The $^{13}CO_2$ (99 atom%) was purchased from Sigma-Aldrich Co.

CARD-FISH of *amoA* mRNA and archaeal 16S rRNA

Clones were fixed in 2% (vol/vol) formaldehyde for 30 min at room temperature, centrifuged, and washed once with PBS and twice with 50% ethanol in PBS. Samples from the soil incubations after 12 weeks of fertilization with 15 µg NH_4^+-N g^{-1} *d.w.s.* were fixed in 4% (vol/vol) formaldehyde for 5 h at 4°C, centrifuged, and washed once with PBS. Cells were then resuspended in 50% ethanol in PBS and stored at -20°C until further processing. Polynucleotide antisense RNA probes of *amoA* were generated by *in vitro* transcription and were simultaneously labeled by incorporating digoxigenin-11-UTP (Roche Molecular Diagnostics, Basel, Switzerland) (3). The hybridization and detection procedures were based on the protocol described by Pernthaler and Amann (4), with some minor modifications. Five microlitres of cell suspensions were applied to each well of a Teflon-coated glass slide (MAGV, Marburg, Germany) and dried at 46°C. Expression clones of *amoA* were permeabilized with lysozyme (5 mg ml^{-1} in Tris-EDTA buffer (TE); Sigma, Taufkirchen, Germany) for 30 min at room temperature. Soil samples were additionally incubated with proteinase K (15 µg ml-1 in TE) for 10 min at room temperature (5). Hybridization was performed overnight at 60.5°C using a formamide concentration of 50% in the hybridization buffer. Subsequent 16S rRNA FISH with horseradish peroxidase (HRP) -labeled oligonucleotide probe was performed as described previously (5, 6). HRP-labeled probe Arch915 (Biomers, Ulm, Germany) was used for detection of *Archaea* in soil samples with Alexa546-labeled tyramide. Finally, slides were stained with 4',6-diamidino-2-phenylindole (DAPI), mounted with the antifading agent Citifluor AF1 (Citifluor, London, UK), and hybridization preparations were visualized by fluorescence microscopy (Axiophot; Carl Zeiss Microimaging GmbH, Jena, Germany).

References

1. Murase J, Noll M, Frenzel P (2006) Impact of protists on the activity and structure of the bacterial community in a rice field soil. *Appl Environ Microbiol* 72: 5436–5444.

2. Conrad R, Klose M, Claus P (2002) Pathway of CH_4 formation in anoxic rice field soil and rice roots determined by ^{13}C-stable isotope fractionation. *Chemosphere* 47: 797–806.

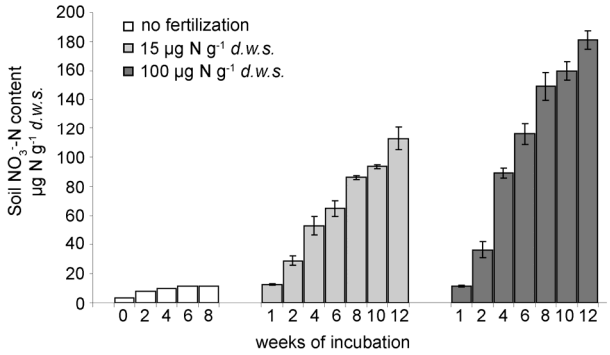
3. Zwirglmaier K, Ludwig W, Schleifer K-H (2003) Improved fluorescence in situ hybridization of individual microbial cells using polynucleotide probes: the network hypothesis. *Syst Appl Microbiol* 26: 327–337.

4. Pernthaler A, Amann R (2004) Simultaneous fluorescence in situ hybridization of mRNA and rRNA in environmental bacteria. *Appl Environ Microbiol* 70: 5426–5433.

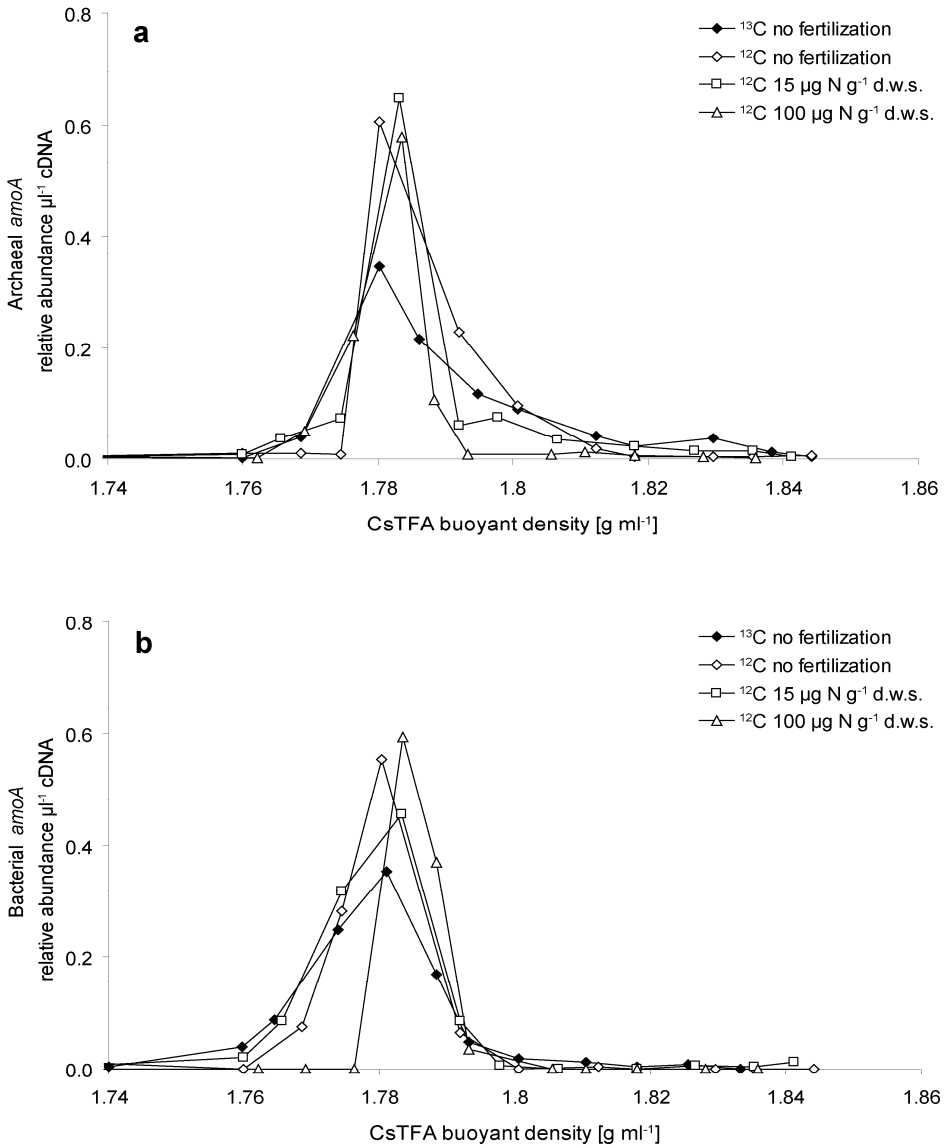
5. Hatzenpichler R, *et al.* (2008) A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proc Natl Acad Sci U S A* 105: 2134–2139.

6. Pernthaler A, Pernthaler J (2007) Fluorescence in situ hybridization for the identification of environmental microbes. *Methods Mol Biol* 353: 153–164.

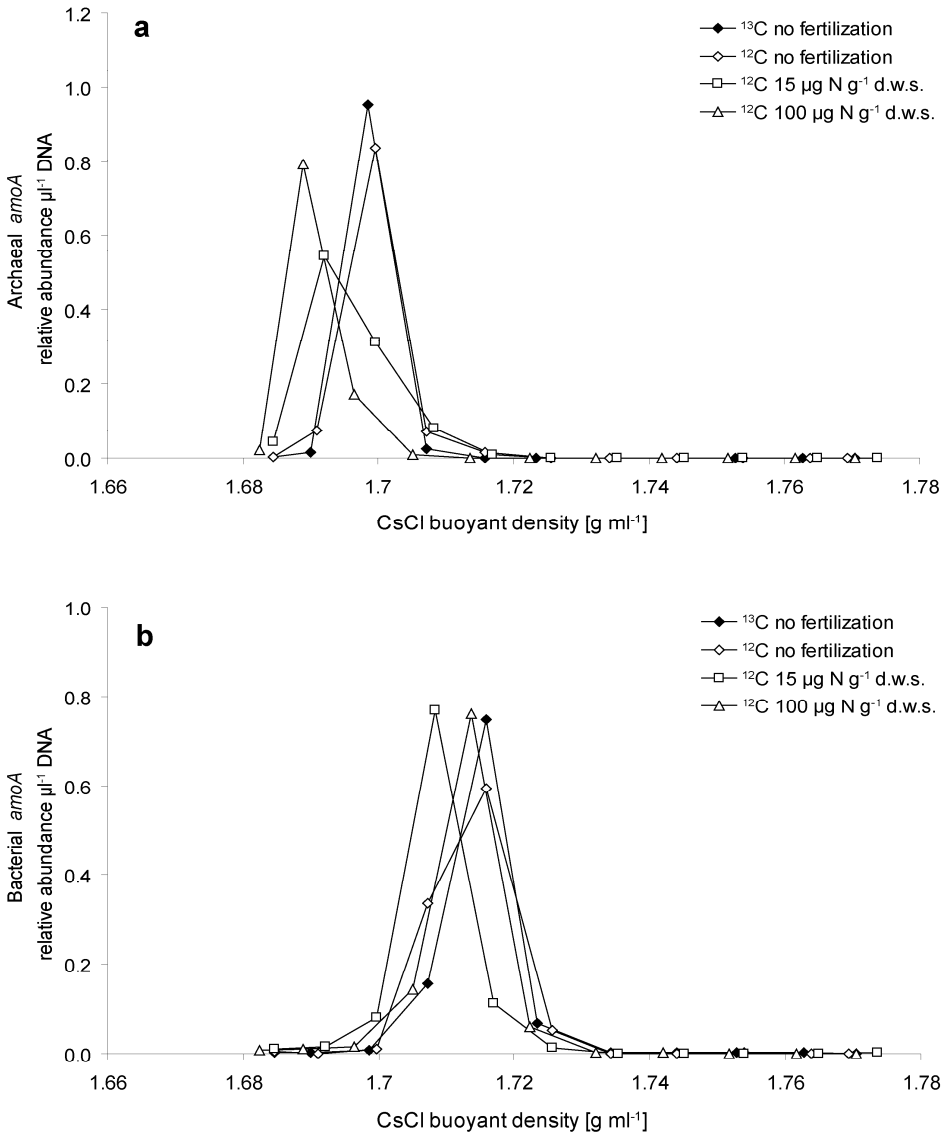
Supplementary figures



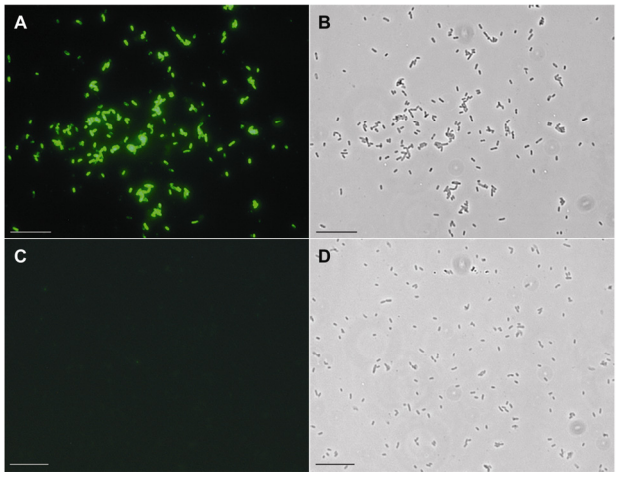
SI figure 1. Changes in nitrate concentration in SIP microcosms over a time course of 12 weeks. The error bars are standard deviations of triplicate incubations.



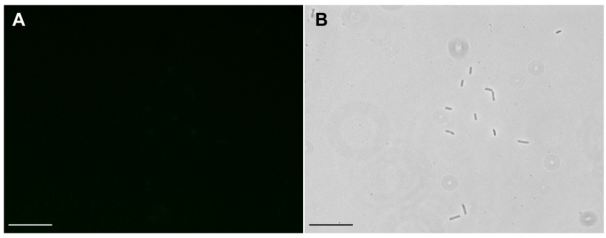
SI figure 2. Distribution of *amoA* transcripts from *Archaea* (a) and *Bacteria* (b) in RNA-SIP control gradients after 12 weeks of incubation with $^{12}CO_2$ and fertilization with either 15 µg or 100 µg $(NH_4)_2SO_4$-N g^{-1} *d.w.s.*, and incubation with ^{12}C- and ^{13}C-CO_2 and no fertilization. Distribution of *amoA* transcripts was measured by real-time PCR of cDNA from gradient fractions.



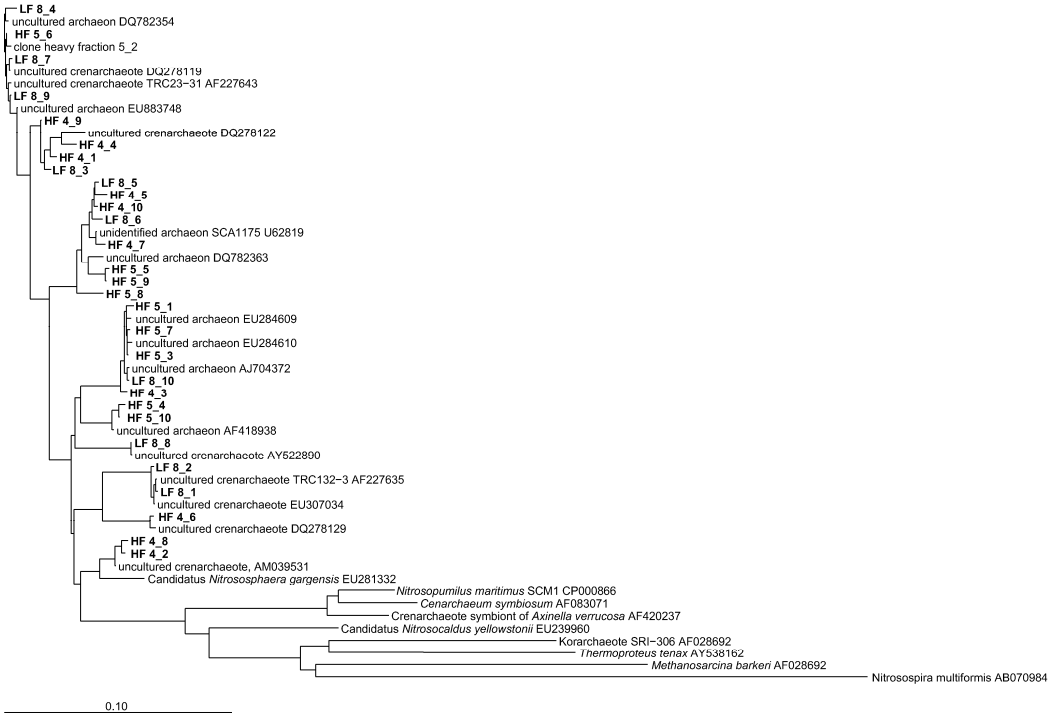
SI figure 3. Distribution of *amoA* genes from *Archaea* (a) and *Bacteria* (b) in DNA-SIP control gradients after 12 weeks of incubation with $^{12}CO_2$ and fertilization with either 15 µg or 100 µg $(NH_4)_2SO_4$-N g^{-1} *d.w.s.*, and incubation with ^{12}C- and ^{13}C-CO_2 and no fertilization. Distribution of *amoA* gene abundance was measured by real-time PCR of DNA from gradient fractions.



SI figure 4. Detection of *amoA* mRNA transcripts by application of CARD-FISH with archaeal *amoA* antisense probe in clones expressing *amoA* of ammonia oxidizing archaea (A and B) or bacteria (C and D). Fluorescence images for *amoA* CARD-FISH (A and C) and respective phase contrast (B and D). Bars = 10 µm.



SI figure 5. Failed detection of *amoA* mRNA transcripts by application of CARD-FISH with archaeal *amoA* antisense probe in uninduced clones harboring but not expressing *amoA* of ammonia oxidizing archaea (A and B). Fluorescence images for *amoA* CARD-FISH (A) and respective phase contrast (B). Bars = 10 µm.



SI figure 6. Phylogenetic affiliation of archaeal 16S rRNA transcript clones derived from $^{13}CO_2$ RNA-SIP 'light' (LF) and 'heavy' (HF) gradient fractions of soil after 8 weeks of incubation and fertilization with 15 µg N g^{-1} *d.w.s.*. Neighbor-joining analysis using 500 bootstrap replicates and Jukes-Cantor correction of distances was used to infer tree topology. The scale bar represents 10% nucleic acid sequence divergence.

IV.7 References

1. Kowalchuk GA, Stephen JR (2001) Ammonia oxidizing bacteria: a model for molecular microbial ecology. *Annu Rev Microbiol* 55: 485–529.

2. Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB (2005) Ubiquity and diversity of ammonia oxidizing archaea in water columns and sediments of the ocean. *Proc Natl Acad Sci USA* 102: 14683–14688.

3. Wuchter C, *et al.* (2006) Archaeal nitrification in the ocean. *Proc Natl Acad Sci USA* 103: 12317–12322.

4. Reigstad LJ, *et al.* (2008) Nitrification in terrestrial hot springs of Iceland and Kamchatka. *FEMS Microbiol Ecol* 64: 167–174.

5. Leininger S, *et al.* (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* 442: 806–809.

6. Di HJ, *et al.* (2009) Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nat Geosci* 2: 621-624.

7. Brochier-Armanet C, *et al.* (2008) Mesophilic Crenarchaeota : proposal for a third archaeal phylum, the Thaumarchaeota. *Nat Rev Microbiol* 6: 245-252.

8. Spang A, *et al.* (2010) Distinct gene set in two different lineages of ammonia-oxidizing archaea supports the phylum Thaumarchaeota. *Trends Microbiol* doi:10.1016/j.tim.2010.06.003

9. Könneke M, *et al.* (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437: 543–546.

10. de la Torre JR, Walker CB, Ingalls AE, Könneke M, Stahl DA (2008) Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. *Environ Microbiol* 10: 810–818.

11. Hatzenpichler R, *et al.* (2008) A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proc Natl Acad Sci U S A* 105: 2134–2139.

12. Wuchter C, Schouten S, Boschker HTS, Sinninghe Damsté JS (2003) Bicarbonate uptake by marine Crenarchaeota. *FEMS Microbiol Lett* 219: 203–207.

13. Dumont MG, Murrell JC (2005) Stable isotope probing – linking microbial identity to function. *Nat Rev Microbiol* 3: 499-504.

14. Whitby CB, *et al.* (2001) ^{13}C incorporation into DNA as a means of identifying the active components of ammonia-oxidizer populations. *Lett Appl Microbiol* 32: 398–401.

15. Freitag TE, Chang L, Prosser JI (2006) Changes in the community structure and activity of betaproteobacterial ammonia-oxidizing sediment bacteria along a freshwater-marine gradient. *Environ Microbiol* 8: 684–696.

16. Jia Z, Conrad R (2009) *Bacteria* rather than *Archaea* dominate microbial ammonia oxidation in an agricultural soil. *Environ Microbiol* 11: 1658-1671.

17. Whiteley AS, Thomson B, Lueders T, Manefield M (2007) RNA stable-isotope probing. *Nat Protoc* 2: 838-844.

18. Buyanovsky GA, Wagner GH (1983) Annual cycles of carbon dioxide level in soil air. *Soil Sci Soc Am J* 47: 1139–1145.

19. Neufeld J, Dumont M, Vohra J, Murrell J (2007) Methodological considerations for the use of stable isotope probing in microbial ecology. *Microb Ecol* 53: 435–442.

20. Lueders T (2009) Stable isotope probing of hydrocarbon-degraders. In: Timmis KN (ed). *Handbook of Hydrocarbon and Lipid Microbiology*. Springer: Berlin, Heidelberg, pp 4011–4026.

21. Lueders T, Manefield M, Friedrich MW (2004) Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* 6: 73–78.

22. Carter C, Britton VG, Haff L (1983) CsTFA: a centrifugation medium for nucleic acid isolation. *Biotechniques* 1: 142–147.

23. Yakimov MM, La Cono V, Denaro R (2009) A first insight into the occurrence and expression of functional *amoA* and *accA* genes of autotrophic and ammonia-oxidizing bathypelagic *Crenarchaeota* of Tyrrhenian Sea. *DEEP-SEA RES PT II* 56: 748-754.

24. Pernthaler A, Amann R (2004) Simultaneous fluorescence in situ hybridization of mRNA and rRNA in environmental bacteria. *Appl Environ Microbiol* 70: 5426–5433.

25. Valentine DL (2007) Adaptations to energy stress dictate the ecology and evolution of the *Archaea*. *Nat Rev Microbiol* 5: 316–323.

26. Schauss K, *et al*. (2009) Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils. *Environ Microbiol* 11: 446-456.

27. Martens-Habbena W, Berube PM, Urakawa H, de la Torre JR, Stahl DA (2009) Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature* 461: 976-979.

28. Di HJ, *et al.* (2010) Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. *FEMS Microbiol Ecol* 72: 386-394.

29. Schramm A, De Beer D, Wagner M, Amann R (1998) Identification and activities in situ of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. *Appl Environ Microbiol* 69: 3480–3485.

30. Utåker JB, Andersen K, Aakra Å, Moen B, Nes IF (2002) Phylogeny and functional expression of ribulose 1,5-bisphosphate carboxylase/oxygenase from the autotrophic ammonia-oxidizing bacterium *Nitrosospira* sp. isolate 40KI. *J Bacteriol* 184: 468–478.

31. Nelson KA, Moin NS, Bernhard AE (2009) Archaeal Diversity and the Prevalence of *Crenarchaeota* in Salt Marsh Sediments. *Appl Environ Microbiol* 75: 4211–4215.

32. Walker CB, *et al*. (2010) *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proc Natl Acad Sci U S A* 107: 8818-8823.

33. Berg IA, *et al.* (2010) Autotrophic carbon fixation in archaea. *Nat Rev Microbiol* 8: 447-460.

34. Jahn U, Huber H, Eisenreich W, Hügler M, Fuchs G (2007) Insights into the autotrophic CO_2 fixation pathway of the archaeon *Ignicoccus hospitalis*: comprehensive analysis of the central carbon metabolism. *J Bacteriol* 189: 4108–4119.

35. Huber H, *et al.* (2008) A dicarboxylate/4-hydroxybutyrate autotrophic carbon assimilation cycle in the hyperthermophilic archaeum *Ignicoccus hospitalis*. *Proc Natl Acad Sci U S A* 105: 7851–7856.

36. Ramos-Vera WH, Berg IA, Fuchs G (2009) Autotrophic carbon dioxide assimilation in *Thermoproteales* revisited. *J Bacteriol* 191: 4286–4297.

37. Berg IA, Ramos-Vera WH, Petri A, Huber H, Fuchs G (2010) Study of the distribution of autotrophic CO_2 fixation cycles in Crenarchaeota. *Microbiology* 156: 256–269.

38. Ishii M, *et al.* (1997) Autotrophic carbon dioxide fixation in *Acidianus brierleyi*. *Arch Microbiol* 166: 368–371.

39. Menendez C, *et al*. (1999) Presence of acetyl coenzyme A (CoA) carboxylase and propionyl-CoA carboxylase in autotrophic Crenarchaeota and indication for operation of a 3-hydroxypropionate cycle in autotrophic carbon fixation. *J Bacteriol* 181: 1088–1098.

40. Berg IA, Kockelkorn D, Buckel W, Fuchs G (2007) A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in Archaea. *Science* 318: 1782–1786.

41. Hallam SJ, *et al.* (2006) Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine *Crenarchaeota*. *PLoS Biol* 4: e95.

42. Venter JC, *et al.* (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304: 66–74.

43. Delong EF, *et al.* (2006) Community genomics among stratified microbial assemblages in the ocean's interior. *Science* 311: 496–503.

44. Mincer TJ, *et al.* (2007) Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre. *Environ Microbiol* 9: 1116–1175.

45. Bürgmann H, Widmer F, Sigler WV, Zeyer J (2003) mRNA extraction and reverse transcription-PCR protocol for detection of *nifH* gene expression by *Azotobacter vinelandii* in soil. *Appl Environ Microbiol* 69: 1928–1935.

46. Neufeld JD, *et al.* (2007) DNA stable-isotope probing. *Nat Protoc* 2: 860-866.

47. Kolb S, Knief C, Dunfield PF, Conrad R (2005) Abundance and activity of uncultured methanotrophic bacteria involved in the consumption of atmospheric methane in two forest soils. *Environ Microbiol* 7: 1150–1161.

48. Horz HP, Barbrook A, Field CB, Bohannan BJM (2004) Ammonia-oxidizing bacteria respond to multifactorial global change. *Proc Natl Acad Sci USA* 101: 15136–15141.

49. Grosskopf R, Janssen PH, Liesack W (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl Environ Microbiol* 64: 960–969.

50. Ludwig W, *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* 32: 1363–1371.

51. Treusch AH, *et al.* (2005) Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ Microbiol* 7: 1985–1995.

52. Avrahami S, Liesack W, Conrad R (2003) Effects of temperature and fertilizer on activity and community structure of soil ammonia oxidizers. *Environ Microbiol* 5: 691–705.

53. Offre P, Prosser JI, Nicol GW (2009) Growth of ammonia-oxidizing archaea in soil microcosms is inhibited by acetylene. *FEMS Microbiol Ecol* 70: 99-108.

54. Stark JM, Hart SC (1997) High rates of nitrification and nitrate turnover in undisturbed coniferous forests. *Nature* 385: 61-64.

V. Streptomycetes contributing to atmospheric molecular hydrogen soil uptake are widespread and encode a putative high-affinity [NiFe]-hydrogenase

Philippe Constant, Soumitra Paul Chowdhury, Jennifer Pratscher and Ralf Conrad

Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany

Environmental Microbiology (2010) **12**(3), 821-829

V.1 Summary

Uptake of molecular hydrogen (H_2) by soil is a biological reaction responsible for ~80% of the global loss of atmospheric H_2. Indirect evidence obtained over the last decades suggests that free soil hydrogenases with an unusually high affinity for H_2 are carrying out the reaction. This assumption has recently been challenged by the isolation of *Streptomyces* sp. PCB7, displaying the high affinity H_2 uptake activity previously attributed to free soil enzymes. While this finding suggests that actinobacteria could be responsible for atmospheric H_2 soil uptake, the ecological importance of H_2-oxidizing streptomycetes remains to be investigated. Here, we show that high affinity H_2 uptake activity is widespread among the streptomycetes. Among 14 streptomycetes strains isolated from temperate forest and agricultural soils, 6 exhibited a high affinity H_2 uptake activity. The gene encoding the large subunit of a putative high affinity [NiFe]-hydrogenase (*hydB*-like gene sequence) was detected exclusively in the isolates exhibiting high affinity H_2 uptake. CARD-FISH experiments targeting *hydB*-like gene transcripts and H_2 uptake assays performed with strain PCB7 suggested that streptomycetes spores catalyzed the H_2 uptake activity. Expression of the activity in term of biomass revealed that 10^6-10^7 H_2-oxidizing bacteria per gram of soil should be sufficient to explain in-situ H_2 uptake by soil. We propose that specialized H_2-oxidizing actinobacteria are responsible for the most important sink term in the atmospheric H_2 budget.

V.2 Introduction

The biogeochemical cycle of molecular hydrogen (H_2) is receiving renewed interest as reaction to controversial modeling studies suggesting that a future H_2-based economy would alter the oxidative capacity of the troposphere (Schultz et al., 2003; Tromp et al., 2003; Warwick et al., 2004). Indeed, current atmospheric H_2 budget estimations have considerable

uncertainties, especially in the case of soil uptake terms (Ehhalt and Rohrer, 2009). Although soil uptake accounts for ~80% of the global H_2 loss, almost nothing is known about the origin and the environmental factors influencing the H_2 oxidation activity (Constant et al., 2009).

The H_2 oxidation activity, which is located within the upper soil layers, displays a biphasic kinetic showing two different $K_{m(app)}$ for H_2 (Schuler and Conrad, 1990; Häring and Conrad, 1994). The "low affinity" H_2 oxidation activity exhibits a high $K_{m(app)}$ (>800 nM) and is catalyzed by a metabolically diverse group of aerobic H_2-oxidizing microorganisms, namely the Knallgas bacteria. These microorganisms are particularly abundant in soil surrounding legume nodules that are lacking uptake hydrogenases and are enriched in H_2-treated soils (Maimaiti et al., 2007; Zhang et al., 2009). Knallgas bacteria are unable to consume atmospheric H_2 due to their elevated H_2 threshold concentration and their low affinity for H_2 (Conrad et al., 1983). Atmospheric H_2 soil uptake is catalyzed by the "high affinity" H_2 oxidation activity. This high affinity activity exhibits a low $K_{m(app)}$ (10-70 nM) and involves microorganisms or enzymes that have not yet been identified.

Indirect evidence obtained over the last decades suggests that the uptake activity of atmospheric H_2 is mediated by free soil hydrogenases (Conrad, 1996). For instance, fumigation of soil with chloroform or acetone is not inhibiting completely the H_2 uptake, suggesting that cells membrane integrity is not required to support the activity (Conrad and Seiler, 1981). Attempts to extract free soil hydrogenases have resulted in recovery of ~2% of the original activity, but the responsible enzymes have neither been purified nor identified (Guo and Conrad, 2008). Interestingly, the recent finding that *Streptomyces* sp. PCB7 exhibits a high affinity H_2 uptake activity has challenged the free soil hydrogenases hypothesis (Constant et al., 2008). H_2 uptake activity by strain PCB7 implies that metabolically-active cells would be responsible for atmospheric H_2 soil uptake, but further investigations are needed to assess the ecological importance of streptomycetes. With the exception of strain PCB7 and a few thermophilic isolates (Gadkari et al., 1990; Kim et al., 1998), H_2 oxidation activity has not been reported for the streptomycetes. Genes encoding a putative [NiFe]-hydrogenase have been observed in the genome of *S. avermitilis* (Ikeda et al., 2003), but experimental evidence is not yet available to confirm its H_2 metabolism.

The main objective of this article is to provide new evidence for the involvement of *Streptomyces* spp. in the H_2 biogeochemical cycle. Therefore, we investigated the high affinity H_2 uptake activity of *S. avermitilis* and developed primers targeting the large subunit of its [NiFe]-hydrogenase (*hydB*-like gene sequence). Strain PCB7 was used as model microorganism to develop a suitable assay to screen new environmental isolates for high affinity H_2 consumption activity.

V.3 Results and discussion

***Streptomyces avermitilis* displays a high affinity H_2 uptake activity**

The presence of genes encoding for accessory (HypA-F, HypX, HupB) and structural (HydA-B) proteins of a putative [NiFe]-hydrogenase was reported in the genome of *S. avermitilis*, but neither gene expression nor H_2 oxidation activity have been analyzed (Ikeda et al., 2003). We evaluated the H_2 uptake capability of *S. avermitilis* 46492^T by growing the strain in sterile soil. After 10 days of incubation, the strain displayed a high affinity H_2 uptake activity, with a $K_{m(app)}$ of 39 nM and a $V_{max(app)}$ of 1.51 nmol min^{-1} g^{-1}. Since the genome of *S. avermitilis* contains a single [NiFe]-hydrogenase-coding region, this hydrogenase represents a potential candidate conferring the high affinity H_2 uptake activity. Primers targeting the gene encoding the large subunit of the putative hydrogenase (*hydB*-like gene) were designed and utilized to detect the presence of this gene in strain PCB7. As anticipated, a *hydB* PCR amplification product was observed, and its expression was confirmed by RT-PCR (data not shown).

Characterization of strain PCB7 H_2 uptake activity

Given that no molecular tools are yet available to detect high affinity hydrogenases, a cultivation-dependent approach combined with *hydB*-like genes detection was an obligatory first step to explore the ecological importance of H_2-consuming streptomycetes. We used strain PCB7 as model microorganism to develop a suitable H_2 oxidation assay. The strain was grown in liquid or agar-solidified R2A minimal medium, and in sterile soil before analyzing its H_2 uptake activity. On R2A agar and sterile soil, strain PCB7 grew following the well-defined series of differentiation that is typical for streptomycetes. Growth began with substrate mycelium development and was followed by the formation of aerial mycelia and then, sporulation. On the other hand, substrate mycelia dominated the biomass when strain PCB7 was grown in R2A broth. Indeed, liquid media do not support sporulation of streptomycetes, with only few exceptions (Karandikar et al., 1996). Spores of strains PCB7 harvested from R2A agar plates displayed a higher H_2 uptake rate than the substrate mycelia grown in R2A broth (Figure 1). This lower activity in liquid media, as well as previous microscopic observation of the development stages of strain PCB7 during the monitoring of its H_2 uptake activity (Constant et al., 2008) suggested that H_2 was consumed at the sporulation stage.

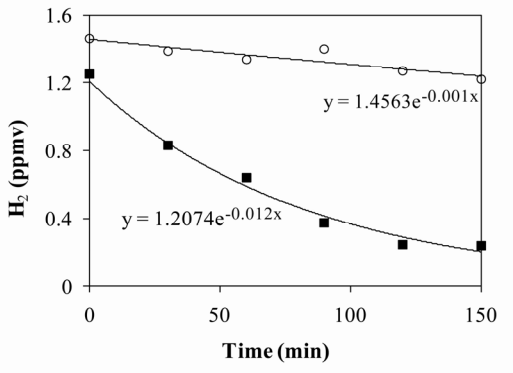


Figure 1. (A) H_2 oxidation activity of strain PCB7 biomass harvested from liquid (○) and agar solidified (■) R2A growth medium.

To visually investigate the expression of *hydB* mRNA in strain PCB7, *hydB* mRNA was detected by CARD-FISH (controls see Supplementary Fig. S1). Hybridizations of strain PCB7, grown on agar-solidified R2A medium, resulted in strong signals for the spores (Figure 2A-B), while no signal was observed for the mycelia (Figure 2C-D). This observation supports the results of the H_2 oxidation assays performed in liquid and agar-solidified R2A medium, indicating that spores rather than mycelia are responsible for the H_2 uptake activity. Cell cycle specific metabolism is typical for the streptomycetes and needs to be considered when assigning H_2 consumption activity to environmental isolates. Consequently, environmental streptomycetes isolates being tested for their H_2 uptake activity should be grown on R2A agar or sterile soil.

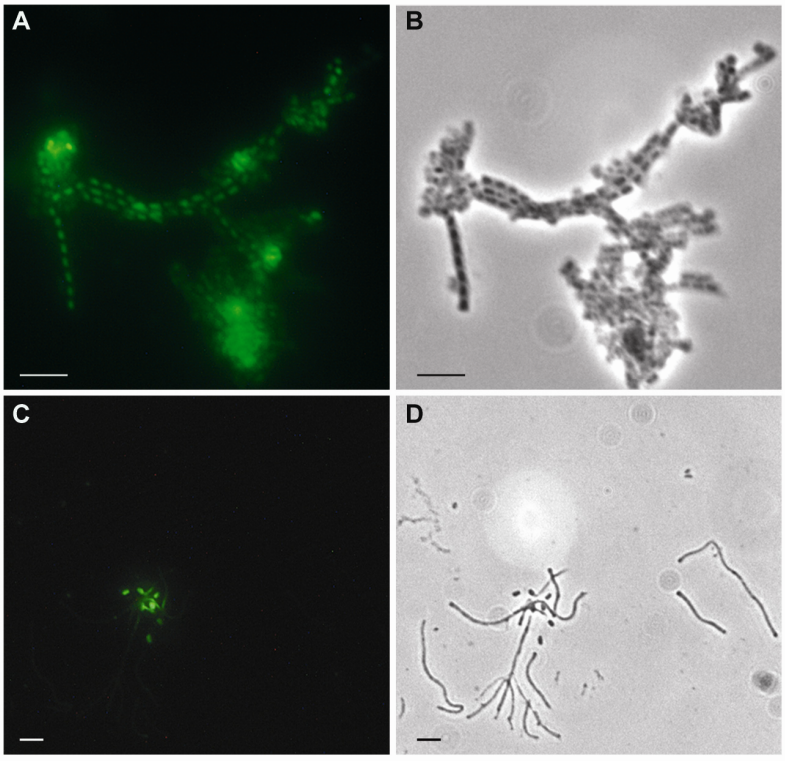


Figure 2. Detection of *hydB*-like mRNA in *Streptomyces* sp. PCB7 by application of CARD-FISH with *hydB* antisense probe. (A, B) Biomass was harvested from R2A Agar and (C, D) R2A broth after 10 days of incubation. Fluorescence images (A, C) and respective phase contrast (B, D). Bars = 2 µm.

H_2-consuming streptomycetes are widespread

The ecological importance of culturable H_2-consuming streptomycetes was explored using soil samples collected from forest and agricultural ecosystems. Presumptive actinobacteria were distinguished from other bacteria by their morphology. Formation of aerial and/or substrate mycelia were the main criteria to select the isolates. A total of 40 presumptive actinobacteria isolates were maintained on starch casein agar and further examined. Analysis of the 16S rRNA gene sequences revealed that 14 different isolates (12 from agricultural soil, 2 from forest soil) belonged to streptomycetes (Figure 3).

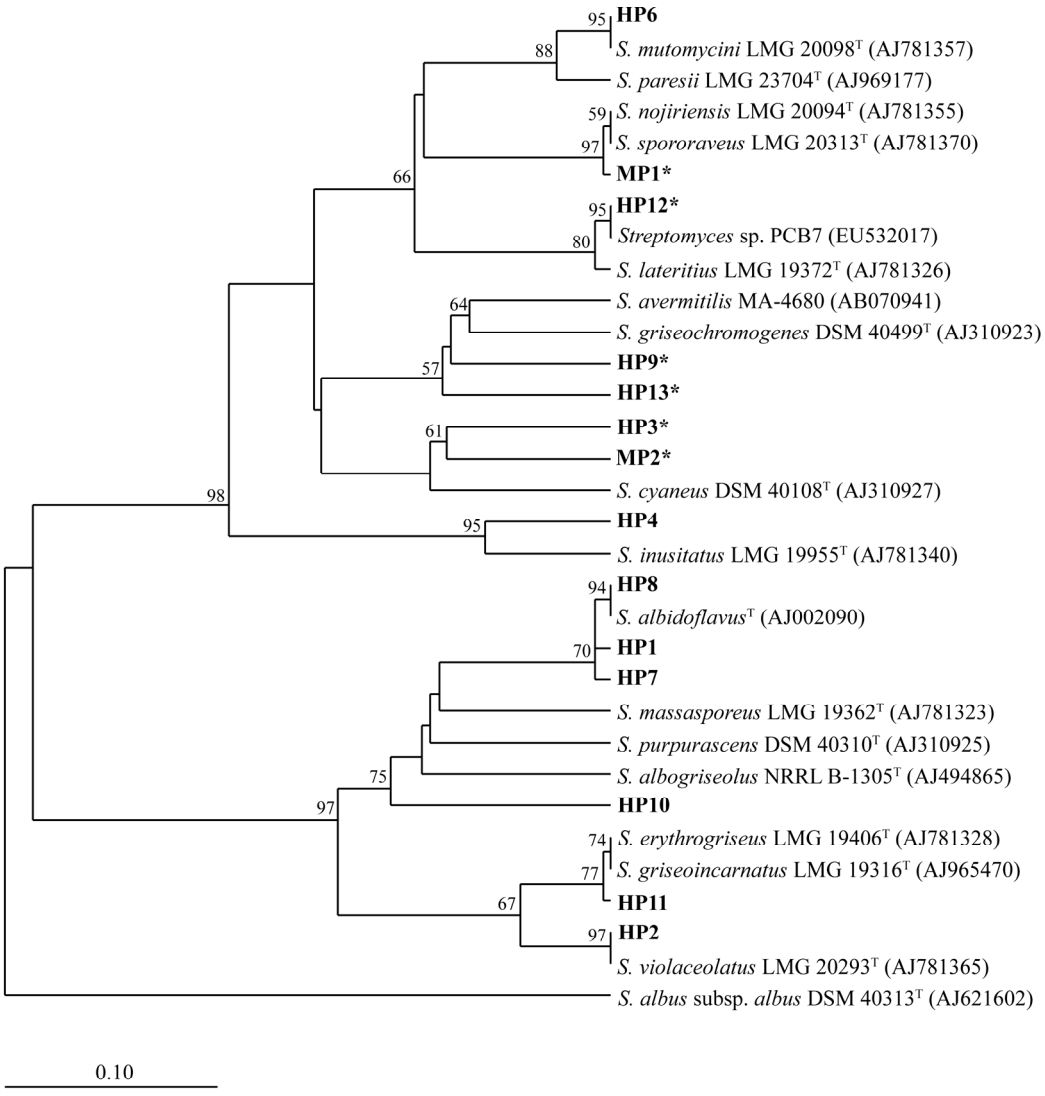


Figure 3. Maximum-likelihood tree based on nearly complete 16S rRNA gene sequence (1374 bp) showing the relationships between the *Streptomyces* isolates and related *Streptomyces* type strains. High affinity H_2 consuming strains are denoted by asterisk (*). The prefixes HP and MP indicate strains isolated from Heidelberg (agricultural soil) and Mainz (forest soil), respectively. The numbers at the branch points are tree puzzle support values. Only values greater than 50 are shown. The scale bar represents 10% sequence divergence.

All of the isolates grew well on R2A agar, but few formed spores under these conditions hampering the analysis of H_2 uptake activity. Therefore, the isolates were inoculated into sterile soil before measuring their H_2 uptake activity. After 10 days of incubation, white aerial sporulating mycelia were visible on soil particles, 6 out of the 14 streptomycetes isolates demonstrated a positive H_2 uptake activity. These isolates displayed moderate to high affinity for H_2 with $K_{m(app)}$ values between 39 nM and 374 nM (Table 1).

Table 1. H_2 uptake kinetic parameters and number of cfu of streptomycete isolates.

Strains	$V_{max(app)}$ [nmol min^{-1} $g^{-1}_{(dw)}$]	$K_{m(app)}$ (nM)	a^0_S[b] (L C-$mol_{Biomass}^{-1}$ h^{-1})	cfu $g^{-1}_{(dw)}$	H_2 oxidation rate [nmol h^{-1} $g^{-1}_{(dw)}$]	Theoretical population[b] [cfu $g^{-1}_{(dw)}$]
Streptomyces sp. MP1	1.13	100	5.29×10^6	$1.8 \pm 0.62 \times 10^7$	1.72	1.06×10^7
Streptomyces sp. MP2	1.10	135	4.70×10^6	$1.5 \pm 0.32 \times 10^7$	1.23	7.60×10^6
Streptomyces sp. HP3	2.01	43	2.80×10^7	$1.4 \pm 0.27 \times 10^7$	1.72	1.06×10^7
Streptomyces sp. HP9	0.18	374	8.43×10^5	$4.8 \pm 0.56 \times 10^6$	0.25	1.52×10^6
Streptomyces sp. HP12	0.92	361	4.86×10^5	$4.4 \pm 1.8 \times 10^7$	0.25	1.52×10^6
Streptomyces sp. HP13	0.17	112	2.64×10^6	$4.8 \pm 0.6 \times 10^6$	0.25	1.52×10^6
Streptomyces sp. PCB7	0.63	47	1.68×10^6	$6.8 \pm 0.1 \times 10^7$	1.47	9.11×10^6
S. avermitilis 46492^T	2.14	39	9.64×10^7	$9.0 \pm 5.0 \times 10^6$	5.40	3.34×10^7
Natural soil[a]	0.6–36	10–70	5.3×10^7		10	6.2×10^7

a. Data from Häring and Conrad (1994), Conrad (1996) and Conrad (1999).

b. See Conrad (1999) for calculations. Specific affinity (a^0_S) is the ratio V_{max}/K_m where V_{max} is expressed in $mol_{(H2)}$ C-$mol_{Biomass}^{-1}$ h^{-1} (derived assuming 1.4 x10^{14} CFU per C-$mol_{Biomass}$) and K_m is expressed in $mol_{(H2)}L^{-1}$.

The oxidation rate of individual isolates exposed to 1.5 ppmv H_2 was used to estimate the maximal population density sustained by H_2. H_2 uptake kinetic parameters of natural soil and theoretical population density sustained by typical H_2 uptake rate are shown for comparison.

It is noteworthy to mention that H_2 uptake activity of the isolates was detected at the sporulation period, as observed with strain PCB7 (data not shown). The kinetic analysis reported in Table 1 showed that the $K_{m(app)}$ values of the H_2-consuming streptomycetes were not clustered into a low affinity (>800 nM) and a high affinity (10-70 nM) group, but instead apparently covered a continuum of $K_{m(app)}$. This continuous range of $K_{m(app)}$ values may be explained by the different ability of the isolates to scavenge H_2, or the variation caused by cells metabolism (Button, 1993; Dunfield and Conrad, 2000). Since estimation of kinetic parameters (V_{max} and K_m) is influenced by substrate diffusion limitation and physiological state of microorganisms, the specific affinity coefficient ($a^0_S = V_{max}/K_m$) is considered as a better index for the ability of the cells to consume limiting substrate (Button, 1993; Dunfield and Conrad, 2000). Comparison of the calculated a^0_S confirmed that isolates differed in their ability to consume H_2. *S. avermitilis* and strain HP3 displayed the highest specific affinity for H_2, with a^0_S values being similar to those of natural soil (Table 1). No relationship was observed between the phylogenetical affiliation of the isolates and their affinity for H_2 (Figure 3).For instance, the 16S rRNA gene sequences of strains HP12 and PCB7 shared 100% similarity but differed in their phenotypic characteristics and H_2 uptake activities.

Based on microbial maintenance energy requirement, it has been estimated that a typical atmospheric H_2 soil uptake rate could support a maximal population of 6.2 x10^7 H_2-oxidizing bacteria per gram of soil (Conrad, 1999). Application of the same calculations to the H_2 uptake rates measured with the individual streptomycetes isolates revealed that the observed H_2 uptake supported the maintenance energy requirement for 10^6-10^7 cells $g^{-1}_{(soil)}$. Interestingly, these estimates are on the same order of magnitude as the agar plate enumerations performed following the H_2 uptake assays (Table 1). Hence, uptake of atmospheric H_2 should be able to sustain the maintenance energy required for the survival of the isolates. The occurrence and the abundance of the streptomycetes have been reported for a broad variety of ecosystems, including desert soils, forests and peatlands (Xu et al., 1996; Okoro et al., 2009; Pankratov and Dedysh, 2009). For instance, cultivation-dependant approaches revealed the presence of 10^5 streptomycetes $g^{-1}_{(soil)}$ in coastal sand dune and rhizospheric soil (Xu et al., 1996; Kurtböke et al., 2007; Yilmaz et al., 2008). Considering their ubiquity and their ecological importance, we propose that H_2-consuming *Streptomyces* spp. are contributing to the atmospheric H_2 soil uptake observed in the environment. The involvement of other nonculturable microorganisms or other bacteria harbouring functional genes encoding for putative high affinity hydrogenase is however not excluded, showing the importance to use specific molecular tools to detect other potential high affinity H_2 consumers.

Detection of putative high affinity [NiFe]-hydrogenases

We used the primers targeting the *hydB*-like gene sequence of *S. avermitilis* and strain PCB7 to test the streptomycete isolates. As anticipated, PCR amplification products were observed exclusively in the six isolates that also demonstrated the moderate-to-high affinity toward H_2. The phylogenetic analysis of the amino acid sequences of *hydB*-like genes from strain PCB7, *S. avermitilis* and these six isolates shows that they are clustered together with the corresponding sequences from *Mycobacterium* spp., *Frankia* spp., *Rhodococcus jostii* RHA1 and *Ralstonia eutropha* H16 (Figure 4). These clustered genes have previously been described as belonging to Group 1 of the [NiFe]-hydrogenases (Vignais and Billoud, 2007). Group 1 contains membrane-bound uptake hydrogenases responsible for either anaerobic or aerobic oxidation of H_2 with the generation of energy. Phylogenetically this group is an assemblage of H_2 uptake hydrogenases from mainly *Bacteria* and some thermophilic and methanogenic *Archaea*.

So far, there has been no report that Group 1 [NiFe]-hydrogenases exhibit a high affinity H_2-uptake activity. Genes of these hydrogenases have been reported in genome sequencing projects and analysis of their G+C content suggests that they originate from lateral transfer of hydrogenase genes from actinobacteria (Cramm, 2009; Leul et al., 2009), but it is unclear what type of hydrogenase activity they code for. Previous experiments have shown that *R. eutropha* H16 displays only a low affinity H_2 uptake activity in soil (Conrad et al., 1983).

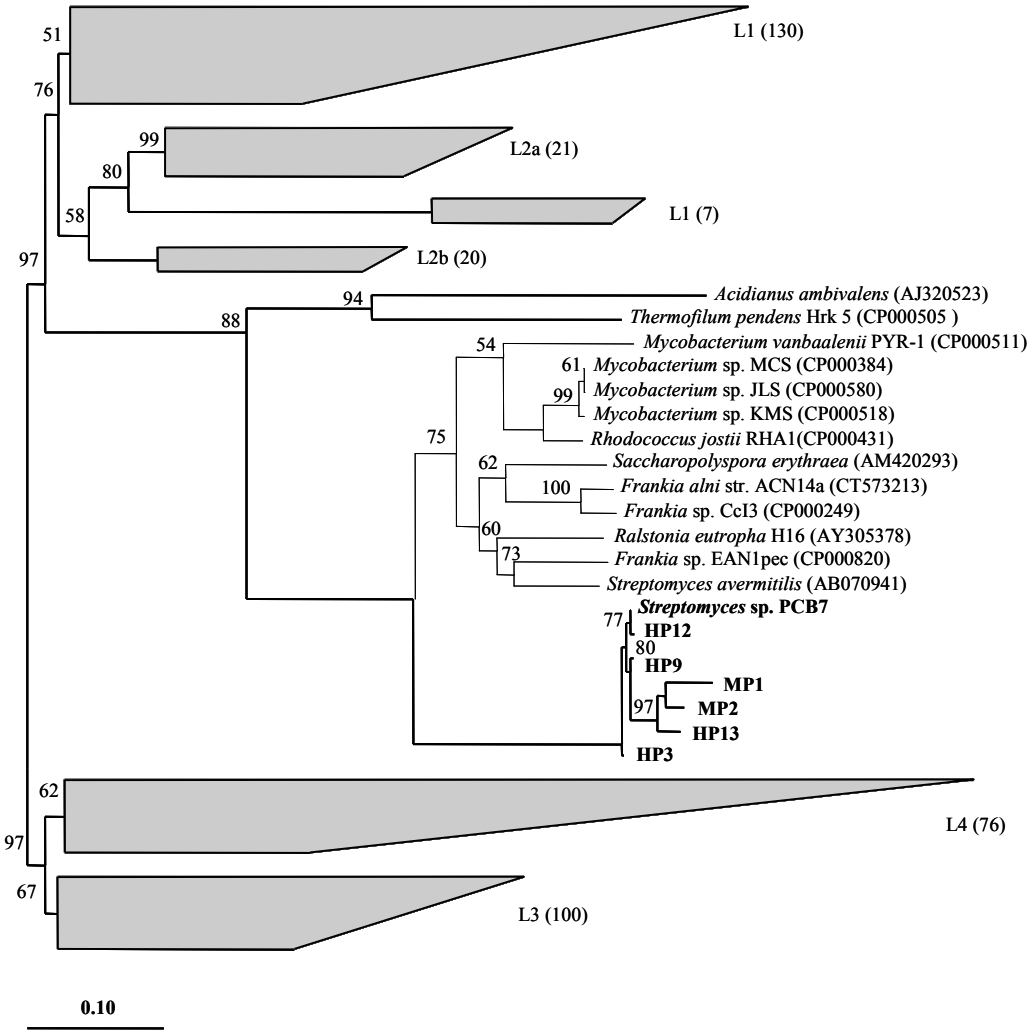


Figure 4. Consensus tree of partial amino acid sequences translated from *hydB*-like gene sequences from *Streptomyces* isolates and large subunits of [NiFe]-hydrogenases gene sequences available in public databases. Different subgroups (L1-L4) were designated according to Vignais and Billoud (2007). The number of sequences in each subgroup has been indicated in brackets. The prefixes HP and MP indicate strains isolated from Heidelberg (agricultural soil) and Mainz (forest soil), respectively. The scale bar represents 10% sequence divergence.

The genome of *R. eutropha* H16 encodes for four different hydrogenases: a soluble (SH) and a membrane-bound (MBH) hydrogenase involved in lithotrophic metabolism, a H_2 sensor hydrogenase activating the MBH and SH gene expression, and a [NiFe]-hydrogenase (*hyd4* DNA region) of which the function has not been known (Schwartz et al., 2003; Cramm, 2009). The physiological role of Hyd4 enzyme is unknown since *R. eutropha* H16 mutant strains deficient in both MBH and SH genes is unable to grow in presence of H_2 and CO_2 (Kleihues et al., 2000). Our results show that the *hyd4* gene is possibly affiliated to the streptomycetes

putative high affinity hydrogenase. Owing to the specificity of hydrogenase maturation systems involving *cis*- and *trans*-acting factors (Vignais and Colbeau, 2004), it is possible that *hyd4* DNA region of *R. eutropha* H16 is not functional. On the other hand, a recent study on the phylogeny of uptake hydrogenases in *Frankia* revealed the presence of two different hydrogenase syntrons (Leul et al., 2009). Our phylogenetic tree provides evidence that the *hydB* like genes in the *Streptomyces* sp. PCB7 and the isolates are closely related to the hydrogenase syntron 1 genes of *Frankia* sp.. These hydrogenase genes have been shown to be quantitatively more expressed in free-living cells than in symbiotic cells that are exposed to an elevated H_2 level, but measurements of the H_2 oxidation activity have not been reported (Leul et al., 2007). Based on our data we propose that the *hydB*-like genes code for a high affinity [NiFe]-hydrogenase that is responsible for uptake of atmospheric H_2 by soil. Considering the phylogenetic analysis presented in Figure 4, it is tempting to hypothesize that some Knallgas bacteria could express high affinity hydrogenase under specific conditions. However, there is currently no evidence for an implication of these microorganisms in the soil sink term of atmospheric H_2. Indeed, Conrad et al. (1983) have shown that Knallgas bacteria, including *Ralstonia eutropha* H16, are unable to consume atmospheric H_2 due to their high H_2 threshold concentration. Further investigation is required to characterize the newly identified putative high affinity [NiFe]-hydrogenases cluster in terms of activity and gene expression.

Our study provides new evidence for the significant role of *Streptomyces* spp. in the H_2 biogeochemical cycle. *Streptomyces* spp. are usually chemoorganotrophs, degrading complex polymeric substrates during their initial development and consuming stored carbon during sporulation. Is uptake of atmospheric H_2 providing a selective advantage for streptomycetes with high affinity H_2 uptake under nutrient-limiting conditions? Identification of the structural and regulatory genes encoding the high affinity hydrogenase system of the streptomycetes isolates described in this study will be crucial to understand the metabolism and the diversity of microorganisms consuming atmospheric H_2. As H_2 uptake activity likely occurs at the sporulation period, environmental factors influencing the development of streptomycetes, including soil water and carbon content as well as C:N ratio may influence atmospheric H_2 soil uptake activity.

Acknowledgements

P.C. was supported by postdoctoral fellowships from the Fonds Québécois de Recherche sur la nature et les technologies and the Max Planck Society. The authors are grateful to Melanie Klose for her excellent technical assistance. This study was partially funded by the European Network for Atmospheric Hydrogen observations and studies (Eurohydros) grant to R.C.

V.4 Experimental procedures

Isolation of streptomycetes from soil

Streptomycetes were isolated from samples of a sandy soil collected from a deciduous forest (*Fagus sylvatica*) located in Mainz, Germany (50.00°N; 08.17°E) and from an agricultural soil collected from areas covered with white clover (*Trifolium repens*) located in Heidelberg, Germany (49.42°N; 08.61°E). Samples were collected from the upper 10 cm of the soil profile after removal of the litter. Mainz soil had a pH of 5.9, total nitrogen content of 0.1% and carbon content of 1.33%, while Heidelberg soil had a pH of 7.3, total nitrogen of 0.1% and carbon content of 0.88%. Soils were sieved (2 mm) before taking 500 mg sub-samples that were subsequently suspended in 5 ml sterile saline water (0.85% NaCl) and thoroughly vortexed at maximum speed to detach cells from soil particles. Soil suspensions were serially diluted (10^{-1} to 10^{-6}) and 0.1 ml of each dilution was inoculated onto casein starch agar supplemented with cycloheximide (50µg ml^{-1}) as antifungal agent (Kuster and Williams, 1964). The agar plates were incubated for 14 days at 30°C. Putative streptomycetes were distinguished based on their colony morphology. Pure cultures were obtained after three sequential transfers of individual colonies onto casein starch agar. Individual colonies were then transferred onto oatmeal agar (60 g oatmeal and 12.5 g agar per litre of water) to determine the morphology characteristics of the isolates, and the biomass was utilized for genomic DNA extraction. Isolates were discriminated and identified on the basis of their morphology features (e.g. diffusive pigments, spores) and their 16S rDNA gene sequence. Stock cultures, prepared by transferring spores and mycelia of each isolate in 20% sterile glycerol, were stored at -80°C. *Streptomyces avermitilis* 46492^T was provided by the German Collection of Microorganisms and Cell Cultures (DSMZ).

DNA extraction

Biomass was collected from oatmeal agar plates and mixed with 500 mg of glass beads (0.17–0.18 mm in diameter), 1.0 ml TEN buffer (50 mM Tris-HCl, 100 mM EDTA, 150 mM NaCl, pH 8.0) and 50 µl 10% SDS. The samples were transferred into a FastPrep™ instrument (Bio101 Thermo Savant) for two cycles of mechanical cell disruption of 45 s at speed 5.5, with a 5-min incubation on ice in-between the cycles. The tubes were centrifuged (10 min, 16 000×g), and 500 µl of the supernatant was used for DNA purification using Wizard® DNA Clean-Up System (Promega, Madison, WI, U.S.A.) according to the manufacturer instructions. Purified DNA was quantified using Nanodrop 1000 (peqlab Biotechnologie GmbH, Erlangen, Germany) and kept frozen at -20°C.

PCR amplification of 16S rRNA and *hydB*-like genes

PCR reactions were performed in 50-µl reaction volumes containing the following concentrations or total amounts: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTP, 10 pM of each primer, 20 mg of bovine serum albumin, 2.5 U of Taq polymerase (Go Taq®, Promega, Madison, WI, U.S.A) and 20 ng DNA. The reactions were performed

using a Primus Thermocycler (MWG-Biotech AG, Ebersberg, Germany) at 94°C for 5 min, 55°C for 5 min, followed by 30 cycles at 72°C for 45 s, 94°C for 45 s, 55°C for 45 s and a final extension period of 10 min at 72°C. 16S rRNA genes were amplified using the PA (5'-AGAGTTTGATCMTGGCTCAG-3') and PH (5'-AAGGAGGTGATCCARCCGCA-3') primer pair that corresponds to the 8–27 and 1521–1541 positions in *Escherichia coli* 16S rRNA gene sequence (Edwards et al., 1989). *hydB*-like genes encoding the putative [NiFe]-hydrogenase large subunit of *Streptomyces avermitilis* 46492^T were amplified using the NiFe-244f (5'-GGGATCTGCGGGGACAACCA-3') and NiFe-1640r (5'-TGCACGGCGTCCTCGTACGG-3') primer pair. Primer NiFe-244f was specific to conserved L1 signature region of the NiFe-hydrogenase large subunit (Vignais et al., 2001). Another forward primer (NiFe-1129f, 5'-ccgcggtggttcgacggcaa-3') was designed to generate shorter PCR amplification products appropriate for reverse transcription reactions and CARD-FISH probe synthesis (see below).

H_2 soil uptake activity assays

H_2 uptake measurements were performed using biomass grown in sterile soil, in liquid or in agar-solidified R2A medium (in g L^{-1}: yeast extract 0.5, proteose peptone 0.5, casein hydrolysate 0.5, glucose 0.5, soluble starch 0.5, sodium pyruvate 0.3, K_2HPO_4 0.3, $MgSO_4.7H_2O$ 0.05, and agar 12). For growth in sterile soil, 25 g of 2-mm sieved soil samples collected in Mainz forest were transferred into a 125-ml glass bottle and heat-sterilized (30 min, 121°C, 1 bar) in two cycles separated by a 24-h time interval. Single isolates were grown 48 hours at 30°C in tryptic soy broth and transferred into the heat-sterilized soil samples to obtain a water content of 20%. Bottles were closed with a butyl rubber stopper and incubated at 25°C for 10 days. Sterile packed cotton wool column fitted to a needle was inserted through the stopper to ensure aerobic conditions in bottle's headspace throughout the incubation period. The packed column was removed during the H_2 soil uptake measurements performed under a static headspace. In other experiments, biomass grown in liquid or agar-solidified R2A medium was aseptically harvested before being suspended in 5 ml modified sterile PBS buffer (phosphate buffered saline; 130 mM NaCl; 7 mM $Na_2HPO_4.12H_2O$; 3 mM $NaH_2PO_4.2H_2O$) (Caracciolo et al., 2005). The cells suspensions were then transferred into 60ml vials for H_2 uptake activity measurements.

Before performing the H_2 uptake activity measurements, glass bottles containing biomass suspension or soil samples were flushed for 30 min with synthetic air (80% N_2 and 20% O_2). Pure H_2 was then added into the headspace of the bottles to obtain an initial mixing ratio of 1.5 – 1.8 ppmv H_2. Decrease of the H_2 mixing ratio was monitored as a function of time by analyzing aliquots (0.5 ml) of the headspace air in a Trace Analytical Reduced Gas Analyzer as previously described (Schuler and Conrad, 1990). In the case of liquid samples, bottles were continuously agitated at 100 rpm on an orbital shaker to enhance the transfer H_2 from the gas into the liquid phase. Apparent first order H_2 uptake rate constants were obtained by integrating the logarithmic decrease of headspace H_2 mixing ratio. Reproducibility of the H_2 analyses was assessed before each set of experiments by repeated analysis of certified H_2

standard gas (2.0 ppmv ±5%, 50 ppmv ±2% and 1000 ppmv ±2% H_2, Messer Schweiz AG, Lenzburg, Switzerland), and standard deviations were typically <5%. No significant H_2 uptake was observed for blank experiments involving sterile soil or phosphate buffer containing no biomass.

H_2 oxidation kinetic parameters ($K_{m(app)}$; $V_{max(app)}$) were obtained by the addition of specified amounts of pure H_2 into the headspace of glass bottles containing inoculated soils samples as previously described (Schuler and Conrad, 1990). Estimation of the kinetic parameters was performed 10 days following soils inoculation (Constant et al., 2008).

Agar plate enumeration

Agar plate enumerations of the streptomycetes strains were performed at the end of each H_2 uptake measurement series. Aliquots of the cell suspension (0.5 ml) or the soil (~1 g) were sampled in triplicate and serially diluted in 0.85% NaCl (10^{-1} to 10^{-6}). An aliquot (0.1 ml) of each dilution was inoculated onto a R2A agar plate for colony-forming unit (CFU) enumerations. CFU enumerations were performed following 10 days of incubation at 25°C.

CARD-FISH of *hydB* mRNA

To generate controls for Catalyzed Reporter Deposition-Fluorescence *In Situ* Hybridization (CARD-FISH) assays, partial *hydB*-like genes from *Streptomyces* sp. PCB7 (positive control) and *Paracoccus denitrificans* (Knallgas bacteria; negative control) were cloned and expressed in *E. coli* by using vector pBAD as previously described (Pernthaler and Amann, 2004). Polynucleotide antisense RNA probes were generated by in vitro transcription and were simultaneously labeled by incorporating digoxigenin-11-UTP (Roche Molecular Diagnostics) (Zwirglmaier et al., 2003). Clones were fixed in 2% (vol/vol) formaldehyde for 30 min at room temperature, centrifuged, and washed once with PBS and twice with 50% ethanol in PBS. Samples of *Streptomyces* sp. PCB7 were fixed in 50% ethanol in PBS for 24 h at 4°C, centrifuged, and washed once with PBS. Cells then were resuspended in 50% ethanol in PBS and stored at -20°C until further processing. The hybridization and detection procedures were based on the protocol described by Pernthaler and Amann (2004), with some minor modifications. 5 µl of cell suspensions were applied to each well of a Teflon-coated glass slide (MAGV, Rabenau-Londorf, Germany) and dried at 46°C. Expression clones of *hydB* were permeabilized with lysozyme (5 mg ml^{-1} in TE; Sigma) for 30 min at room temperature. *Streptomyces* sp. PCB7 samples were first incubated with lysozyme (10 mg ml^{-1} in TE; Sigma) for 1 h at 37°C, subsequently washed in Milli-Q water, and then incubated with achromopeptidase (60 units ml^{-1}, dissolved in 0.01 M NaCl and 0.01 M Tris-HCl; Sigma) for 30 min at 37°C (Sekar et al., 2003). Hybridization was performed overnight at 58°C using a formamide concentration of 50% in the hybridization buffer. For microscopic evaluation, slides were mounted with the antifading agent Citifluor AF1 (Citifluor, UK) and hybridization preparations were visualized by fluorescence microscopy (Axiophot; Carl Zeiss Microimaging GmbH, Goettingen, Germany).

Phylogenetic analysis

PCR-amplified 16S rRNA and *hydB* genes from isolates were sequenced using the Applied Biosystems 3730XL Genetic Analyser (Max Planck Institute for Plant Breeding Research). Gene sequences were edited and assembled using BioEdit software (Hall, 1999). Gene sequences were compared with GenBank database using standard nucleotide-nucleotide BLAST search (Altschul et al., 1997). Phylogenetic analyses of 16S rRNA gene sequences were performed with ARB software package (Ludwig et al., 2004) and SILVA database containing aligned 16S ribosomal RNA sequences with a minimum length of 1200 bp for bacteria (Pruesse et al., 2007). Phylogenetic trees were constructed using Tree-Puzzle maximum-likelihood (Schmidt et al., 2002) and maximum-parsimony (Fitch, 1971) tree calculating algorithms. Robustness of the calculated trees was evaluated by 1000 puzzling steps and by bootstrap analysis based on 1000 resamplings. 16S rRNA gene sequences of the isolates were compared with those of *Streptomyces* type strains. The translated amino acid sequences of the *hydB*-like genes were aligned using the MUSCLE sequence alignment tool (Edgar, 2004). The alignment was corrected manually and trees were constructed using the aligned sequences by RAxML (Stamatakis et al., 2008), neighbor joining (Saitou and Nei, 1987) and maximum parsimony algorithms. Robustness of the calculated trees was evaluated by bootstrap analysis based on 1000 resamplings. Consensus trees were obtained depicting the trees with most frequent branching supported by all the analyses. All sequences were deposited in the GenBank with accession numbers GQ867021 to GQ867035 for 16S rRNA genes and GQ867036 to GQ867042 for *hydB* gene sequences.

V.5 Supplementary material

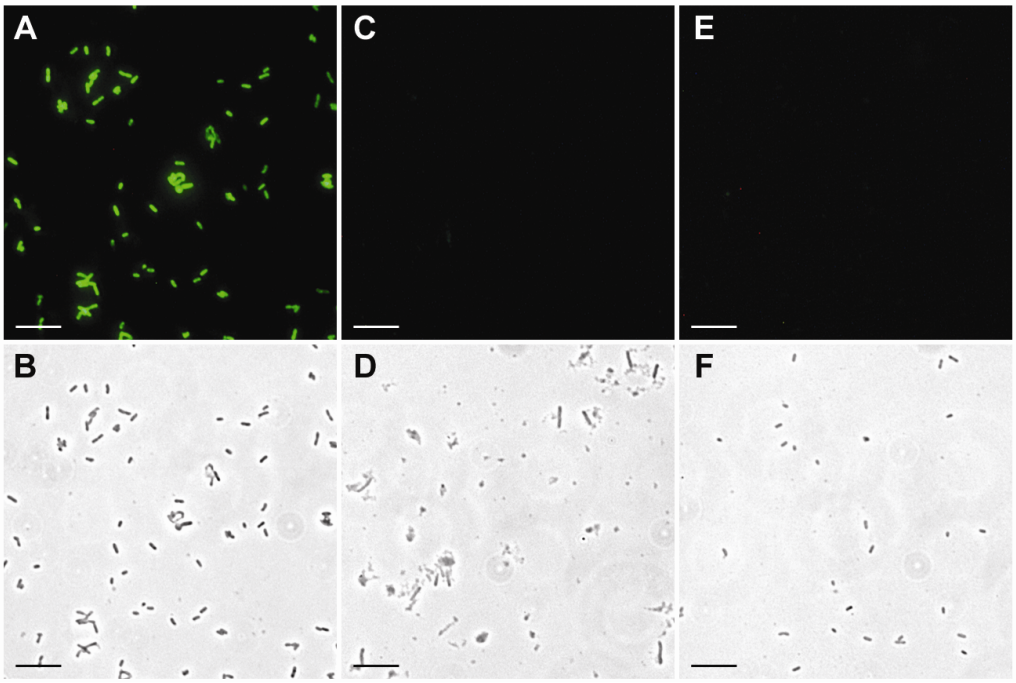


Figure S1. Hybridization of various expression clones with *hydB* mRNA antisense probe as controls for specificity. (A) *E. coli* clone expressing *hydB* of *Streptomyces* sp. PCB7. (C) Uninduced *E. coli* clone harbouring partial *hydB* gene of *Streptomyces* sp. PCB7. (E) *E. coli* clone expressing *hydB* of *Paracoccus denitrificans*. Panels B, D, and F show respective phase contrast images. Bars = 5 µm.

V.6 References

Altschul, S., Madden, T., Schaffer, A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389-3402.

Button, D.K. (1993) Nutrient-limited microbial growth kinetics: overview and recent advances. *Antonie van Leeuwenhoek* 63: 225-235.

Caracciolo, A.B., Grenni, P., Cupo, C., and Rossetti, S. (2005) *In situ* analysis of native microbial communities in complex samples with high particulate loads. *FEMS Microbiol Lett* 253: 55-58.

Conrad, R. (1996) Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). *Microbiol Rev* 60: 609-640.

Conrad, R. (1999) Soil microorganisms oxidizing atmospheric trace gases (CH_4, CO, H_2, NO). *Ind J Microbiol* 39: 193-203.

Conrad, R., and Seiler, W. (1981) Decomposition of atmospheric hydrogen by soil-microorganisms and soil enzymes. *Soil Biol Biochem* 13: 43-49.

Conrad, R., Aragno, M., and Seiler, W. (1983) The inability of hydrogen bacteria to utilize atmospheric hydrogen is due to threshold and affinity for hydrogen. *FEMS Microbiol Lett* 18: 207-210.

Constant, P., Poissant, L., and Villemur, R. (2008) Isolation of *Streptomyces* sp. PCB7, the first microorganism demonstrating high-affinity uptake of tropospheric H_2. *ISME J* 2: 1066-1076.

Constant, P., Poissant, L., and Villemur, R. (2009) Tropospheric H_2 budget and the response of its soil uptake under the changing environment. *Sci Tot Environ* 407: 1809-1823.

Cramm, R. (2009) Genomic view of energy metabolism in *Ralstonia eutropha* H16. *J Mol Microbiol Biotechnol* 16: 38–52.

Dunfield, P.F., and Conrad, R. (2000) Starvation alters the apparent half-saturation constant for methane in the type II methanotroph *Methylocystis* strain LR1. *Appl Environ Microbiol* 66: 4136-4138.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32: 1792-1797.

Edwards, U., Rogall, T., Blocker, H., Emde, M., and Bottger, E.C. (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* 17: 7843-7853.

Ehhalt, D.H., and Rohrer, F. (2009) The tropospheric cycle of H_2: a critical review. *Tellus* 61B: 500-535.

Fitch, W.M. (1971) Towards defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* 20: 406-416.

Gadkari, D., Schricker, K., Acker, G., Kroppenstedt, R.M., and Meyer, O. (1990) *Streptomyces thermoautotrophicus* sp. nov., a thermophilic CO- and H_2-oxidizing obligate chemolithoautotroph. *Appl Environ Microbiol* 56: 3727-3734.

Guo, R.B., and Conrad, R. (2008) Extraction and characterization of soil hydrogenases oxidizing atmospheric hydrogen. *Soil Biol Biochem* 40: 1149-1154.

Hall, T.A. (1999) BioEdit: a user friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp Ser* 41: 95-98.

Häring, V., and Conrad, R. (1994) Demonstration of two different H_2-oxidizing activities in soil using an H_2 consumption and a tritium exchange assay. *Biol Fertil Soils* 17: 125-128.

Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T. et al. (2003) Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nat Biotech* 21: 526-531.

Karandikar, A., Sharples, G.P., and Hobbs, G. (1996) Influence of medium composition on sporulation by *Streptomyces coelicolor* A3(2) grown on defined solid media. *Biotechnol Tech* 10: 79-82.

Kim, S.B., Falconer, C., Williams, E., and Goodfellow, M. (1998) *Streptomyces thermocarboxydovorans* sp. nov. and *Streptomyces thermocarboxydus* sp. nov., two moderately thermophilic carboxydotrophic species from soil. *Int J Syst Bacteriol* 48: 59-68.

Kleihues, L., Lenz, O., Bernhard, M., Buhrke, T., and Friedrich, B. (2000) The H_2 sensor of *Ralstonia eutropha* is a member of the subclass of regulatory [NiFe] hydrogenases. *J Bacteriol* 182: 2716-2724.

Kurtböke, D., Neller, R., and Bellgard, S. (2007) Mesophilic actinomycetes in the natural and reconstructed sand dune vegetation zones of Fraser Island, Australia. *Microb Ecol* 54: 332-340.

Kuster, E., and Williams, S.T. (1964) Selection of media for isolation of streptomycetes. *Nature* 202: 928-929.

Leul, M., Normand, P., and Sellstedt, A. (2007) The organization, regulation and phylogeny of uptake hydrogenase genes in *Frankia*. *Physiol Plant* 130: 464-470.

Leul, M., Normand, P., and Sellstedt, A. (2009) The phylogeny of uptake hydrogenases in *Frankia*. *Int Microbiol* 12: 23-28.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar et al. (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* 32: 1363-1371.

Maimaiti, J., Zhang, Y., Yang, J., Cen, Y.-P., Layzell, D.B., Peoples, M., and Dong, Z. (2007) Isolation and characterization of hydrogen-oxidizing bacteria induced following exposure of soil to hydrogen gas and their impact on plant growth. *Environ Microbiol* 9: 435-444.

Okoro, C., Brown, R., Jones, A., Andrews, B., Asenjo, J., Goodfellow, M., and Bull, A. (2009) Diversity of culturable actinomycetes in hyper-arid soils of the Atacama Desert, Chile. *Antonie van Leeuwenhoek* 95: 121-133.

Pankratov, T., and Dedysh, S. (2009) Cellulolytic streptomycetes from *Sphagnum* peat bogs and factors controlling their activity. *Microbiology* 78: 227-233.

Pernthaler, A., and Amann, R. (2004) Simultaneous fluorescence *in situ* hybridization of mRNA and rRNA in environmental bacteria. *Appl Environ Microbiol* 70: 5426-5433.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., and Glockner, F.O. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* 35: 7188-7196.

Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406-425.

Schmidt, H.A., Strimmer, K., Vingron, M., and von Haeseler, A. (2002) TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* 18: 502-504.

Schuler, S., and Conrad, R. (1990) Soils contain two different activities for oxidation of hydrogen. *FEMS Microbiol Lett* 73: 77-83.

Schultz, M.G., Diehl, T., Brasseur, G.P., and Zittel, W. (2003) Air pollution and climate-forcing impacts of a global hydrogen economy. *Science* 302: 624-627.

Schwartz, E., Henne, A., Cramm, R., Eitinger, T., Friedrich, B., and Gottschalk, G. (2003) Complete nucleotide sequence of pHG1: a *Ralstonia eutropha* H16 megaplasmid encoding key enzymes of H_2-based lithoautotrophy and anaerobiosis. *J Mol Biol* 332: 369-383.

Sekar, R., Pernthaler, A., Pernthaler, J., Warnecke, F., Posch, T., and Amann, R. (2003) An improved protocol for quantification of freshwater *Actinobacteria* by fluorescence *in situ* hybridization. *Appl Environ Microbiol* 69: 2928-2935.

Stamatakis, A., Hoover, P., and Rougemont, J. (2008) A rapid bootstrap algorithm for the RAxML Web servers. *Syst Biol* 57: 758-771.

Tromp, T.K., Shia, R.-L., Allen, M., Eiler, J.M., and Yung, Y.L. (2003) Potential environmental impact of a hydrogen economy on the stratosphere. *Science* 300: 1740-1742.

Vignais, P.M., and Colbeau, A. (2004) Molecular biology of microbial hydrogenases. *Curr Issues Mol Biol* 6: 159-188.

Vignais, P.M., and Billoud, B. (2007) Occurrence, classification, and biological function of hydrogenases: an overview. *Chem Rev* 107: 4206-4272.

Vignais, P.M., Billoud, B., and Meyer, J. (2001) Classification and phylogeny of hydrogenases. *FEMS Microbiol Rev* 25: 455-501.

Warwick, N.J., Bekki, S., Nisbet, E.G., and Pyle, J.A. (2004) Impact of a hydrogen economy on the stratosphere and troposphere studied in a 2-D model. *Geophys. Res. Lett.* 31, doi: 10.1029/2003GL019224.

Xu, L., Li, Q., and Jiang, C. (1996) Diversity of soil actinomycetes in Yunnan, China. *Appl Environ Microbiol* 62: 244-248.

Yilmaz, E., Yavuz, M., and Kizil, M. (2008) Molecular characterization of rhizospheric soil streptomycetes isolated from indigenous Turkish plants and their antimicrobial activity. *World J Microbiol Biotechnol* 24: 1461-1470.

Zhang, Y., He, X., and Dong, Z. (2009) Effect of hydrogen on soil bacterial community structure in two soils as determined by terminal restriction fragment length polymorphism. *Plant and Soil* 320: 295-305.

Zwirglmaier, K., Ludwig, W., and Schleifer, K.H. (2003) Improved fluorescence *in situ* hybridization of individual microbial cells using polynucleotide probes: the network hypothesis. *Syst Appl Microbiol* 26: 327-337.

VI. Discussion

VI.1 Upland Soil Cluster α in Marburg forest soil

Forest soils are considered to represent the most effective biological sink for atmospheric methane, but to date it remains elusive which microorganisms process this uptake and whether they rely on methane as their sole carbon and energy source. The physiological properties of these atmospheric CH_4 oxidizers remain unknown because so far organisms harbouring the key forest soil *pmoA* genotypes resisted cultivation. This applies particularly to the upland soil cluster α (USCα), proposed to exhibit a crucial role in atmospheric methane oxidation (Kolb, 2009).

VI.1.1 Methane oxidation and use of alternative carbon sources

The aim of this part of the thesis was to investigate the potential oxidation of methane and furthermore assimilation of acetate by upland soil cluster α in the acidic Marburg forest soil using RNA-SIP and DNA-SIP in comparison. Expression of USCα *pmoA* by mRNA catalyzed reporter deposition – fluorescence *in situ* hybridization (CARD-FISH) in the soil incubations was further monitored.

Active assimilation of acetate by USCα was shown by RNA-SIP, while incorporation of methane could not be detected, although *pmoA* was constantly expressed. These results indicate that USCα (and also other potential atmospheric methane oxidizers) might not be restricted to methane as sole carbon and energy source. Furthermore, they might utilize additional carbon compounds for growth, such as acetate, and could use CH_4 mainly as energy replenishment and survival strategy under limited conditions, suggesting the USCα represents rather facultative than obligate methanotrophs.
(For detailed discussion see chapter III.1)

VI.1.2 Applicability of RING-FISH with coupled cell sorting to target cells of USCα

In this thesis, recognition of individual genes – fluorescent *in situ* hybridization (RING-FISH) was tested to target and, in combination with cell sorting, isolate cells possessing *pmoA* genes of USCα from Marburg forest soil (chapter III.2.1). Although all of the generated polynucleotide and monospecific oligo-oligonucleotide RNA probes showed a high potential to form secondary structures, required for the formation of a probe network around the cell during hybridization, and were checked for specificity, no specific hybridization could be achieved. Independent from formamide concentration and incubation time, cells of positive and negative controls both showed whole cell fluorescence and could not be discriminated. Halo signals were only observed sporadically and in single areas on the well. These

observations indicate that formation of the probe network did not take place and probes mainly accumulated inside the cells. Unfortunately, the exact conditions needed for a successful and specific network formation still remain unclear (Zwirglmaier, 2005), thus no explanation can be given, why these probes were not able to form specific probe networks.

A comparison of both the negative controls also showed that cells of *Methylocapsa acidiphila* gave different hybridization results than *E. coli* clones harbouring the same *pmoA* gene. This indicates that, in contrast to CARD-FISH (chapter III.1), clones might not be suitable as general controls for RING-FISH because different cell morphologies between clones and “real” target cells might have a significant impact on the hybridization results. In particular in case of the methanotrophic bacteria, which possess extensive intercytoplasmatic membrane arrangements (see figure VI.1-1; Wartiainen *et al*., 2006; Dalton 2005; Dedysh *et al*., 2002), probably influencing or even inhibiting the formation of a potential probe network around the cell in RING-FISH. As fluorescent signals in CARD-FISH form and appear directly inside the cells, intracellular structures generally show no significant influence on the hybridization efficiency.

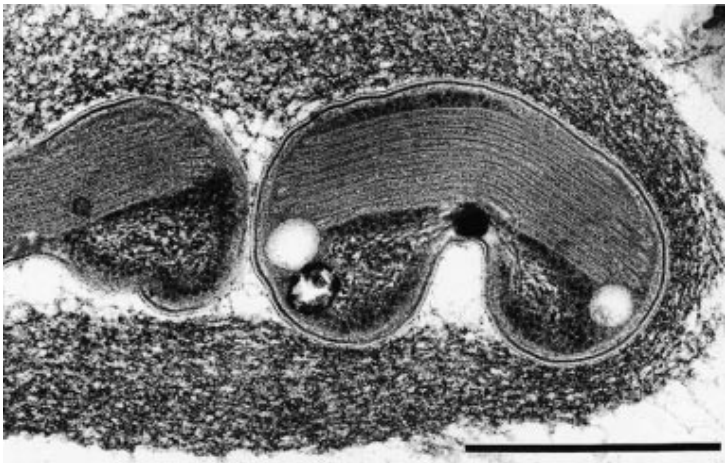


Figure VI.1-1: Intercytoplasmatic membrane arrangements in *Methylocapsa acidiphila*. Adopted from Dedysh *et al.* (2002). Scale bar = 0.5 µm.

Sorting of cells using microplates coated with *pmoA* DNA of USCα in combination with RING-FISH in solution also proved to be unspecific. Cells of positive and negative control clones could not be separated, probably due to the missing formation of a specific probe network around the target cells during RING-FISH. The random and unspecific detection of PCR products most likely emerged from unhybridized cell aggregates inside the wells, which were not washed out completely and led to different results in every immobilization. A possible solution for this could be the use of sonication prior to hybridization, to separate cells, although care has to be taken not to alter or damage the membrane structure and integrity, since this could lead to unspecific formation of the probe network and therewith to false positive hybridization results (Fichtl, 2005).

VI.1.3 Enrichment strategies used for USCα

A further attempt to unravel the phylogeny of the upland soil cluster α was to enrich these microorganisms using two different strategies (chapter III.2.2). The first approach consisted of a soil substrate membrane system (SSMS), set up using Marburg forest soil and incubated with 20 ppmv CH_4. This system closely mimics the natural terrestrial environment for soil bacteria (Ferrari *et al.*, 2008). However, no microcolonies of USCα could be detected on the filter pieces by *pmoA* mRNA CARD-FISH even after 6 weeks of incubation. On the other hand, expression of USCα *pmoA* in single cells was still observed and the SSMS consumed methane throughout the whole incubation period. These results indicate that the USCα did survive on methane and transcribed *pmoA*, as also observed in the $^{13}CH_4$ RNA-SIP (chapter III.1) but probably still lacked compounds (e.g. other carbon sources) for growth (Degelmann *et al.*, 2010), or were inhibited by other soil compounds diffusing through the membrane.

The second enrichment approach implied an incubation of the Marburg forest soil in microcosms and slurries incubated with methane (2 ppmv, 20 ppmv, 100 ppmv) and pulses with acetate (100, 400, 800 µg g^{-1} *d.w.s.*). This strategy was based on recent publications showing that methanotrophs are capable of using acetate as carbon source (West & Schmidt, 1999; Dunfield *et al.*, 2010). In this approach, however, also no growth of USCα could be observed, even after 12 weeks of incubation. The amount of acetate added through the pulses was probably not sufficient, especially due to the strong competition for acetate by the remaining bacterial soil community, to allow growth of USCα. This suggests they might have survived on the methane provided, at least in the microcosms, but did not replicate, as also indicated by qPCR of *pmoA* genes. Copy numbers remained stable (14.2±0.11 $x10^5$ [copy number g^{-1} *d.w.s.*]) and fit to the population size of USCα reported before in Marburg forest soil collected in summer by Kolb *et al.* (2005; 21.3±7.3 $x10^5$ [copy number g^{-1} *d.w.s.*]). Instead, other microoganisms, like the enriched *Burkholderia*, showed faster growth on acetate and completely outcompeted and overgrew USCα. Therefore, acetate might be rather unsuitable for enriching USCα from forest soils, as already indicated by results of the ^{13}C-acetate SIP (chapter III.1). Furthermore, only very low consumption of methane could be observed in the slurry incubations in contrast to the “normal” soil microcosms, possibly suggesting that shaking disturbed methane oxidation and USCα use this process for energy replenishment only when exposed to air on a solid surface.

VI.1.4 Outlook

RNA-SIP using ^{13}C-labeled acetate showed that the upland soil cluster α (USCα), assumed to represent methanotrophic bacteria adapted to the trace level of atmospheric methane and to play an essential part in the removal of this greenhouse gas from the atmosphere, are able to utilize other carbon compounds than methane (chapter III.1). We also know that they are predominantly abundant in a lot of upland soils, where uptake of atmospheric methane occurs, and seem to constitutively express genes encoding for a particulate methane

monooxygenase (Kolb *et al.*, 2009). However, the involvement of USCα in atmospheric methane oxidation still remains unclear. To finally answer this question, isolation of a pure culture from soil would be required, followed by purification of the USCα pMMO enzyme and activity measurements in this culture. Acetate showed to be rather unsuitable for isolation or enrichment of USCα from forest soil, due to the large competition and better growth response of other soil microorganisms (chapter III.1 and III.2.2). However, further testing of other carbon sources that could be used by USCα might open the opportunity to obtain a carbon compound which allows for a more selective enrichment of these potential atmospheric methane oxidizers. In addition, the application of novel high-throughput sequencing approaches to investigate the microbial diversity in this soil could lead to identification of the 16S rRNA phylogeny of USCα (Hirsch *et al*, 2010).

More information regarding the activity of the USCα pMMO could also be gained by investigating the proteins present in the soil at the time point of atmospheric methane oxidation by environmental proteomics. This approach allows the proteome analyses of environmental samples (Keller and Hettich, 2009; Schneider and Riedel, 2010) and could answer the question, whether USCα pMMO is actually active (and when) or if expression of *pmoA* is uncoupled from enzyme activity. However, the fact that pMMO is a membrane-bound enzyme could present a problem in a proteomics approach.

The observation that methane is incorporated into PLFAs of USCα (Chen *et al.*, 2008), which could also be true for acetate, could be used to specifically enrich USCα cells in a soil sample. But so far, no PLFA-targeted cell sorting methods have been reported. However, even a slight enrichment in USCα cells might allow the application of NanoSIMS, a secondary ion mass spectroscopy technique (reviewed in Wagner, 2009), to study the metabolism of labeled (or unlabeled) compounds.

VI.2 Autotrophic CO_2 fixation coupled to ammonia oxidation of archaea and bacteria in an agricultural soil

This study was performed to investigate the potential fixation of CO_2 coupled to oxidation of ammonia by ammonia oxidizing archaea (AOA) and their bacterial counterparts (AOB) in the Rauischholzhausen agricultural soil using RNA-SIP and DNA-SIP in comparison. Gradients were further analyzed regarding possible CO_2 fixation pathways for the AOA. Expression of archaeal *amoA* by mRNA catalyzed reporter deposition – fluorescence *in situ* hybridization (CARD-FISH) in the soil incubations was visualized and compared with qPCR data to evaluate the abundance of AOA within the archaeal and overall microbial community in this soil.

RNA-SIP demonstrated active fixation of CO_2 coupled to ammonia oxidation by AOA and AOB in the agricultural soil. In contrast to the AOB, activity of AOA seemed to be inhibited by higher concentrations of ammonia. Furthermore, the detection of labeled archaeal

transcripts of a functional marker gene (*accA*) for the 3-hydroxypropionate-4-hydroxybutyrate cycle CO_2 fixation pathway, recently proposed to function also within the *Crenarchaeota*, showed that ammonia oxidizing archaea in upland soils are able to autotrophically fix CO_2 using the hydroxypropionate-hydroxybutyrate cycle, hence providing an additional sink for CO_2 in terrestrial environments. CARD-FISH targeting *amoA* mRNA of AOA and archaeal 16S rRNA also revealed a high abundance of ammonia oxidizing archaea (~ 50%) within the *Archaea* in the agricultural soil. These observations give new insights into the function and characteristics of ammonia oxidizing archaea in soil.

Nevertheless, to closer investigate the function of this 3-hydroxypropionate-4-hydroxybutyrate cycle in archaeal ammonia oxidizers in terrestrial environments, obtaining pure cultures of AOA from soil could be beneficial. But so far, no soil isolates have been published. More information might also be gained by using the RNA-SIP approach to compare various upland soils (unfertilized grasslands, forest soils) regarding the activity of autotrophic ammonia oxidizing archaea.

(For detailed discussion see chapter IV)

VI.3 Localized expression of *hydB* in H_2-oxidizing streptomycetes in soil

The aim of this study was to investigate the high-affinity H_2 uptake activity of *Streptomyces* sp., to provide new evidence for the involvement of the streptomycetes in the H_2 biogeochemical cycle. Therefore, the assignment for this part of the PhD thesis was to localize the uptake activity within cells and spores of *Streptomyces* PCB7 by visualizing the expression of *hydB*, encoding the large subunit of the [NiFe]-hydrogenase, by mRNA catalyzed reporter deposition – fluorescence *in situ* hybridization (CARD-FISH).

The study revealed that high-affinity H_2 uptake is widespread among the streptomycetes. Furthermore, analyses showed that streptomyces spores instead of the mycelia catalysed the high-affinity H_2 uptake activity. This indicates that H_2-oxidizing streptomycetes, or actinobacteria in general, are essential for the uptake of atmospheric H_2 in upland soils.

Future research on this topic could focus on the high-affinity enzyme of this process and its control pathways regarding future application of these microorganisms in H_2 fuel cell technologies (Constant *et al*, 2009).

(For detailed discussion see chapter V)

VI.4 General discussion & outlook

This thesis focused on the investigation of microbial groups in terrestrial environments, responsible for or proposed to be involved in the uptake of atmospheric trace gases (CH_4, CO_2, H_2), namely the potential atmospheric methane oxidizer upland soil cluster α (USCα), the autotrophic ammonia oxidizing archaea (AOA), and the hydrogen oxidizing streptomycetes. While for some of these groups, uptake or oxidation of atmospheric trace gases showed to be mainly an energy replenishment and/or a survival strategy under limited conditions (USCα, chapter III.1; spores of *Streptomyces* sp. PCB7, chapter V), others seemed to depend on the utilization (autotrophic ammonia oxidizing archaea, chapter IV). Except for the H_2 oxidizing streptomycetes, these groups so far resisted conventional isolation procedures and also did not show any growth in the incubation and enrichment experiments applied in this study. This indicates that the USCα and the AOA in terrestrial environments either grow very slowly or require yet unknown additional compounds or conditions for replication.

Several methods were tested to investigate the function of these microbial groups in terrestrial environments and the incorporation of labeled substrate, and to monitor the expression of their functional marker genes, namely *pmoA* for the high-affinity particulate methane monooxygenase of USCα, *amoA* for the ammonia monooxygenase of AOA, and *hydB* for the high-affinity [NiFe]-hydrogenase of *Streptomyces* sp. PCB7.

Stable isotope probing of nucleic acids was successfully applied in this study to monitor incorporation of labeled ^{13}C-compounds (CH_4, acetate, and CO_2) into DNA and RNA of selected microbial groups, namely the methanotrophic community in an acidic forest soil (chapter III.1) and the ammonia oxidizing prokaryotes in an agricultural soil (chapter IV). RNA-SIP proved to be competent to link expression of mRNA to function, but only if the labeled substrate was incorporated and used as carbon source. In cases where the labeled substrate might only serve as energy source, as presumed for the oxidation of atmospheric methane by USCα, SIP can not provide any information about utilization of this compound. In comparison to RNA-SIP, DNA-SIP, because of its restriction to label incorporation only during replication, showed labeling only when cells were actively growing. Therefore, RNA-SIP seems to be a more sensitive and effective approach for natural environments harboring slow growing but ecologically essential microbial groups. For RNA-SIP, a probable way to enhance the sensitivity regarding mRNA could be to exclude rRNA prior to density gradient centrifugation using commercially available kits. This pure mRNA-SIP could then serve for e.g. high-throughput sequencing of labeled and unlabeled gradient fractions to gain more information about the metatranscriptome of a selected sample following label incorporation. These kits, however, have to be closely evaluated first regarding the introduction of biases during the selection procedure. In general, SIP provides a broad range of opportunities for further analyses of gradients. Once label incorporation took place and samples were subjected to density gradient centrifugation, gradient fractions can be used for quantitative PCR (qPCR) targeting functional marker genes or rRNA, cloning, or even high-throughput sequencing as

mentioned above. When designing a SIP experiment, care should be taken to incorporate adequate negative controls to exclude the possibility of cross-feeding or natural shifts in DNA density as a reason for label detection (Neufeld, 2007).

Catalyzed reporter deposition – fluorescence *in situ* hybridization (CARD-FISH) targeting transcripts of functional marker genes (*pmoA*, *amoA*, and *hydB*) showed to be a reliable method for specific detection and localization of mRNA expression in pure cultures and environmental samples. Expression clones could successfully be used as general controls, which is especially advantageous when the targeted microorganisms have not been isolated yet and therefore no pure culture can serve as control, as in the case of the ammonia oxidizing archaea in soil and the upland soil cluster α. Furthermore, the coupling of mRNA CARD-FISH with normal 16S rRNA FISH or 16S rRNA targeted CARD-FISH provides an opportunity to specifically identify cells and link function (or at least expression) of important groups of biochemical processes in the environment to 16S rRNA phylogeny. Care should be taken, however, in the choice of a permeabilization procedure specific for the desired target microorganisms, as an insufficient or excessive permeabilization could lead to either false negative or false positive hybridization results, respectively.

In contrast, a different FISH approach tested in this study, the recognition of individual genes – fluorescence *in situ* hybridization (RING-FISH), could not be effectively applied to detect *pmoA* genes of USCα in clones and soil samples. Hybridization results were highly unspecific and control clones exhibited different results than pure cultures harboring the same gene, indicating that clones are no suitable general controls for RING-FISH. Although RING-FISH had previously been used to successfully detect genes of the nitrite reductase (*nirK*) in denitrifiers in pure culture and environmental samples (Pratscher *et al.*, 2009), and glycerol aldehyde 3-phosphate dehydrogenase (GAPDH) in *E. coli* (Zwirglmaier *et al.*, 2004), it did not show specific results for USCα *pmoA* (chapter III.2), presumably because formation of the probe network was insufficient. Since the precise conditions required for this network formation are still unknown, RING-FISH proves to be a rather unreliable method that only seems to work for specific genes or microbial groups. Novel FISH methods targeting genes in microorganisms like geneFISH, first applied to detect crenarchaeotal *amoA* genes in seawater samples (Moraru *et al.*, 2010), or *in situ* rolling circle amplification – fluorescence *in situ* hybridization (RCA-FISH), previously used for detection of denitrification genes in bacterial cells (Hoshino and Schramm, 2010), could provide a better approach to link gene presence to cell identity in microorganisms.

Expression of mRNA is generally regarded as a marker for function and activity because mRNA is supposed to be very short-lived and thus should represent the active community at the time of extraction. In this study, however, *pmoA* transcripts of general methanotrophs could be detected in soil incubations even after 10 weeks, although there was no sign for activity of these microorganisms, indicating that transcripts presumed to be involved in the uptake of atmospheric trace gases in upland soils might exhibit a general high stability and longer half life than other gene transcripts. This has also been observed for

pmoA transcripts in lake sediments (Dumont *et al.*, unpublished) and for transcripts of a high-affinity naphthalene dioxygenase of *Acidovorax* in a groundwater system (Huang *et al.*, 2009). In case of USCα, *pmoA* mRNA could also constantly be detected, although ^{13}C of labeled methane was not incorporated into nucleic acids. Thus, despite the observed expression, a link to function could not be made. Of course, an irrefutable proof of enzyme activity can only be given by isolation of a certain pure culture from soil, followed by purification of the desired enzyme and activity measurements in this culture. Unfortunately, only an estimated 1% of all soil bacteria can be cultured using conventional laboratory procedures (Skinner *et al.*, 1952), thus demanding culture-independent techniques to unravel questions important for microbial ecology.

Application of DNA or RNA microarrays, for example, could give more detailed insight into the community structure and expression of important marker genes in soil environments. Microarrays are based either on 16S rRNA phylogeny like the PhyloChip, which targets nearly 9,000 operational taxonomic units (OTUs) with an average of 24 probes each 25 bp long and was already successfully applied to soil (Brodie *et al.*, 2006), or on functional gene sequences like the GeoChip, which can detect >10,000 genes in >150 functional groups (He *et al.*, 2007). However, these microarrays give no information on single-cell level or about actual activity, and might miss yet unknown groups, since no DNA sequences are available for probe design. A novel technique allows the connection of microarrays with SIP. CHIP-SIP represents a combination of high-density phylogenetic microarrays (“chips”) and stable isotope probing (SIP) (Mayali *et al.*, 2010). RNA extracted from incubations with stable isotope-enriched substrate is hybridized onto a microarray synthesized on a conductive surface and the array is subsequently imaged using high resolution secondary ion mass spectrometry (SIMS) with a NanoSIMS to detect isotopic enrichment. Nevertheless, the resolution of this method has to be evaluated, as this approach is not able to differentiate between fully and only partially labeled RNA.

A new generation of single-cell approaches to study the function of microbial communities by uptake of stable-isotope-labeled compounds, notably the Raman microspectroscopy and secondary ion mass spectrometry (NanoSIMS) (both reviewed in Wagner, 2009), might also prove powerful to link phylogeny to function in various environments.

The use of environmental proteomics, which allows the proteome analyses of environmental samples (Keller and Hettich, 2009; Schneider and Riedel, 2010), could further give more information regarding the activity of USCα or AOA in soil at the time point of atmospheric methane oxidation or ammonia oxidation and CO_2 fixation, respectively. A different screening method, the high-throughput sequencing of 16S rRNA or functional genes/transcripts (MacLean *et al.*, 2009; Hirsch *et al*, 2010), coupled to specific incubations might provide additional knowledge of phylogeny or behavior of these groups in soil. These high-throughput screening methods, however, demand a vast amount of bioinformatic sequence data analysis and are not able to detect less abundant microorganisms without pre-selection.

To summarize, stable isotope probing of RNA and DNA and the use of mRNA CARD-FISH revealed novel and significant insights into the phylogeny and function of microbial key players in the uptake of atmospheric trace gases in soils. Nevertheless, various questions concerning these groups yet remain open, thus new methodological developments could support further investigations. But despite the advancement of high-end single cell analyses and high-troughput screening methods, there is still need for well designed and specific experiments, particularly when investigating the activity and function of microbial groups showing high abundance but maybe only slow growth, like the microorganisms involved in the uptake of atmospheric trace gases in terrestrial environments.

VI.5 References

Brodie EL, DeSantis TZ, Joyner DC, Baek SM, Larsen JT, Andersen GL, Hazen TC, Richardson PM, Herman DJ, Tokunaga TK, Wan JM, Firestone MK (2006) Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. *Appl Environ Microbiol* 72: 6288–6298.

Chen Y, Dumont MG, McNamara NP, Chamberlain PM, Bodrossy L, Stralis-Pavese N, Murrell JC (2008) Diversity of the active methanotrophic community in acidic peatlands as assessed by mRNA and SIP-PLFA analyses. *Environ Microbiol* 10: 446–459.

Constant P, Poissant L, Villemur R (2009) Tropospheric H_2 budget and the response of its soil uptake under the changing environment. *Sci Tot Environ* 407: 1809–1823.

Dalton H (2005) The Leeuwenhoek Lecture 2000 - The natural and unnatural history of methane-oxidizing bacteria. *Philosophical Transactions of the Royal Society B-Biological Sciences* 360: 1207-1222.

Dedysh SN, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Liesack W, Tiedje JM (2002) *Methylocapsa acidiphila* gen. nov., sp nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from *Sphagnum* bog. *Int J Syst Evol Microbiol* 52: 251-261.

Dumont MG, Pommerenke B, Casper P, Conrad R (2010) DNA, rRNA and mRNA stable isotope probing of aerobic methanotrophs in lake sediment. Submitted to *Environ Microbiol*

Dunfield PF, Belova SE, Vorob'ev AV, Cornish SL, Dedysh SN (2010) *Methylocapsa aurea* sp. nov., a facultatively methanotrophic bacterium possessing a particulate methane monooxygenase. *Int J Syst Evol Microbiol* (in press): doi: 10.1099/ijs.0.020149-0.

Ferrari BC, Winsley T, Gillings M, Binnerup S (2008) Cultivating previously uncultured soil bacteria using a soil substrate membrane system. *Nat Protoc* 3: 1261-1269.

Fichtl K (2005) Polynucleotide probe based enrichment of bacterial cells: development of probes for species of clinical relevance. PhD thesis, Technical University Munich

He Z, Gentry TJ, Schadt CW, Wu L, Liebich J, Chong SC, Huang Z, Wu W, Gu B, Jardine P, Criddle C, Zhou J (2007) GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. *ISME J* 1: 67-77.

Hirsch PR, Mauchline TH, Clark IM (2010) Culture-independent molecular techniques for soil microbial ecology. *Soil Biol Biochem* 42: 878-887.

Huang WE, Ferguson A, Singer AC, Lawson K, Thompson IP, Kalin RM, Larkin MJ, Bailey MJ, Whiteley AS (2009) Resolving genetic functions within microbial populations: in situ analyses using rRNA and mRNA stable isotope probing coupled with single-cell Raman-fluorescence in situ hybridization. *Appl Environ Microbiol* 75: 234-241.

Hoshino T, Schramm A (2010) Detection of denitrification genes by *in situ* rolling circle amplification-fluorescence *in situ* hybridization to link metabolic potential with identity inside bacterial cells. *Environ Microbiol* 12: 2508–2517.

Keller M, Hettich R (2009) Environmental proteomics: a paradigm shift in characterizing microbial activities at the molecular level. *Microbiol Mol Biol R* 73: 62-70.

Kolb S, Knief C, Dunfield PF, Conrad R (2005) Abundance and activity of uncultured methanotrophic bacteria involved in the consumption of atmospheric methane in two forest soils. *Environ Microbiol* 7: 1150–1161.

Kolb S (2009) The quest for atmospheric methane oxidizers in forest soils. *Environ Microbiol Rep* 1: 336–346.

MacLean D, Jones JDG, Studholme DJ (2009) Application of 'next-generation' sequencing technologies to microbial genetics. *Nat Rev Micro* 7: 287-296.

Mayali X, Weber PK, Brodie EL, Mabery S, Hoeprich PD, Hutcheon ID, Pett-Ridge J (2010) Stable isotope probing of RNA combining phylogenetic microarrays and high resolution secondary ion mass spectrometry to link composition and function in microbial systems. *Microsc Microanal* doi:10.1017/S1431927610061519

Moraru C, Lam P, Fuchs BM, Kuypers MM, Amann R (2010) GeneFISH – an *in situ* technique for linking gene presence and cell identity in environmental microorganisms. *Environ Microbiol* doi:10.1111/j.1462-2920.2010.02281.x

Neufeld J, Dumont M, Vohra J, Murrell J (2007a) Methodological considerations for the use of stable isotope probing in microbial ecology. *Microb Ecol* 53: 435–442.

Pratscher J, Stichternoth C, Fichtl K, Schleifer K-H, Braker G (2009) Application of recognition of individual genes-fluorescence in situ hybridization (RING-FISH) to detect nitrite reductase genes (*nirK*) of denitrifiers in pure cultures and environmental samples. *Appl Environ Microbiol* 75: 802-810.

Schneider T, Riedel K (2010) Environmental proteomics: Analysis of structure and function of microbial communities. *Proteomics* 10: 785–798.

Skinner, A., Jones, P.C.T., Mollison, J.E., 1952. A comparison of a direct and a plate-counting technique for the quantitative estimation of soil microorganisms. *J Gen Microbiol* 68: 261-271.

Wagner M (2009) Single-cell ecophysiology of microbes as revealed by Raman microspectroscopy or secondary ion mass spectrometry imaging. *Annu Rev Microbiol* 63: 411–29.

Wartiainen I, Hestnes AG, McDonald IR, Svenning MM (2006) *Methylobacter tundripaludum* sp nov., a methane-oxidizing bacterium from Arctic wetland soil on the Svalbard islands, Norway (78° N). *Int J Syst Evol Microbiol* 56: 109-113.

West AE, Schmidt SK (1999) Acetate stimulates atmospheric CH_4 oxidation by an alpine tundra soil. *Soil Biol Biochem* 31: 1649-1655.

Zwirglmaier K, Ludwig W, Schleifer K-H (2004) Recognition of individual genes in a single bacterial cell by fluorescence *in situ* hybridization – RING-FISH. *Mol Microbiol* 51: 89-96.

Zwirglmaier K (2005) Fluorescence *in situ* hybridization (FISH) – the next generation. *FEMS Microbiol Lett* 246: 151-158.

Contribution to national and international conferences

Pratscher, J., Braker, G. RING-FISH as a tool to detect nitrite reductase (*nirK*) genes in denitrifiers. VAAM annual conference 2008, Frankfurt, Germany (Poster presentation).

Pratscher, J., Conrad, R. Oxidation of atmospheric methane in upland forest soils. VAAM annual conference 2009, Bochum, Germany (Poster presentation).

Pratscher, J., Dumont, M.G., Conrad, R. Investigating the activity of ammonia-oxidizing archaea and bacteria in an agricultural soil by stable isotope probing. ISME 13 Symposium 2010, Seattle, USA (Poster presentation).

Abgrenzung der Eigenleistung

Soweit nicht anders erwähnt, wurden alle Experimente dieser Arbeit von mir selbst geplant und durchgeführt, sowie anschließend in Form eines Manuskriptes ausgewertet. Das Verfassen der Manuskripte erfolgte zusammen mit meinem Betreuer Prof. Dr. Ralf Conrad.
Die Planung der SIP Experimente (Kapitel III.1 und IV) erfolgte unter Beratung und Betreuung von Dr. Marc Dumont.
Ausser der CARD-FISH Analyse wurden die weiteren Analysen der H_2-oxidierenden Streptomyzeten von Dr. Philippe Constant und Dr. Soumitra Paul Chowdhury durchgeführt.

Curriculum vitae

Jennifer Pratscher | geboren am 19.03.1983 in Jülich

Promotion

Seit 01/2008	Doktorarbeit am Max-Planck-Institut für terrestrische Mikrobiologie in Marburg/Lahn in der Arbeitsgruppe von Prof. Dr. Ralf Conrad

Studium

09/2007	Diplom
01 - 09/2007	Diplomarbeit am Max-Planck-Institut für terrestrische Mikrobiologie in Marburg/Lahn in der Arbeitsgruppe von Dr. Gesche Braker Thema: "*Detektion denitrifizierender Mikroorganismen in Laborkulturen und Umweltproben anhand des Gens für die kupferhaltige Nitrit-Reduktase (nirK) durch Anwendung von RING (recognition of individual genes) - FISH*"
09/2002 - 09/2007	Studium der Biologie an der Rheinischen Friedrich-Wilhelms-Universität Bonn

Schulbildung

06/2002	Abitur
08/1993 - 06/2002	Mädchengymnasium Jülich

Danksagung

Ich danke...

...ganz besonders Prof. Dr. Ralf Conrad für die Chance, diese Doktorarbeit unter seiner Anleitung anzufertigen und für das Vertrauen und die große wissenschaftliche Freiheit, die er mir hat zuteil werden lassen. Vielen Dank auch für die Geduld und Zuversicht!

...Prof. Dr. Erhard Bremer für die Übernahme des Zweitgutachtens.

...Gesche Braker für ihre Unterstützung, den Laborplatz und viele aufschlussreiche und unterhaltsame Diskussionen.

…Many thanks also to Philippe Constant & Marc Dumont for their great scientific ideas, discussions, and positive energy.

...den Mitgliedern der gesamten Abteilung Biogeochemie für die angenehme Arbeitsatmosphäre.

...Peter Claus, Melanie Klose, Katja Meuser und Bianca Pommerenke für die technische Unterstützung.

...Christian Bengelsdorff, Markus Drescher und Carmen Stubner für ihren Einsatz und ihr Organisationstalent.

...Reiner Platt für die mehrmalige Rettung meiner Mobilität und für eine gehörige Portion Fernweh.

...Frauke Skiba und Wolfgang Bönigk, ohne die ich wohl nie in der Biologie gelandet wäre.

...Maren Schütz und Tanja Krause für die Verbindung zur Außenwelt. Und dafür, dass ich euch schon so lange kenne.

...Carsten Dietrich für seinen offensiven Schwachsinn und das beste Mittel gegen Soziophobie.

...Anne Kaster und Judith Pump für ihre Freundschaft und ihr Interesse.

...Dennis Gövert für das Korrekturlesen dieser Arbeit und sämtlicher Abstracts, Poster und Vorträge in Rekordzeit. Und dafür, dass du es so lange mit mir ausgehalten hast. Tut mir leid wegen der ganzen grauen Haare.

...Sabine „TTAT“ Tippmann für ihren unerschütterlichen Optimismus und dafür, dass alles irgendwann einen Sinn macht. Ob aus Schicksal oder Zufall, darüber ließe sich nochmal diskutieren.

...meiner Familie für ihre Unterstützung.

Erklärung

Ich versichere, dass ich meine Dissertation

„Investigation of microbial groups involved in the uptake of atmospheric trace gases in upland soils“

selbständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Diese Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, Oktober 2010