Novel deep branching Cu-containing membrane-bound monooxygenases: distribution and function

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Table of contents

Summary

The key enzyme of the aerobic methane oxidation is the particulate methane monooxygenase (pMMO) pMMOs are members of the great family of Cu-containing membrane-bound monooxygenases (CuMMO). Genes of the pMMO operon can occur in multiple copies within the genome of methanotrophic bacteria. Some of them encode pMMO isoenzymes with alternative functions. A new isoenzyme (pXMO) has been recently found in some alpha- and gamma-proteobacterial methanotrophs. *pxmA* sequences of this isoenzyme do not cluster within groups of characterized *pmoA* sequences but within the environmental group (M84_P105) that belongs to the distantly related intermediate CuMMO (iCuMMO). To analyze the distribution of *pxmA* sequences in methanotrophic pure cultures and nature primers were designed that target several iCuMMO groups (including M84_P105). The *pxmA* could be detected in several strains of the methylotrophic genera *Methylomonas*, *Methylobacter* and *Methylosarcina*. Additionally, it could be shown that *pxmA* sequences are widespread and numerous in different environment. Almost all iCuMMO groups are not represented by pure cultures. Hence, little sequence information is available which makes the study of the iCuMMOs difficult. A magnetic capture hybridization method (MCH) was established to gain more sequence information of the iCuMMOs. MCH avoids the use of specific primers and may provide long target sequences and information about operon structures of the iCuMMOs.

The physiological functions of the iCuMMOs are unknown. Due to a phylogenetic relationship of *pxmA* sequences to sequences of alkane oxidizers we suggested that they might be involved in alkane degradation, too. However, incubation experiments of pure cultures and environmental indicate that the analyzed iCuMMOs are not involved in alkane degradation. Pure culture incubations indicate that the *pxmA* of the environmental group M84_P105 might be involved in methane oxidation. But further studies need to be performed to confirm this hypothesis. The physiological function of the other iCuMMO groups remains still unknown. iCuMMOs were underestimated for a long time but this study shows that are widely distributed and may play an important role global element cycles.

Methanotrophic bacteria has been believed to be obligate but facultative methanotrophs has been found among the type II methanotrophs that grow on substrates with carbon-carbon bounds like acetate, pyruvate, succinate, malate and ethanol. In this study we could show that type II methanotrophs play a role in the degradation of short chained alkanes in rice field soils. If they use the alkanes directly or if they use metabolic products provided by other bacteria needs to be analyzed. But these findings show that the restricted role of the methanotrophs to certain substrates and specific functions needs to be expended.

Zusammmenfassung

Das Schlüsselenzym der aeroben Methan Oxidation ist die partikuläre Methan Monooxygenase (pMMO). Sie gehört zur großen Familie der Kupfer-abhängigen membran-gebundenen Monooxygenasen (CuMMO). Die Gene des pMMO Operons können in mehreren Kopien im Genom von methanotrophen Bakterien vorliegen. Einige davon kodieren für pMMO Isoenzyme mit alternativen Funktionen. A neues Isoenzym (pXMO) wurde vor kurzem in einigen Alpha- und Gammaproteobacterien gefunden. *pxmA* Sequenzen dieses Enzyms fallen nicht in phylogenetische Gruppen charakterisierter *pmoA* Sequenzen, sondern in eine Gruppe von Umweltsequenzen (M84_P105), die zu den entfernt verwandten CuMMOs (iCuMMO) gehört. Um die Verbreitung von *pxmA* Sequenzen in methanotrophen Reinkulturen und in der Umwelt zu untersuchen, wurden Primer hergestellt, die spezifisch für einige iCuMMO Gruppen (inklusive der M84_P105 Gruppe) sind. Mit diesen neuen Primern konnte die *pxmA* in mehreren *Methylomonas*, *Methylobacter* und *Methylosarcina* Stämmen methylotropher Gattungen nachgewiesen werden. Zusätzlich konnte gezeigt werden, dass *pxmA* Sequenzen in verschieden Umwelten weit verbreitet sind und häufig vorkommen. Fast alle iCuMMO Gruppen besitzen keine Vertreter von Reinkulturen. Darum sind nur wenige Sequenzinformationen vorhanden, was die Untersuchungen der iCuMMO schwierig gestaltet. Eine Hybridisierungsmethode basieren auf magnetischen Sonden (magnetic capture hybridization, MCH) wurde entwickelt, um zusätzliche Sequenzinformationen zu erhalten. Diese Methode umgeht den Gebrauch von Primern und kann im Idealfall lange Sequenzen liefern, die auch Informationen über die Operonstruktur der iCuMMOs enthalten können.

Die physiologische Funktion der iCuMMO ist nicht bekannt. Aufgrund einer phylogenetischen Verwandtschaft von *pxmA* Sequenzen zu Sequenzen, die mit der Alkane Oxidation in Verbindung gebracht werden, wurde vermutet, dass *pxmA* Sequenzen auch darin involviert sein könnten. Inkubationsexperimente mit Reinkulturen und Umweltproben lassen jedoch darauf schließen, dass dies nicht der Fall ist. Reinkultur Inkubationen weisen vielmehr darauf hin, dass *pxmA* Sequenzen an der Oxidation von Methan beteiligt sind. Um diese Hypothese zu bekräftigen müssen allerdings weitere Experimente durchgeführt werden. Die physiologische Funktion andere iCuMMO ist weiterhin unbekannt. Die iCuMMO wurden bislang wenig beachtet. Diese Studie zeigt allerdings, dass sie weit verbreitet sind und möglicherweise eine wichtige Rolle im globalen Elementkreislauf spielen.

Methanotrophe Bakterien wurden lange für obligat gehalten. Aber fakultative Methanotrophe wurden gefunden, die zu den Typ II Methanotophen gehören und Substrate mit Kohlenstoff-

Kohlenstoff-Bindungen wie Acetat, Pyruvat, Succinat, Malat und Ethanol verwerten können. In dieser Studie konnten wir zeigen, dass Typ II Methanotrophe eine Rolle beim Abbau von kurzkettigen Alkanen in Reisfeldböden spielen. Ob sie diese direkt verwerten oder ob sie Abbauprodukte der Alkane verwerten, die von anderen Bakterien zur Verfügung gestellt wurden, kann nicht abschließend geklärt werden. Jedoch zeigt diese Studie, dass die Beschränkung der Rolle der Methanotrophen auf wenige Substrate und spezifische physiologische Funktionen erweitert werden muss.

Introduction

1.1 Athmospheric methane

Methane is next to carbon dioxide and water vapor the most prevalent greenhouse gas in earth's atmosphere (Change, 2007). Though the atmospheric concentration of this simple alkane, consisting of one carbon- and four hydrogen atoms, is much lower than CO_2, its global warming potential is 33-times higher and makes methane a very potent greenhouse gas (Shindell *et al.*, 2009). The concentration of methane in the atmosphere stagnated for nearly a decade (Dlugokencky *et al.*, 2003) but a renewed growth of methane in the atmosphere has been reported (Rigby *et al.*, 2008, Bergamaschi *et al.*, 2013). Atmospheric methane derives from biogenic sources including natural wetlands, rice agriculture, landfills, termites, freshwater sediments and oceans and non-biogenic sources including burning of fossil fuel, waste treatment, biomass burning and geological sources such as geothermal or volcanic methane (Chen & Prinn, 2005, Wuebbles & Hayhoe, 2002, Change, 2007). The largest sink for methane is the troposphere. Methane reacts with hydroxyl radicals forming mainly water and carbon dioxide. This photochemical reaction accounts for 90% of the total methane oxidation (Change, 2007). Other sinks of atmospheric methane are the diffusion of methane into the stratosphere and the microbial oxidation in upland soils (Conrad, 1996, Bender & Conrad, 1992). About 75% of the atmospheric methane originates from a group of anaerobic microbes, the methanogenic archaea. This biogenic methane is produced in a multistep process, the methanogenesis, as an end product in the anaerobic decomposition

of organic matter (Chen & Prinn, 2005, Conrad & Frenzel, 2002, Thauer *et al.*, 2008). Main substrates that are used for methane formation are acetate or carbon dioxide and hydrogen.

A specialized group of microorganisms is able to use methane as a sole carbon and energy source: the methanotrophs. They can be found both in aerobic and anaerobic environments (Hanson and Hanson, 1996, Conrad, 2009, Boetius *et al.*, 2000, Raghoebarsing *et al.*, 2006). Due to the methanotrophs, only a part of the produced methane is released into the atmosphere. They act as biofilters (Reeburgh, 2003, Reim *et al.*, 2012, Conrad & Frenzel, 2002). It is considered that 80% of the CH_4 produced in soil by methanogenic archaea is consumed by methanotrophic bacteria at oxic-anoxic interfaces (Hanson and Hanson, 1996, Conrad *et al*., 2007). By the interfering with the global methane cycle and reducing of the produced methane the methanotrophs play an important role in the global methane cycle.

1.2 Methanotrophs

Methanotrophs are a diverse and specialized subgroup of the methylotrophic prokaryotes that have the unique ability to use methane as their sole carbon and energy source (Trotsenko & Murrell, 2008). Methanotrophs are widespread in nature. The methanotrophic bacteria that oxidize methane aerobically can be found at oxic–anoxic interfaces in a variety of environments like wetlands, soils, rice paddies, marine and freshwater sediments, landfills, peatlands (e.g. Knief *et al.*, 2003, Krause *et al.*, 2010, Liebner *et al.*, 2009, Morris *et al.*, 2002, Nercessian *et al.*, 2005, Reay *et al.*, 2001, Tuomivirta *et al.*, 2009, Dumont *et al.*, 2011). Most of the known methanotrophic bacteria grow best at moderate conditions (neutral pH, mesophilic temperature and low salinity). However, methanotrophs were found that are thermotolerant (Bodrossy *et al.*, 1997, Bodrossy *et al.*, 1999, Islam *et al.*, 2008),

psychrotolerant (Omelchenko *et al.*, 1993, Kalyuzhnaya *et al.*, 1999, Wartiainen *et al.*, 2006), halotolerant (Heyer *et al.*, 2005), alkalitolerant (Khmelenina *et al.*, 1997) and acidophilic (Dedysh *et al.*, 1998, Dedysh *et al.*, 2004, Pol *et al.*, 2007, Dunfield *et al.*, 2007).

Three phyla are known to include methanotrophic bacteria: Proteobacteria, Verrucomicrobia, and NC10. Methanotrophic proteobacteria were classically divided into two groups, type I and type II, based on physiological, morphological and phylogenetical characteristics (Bowman, 2006, Trotsenko & Murrell, 2008, Whittenbury, 1975). This historically grown differentiation corresponds well to molecular phylogeny (Lüke & Frenzel, 2011). Type I methanotrophs are grouped within the gammaproteobacteria. All genera belong to the family of *Methylococcaceae*. Type I methanotrophs consist of the subgroups: type Ia (e.g. *Methylomonas*, *Methylovolum, Methylobacter*, *Methylosarcina*, *Methylomicrobium, Methylomarinum, Methylosoma, Methylohalobius*), type Ib (e.g. *Methylococcus*, *Methylocaldum, Methylogaea, Methylothermus, Methylohalobius*) and type Ic. Type Ic was previously described as type X (Bowman, 2006, Geymonat *et al.*, 2011, Hanson & Hanson, 1996). Type Ic is represented by one cultivated ammonium oxidizer, *Nitrosococcus oceani* (Ward, 1990, Holmes *et al.*, 1995, Klotz *et al.*, 2006), environmental sequences encoding for monooxygenases with unknown substrate specificity (Lüke & Frenzel, 2011) and putative methane monooxygenases (USCγ; Knief *et al.*, 2003). Unusual filamentous methanotrophs have been found within the genera *Crenothrix* and *Clonotrix* belonging to type I and the family of *Methylococcaceae* (Stoecker *et al.*, 2006, Vigliotta *et al.*, 2007). The alphaproteobacterial type II methanotrophs include the families *Methylocystaceae* and *Beijerinckiaceae* with the genera *Methylocystis*, *Methylosinus*,

Methylocapsa, *Methylocella*, and *Methyloferula*. Another phylum containing methanotrophs are the *Verrucomicrobia.* They were first isolated from extreme environments growing at low pH and high temperatures (Dunfield *et al*., 2007, Islam *et al*., 2008, Pol *et al*., 2007) but recently species able to grow at moderate growth conditions were found, too (Sharp *et al.*, 2014, van Teeseling *et al.*, 2014). The phylum NC10 represents bacteria able to oxidize methane aerobically coupled to denitrification under anoxic conditions (Ettwig *et al.*, 2009).

The methanotrophic bacteria were thought to be obligate methylotrophs that could only grow on methane, methanol and in some cases at a narrow range of C1 compounds like formaldehyde, formate and methylamine (e.g. Bowman, 2006). However, the ability of methanotrophs to use compounds with carbon-carbon was shown by Dedish and colleagues. The facultative methanotrophic *Methylocella palustris* strain was able to utilize the multicarbon substrates acetate, pyruvate, succinate, malate and ethanol (Dedysh *et al.*, 2005). Furthermore, *Methylocapsa* and *Methylocystis* species were found that were able to grow on acetate as substrate (Dunfield *et al.*, 2010, Belova *et al.*, 2011). Facultative methanotrophs might be more common than thought until now.

While methane is oxidized aerobically by methanotrophic bacteria, the anaerobic methane oxidation by methanotrophic archaea depends on alternative electron acceptors: SO_4^{2-}, Fe^{3+}, Mn^{4+}, NO^{2-} and NO^{3-}. Sulfate dependent methane oxidation is performed by a consortium of sulfate reducing bacteria and methanotrophic archaea (Hoehler *et al.*, 1994, Knittel & Boetius, 2009). The methanotrophic archaea are clustered in three distinct groups (ANME-1, ANME-2, ANME-3) that are related to methanogens (Niemann *et al.*, 2006, Orphan *et al.*, 2002). Methane oxidation of a microbial consortium coupled to denitrification was detected

in anoxic sediments. Both nitrate and nitrite could act as an electron acceptor (Raghoebarsing *et al.*, 2006, Haroon *et al.*, 2013). Candidatus *Methylomirabilis oxyfera* that belongs to the NC10 bacteria is able to perform the anaerobic oxidation without methanotrophic archaea. Candidatus *Methylomirabilis oxyfera* produces its own oxygen supply in an intra-aerobic metabolism by metabolizing nitrite via nitric oxide into oxygen and dinitrogen gas (Ettwig *et al.*, 2010, Ettwig *et al*., 2009, Wu *et al.*, 2011). Candidatus *Methanoperedens nitroreducens* is affiliated with ANME and may be able to oxidize methane anaerobically through a reverse methanogenesis pathway (Haroon *et al.*, 2013). In marine sediments the oxidation of methane in the presence of Fe^{3+} and Mn^{4+} by a microbial population could be detected (Beal *et al.*, 2009). The methanotrophic archaea are globally distributed in many environments like marine and limnic water columns and sediments, landfills and soils and play a significant role as a methane sink (Cadillo-Quiroz *et al.*, 2008, Castro *et al.*, 2004, Eller *et al.*, 2005, Grossman *et al.*, 2002, MacLean *et al.*, 2007).

In aerobic methanotrophs methane is oxidized via the intermediates methanol, formaldehyde and formate to carbon dioxide in the dissimilatory pathway (Figure 1). The key enzyme of this pathway is the methane monooxygenase (MMO). Two types of the MMO are described, one is located in the cytoplasma of the cell (soluble MMO, sMMO), the other is attached to the cytoplasmic membrane in a particulate form (particulate MMO, pMMO). Nearly all MOB possess a pMMO while some have an additional sMMO. The latter is the only MMO in the genera *Methylocella* and *Methyloferula* (Dedysh *et al.*, 2000, Vorobev *et al.*, 2011, Dunfield *et al.*, 2003). Gene expression in methanotrophs containing both MMOs is regulated by copper concentration. Under low copper conditions, when the copper-biomass

ratio is low, sMMO is expressed (Murrell *et al.*, 2000, Nielsen *et al.*, 1996, Prior & Dalton, 1985). The genes of the MMOs are located on the chromosome of methanotrophs.

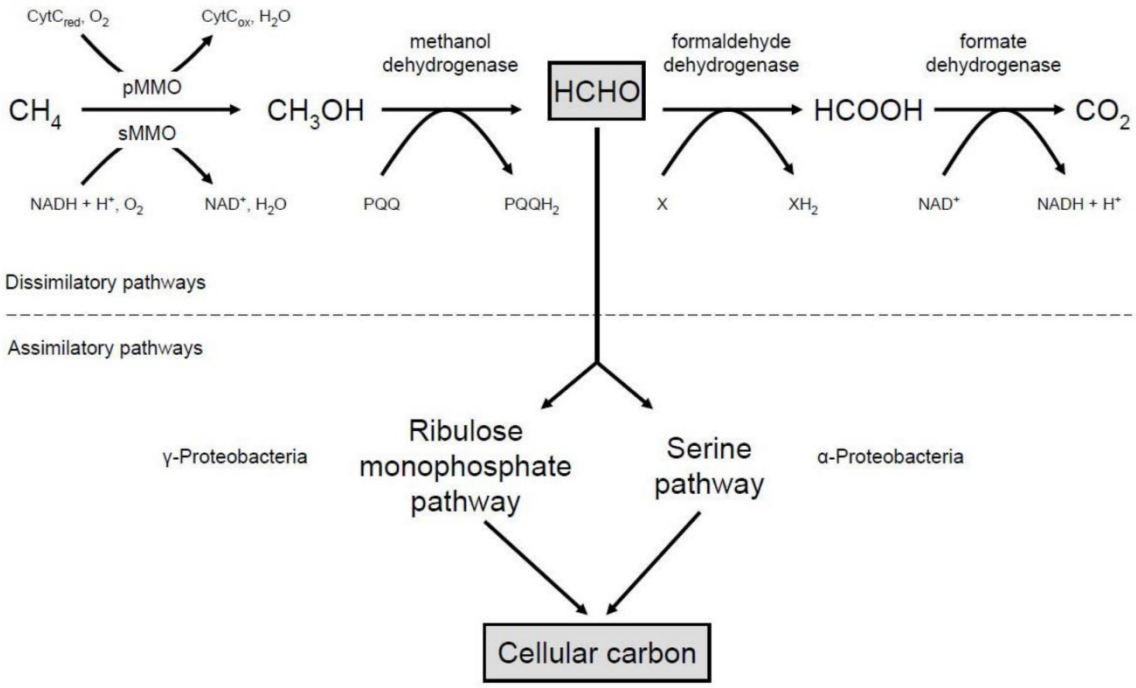

Figure 1: Assimilatory and dissimilatory methane oxidation pathways. Abbreviations: Cytc =Cytochrome c; PQQ = pyrroloquinoline quinine; X = NADP+ or cytochrome linked. Modified from Hanson and Hanson 1996; Lüke, 2009; Mancinelli, 1995. Adopted from Ho, 2010

The sMMO consists of three components, a reductase, a hydroxylase, and a regulatory protein that are encoded by the genes *mmoX*, *mmoY*, *mmoB*, *mmoZ*, *orfY*, and *mmoC* (Cardy *et al.*, 1991, McDonald *et al.*, 1997, Stainthorpe *et al.*, 1990). The three subunits of the pMMO are encoded by the genes *pmoA*, *pmoB* and *pmoC* that are organized canonically in an operon as *pmoCAB* (Gilbert *et al.*, 2000, Semrau *et al.*, 1995, Stolyar *et al.*, 1999). Genes of the pMMO operon can occur in multiple copies within the genome of methanotrophs. (Stolyar *et al*., 1999, Dunfield *et al.*, 2002, Ricke *et al.*, 2004, Op den Camp *et al.*, 2009, Baani & Liesack, 2008, Tavormina *et al.*, 2008, Stoecker *et al*., 2006). Some of these copies are nearly sequence identical. *Methylococcus capsulatus* (Bath), for example, possess two

virtually identical copies of the complete *pmoCAB* operon and an additional copy of *pmoC* (Stolyar *et al*., 1999). This distribution is similar to the system of ammonium oxidizers that can also contain two copies of the gene encoding the ammonia monooxygenase, *amoCAB,* and a third *amoC* gene (Sayavedra-Soto *et al.*, 1998). Two nearly identical copies of the *pmoCAB* could be shown in several other methanotrophs including strains of *Methylocystis*, *Methylosinus* and the *Verrucomicrobia*. Sequence divergent copies of the *pmoCAB* operon could be detected in several type II methanotrophs: *pmoCAB*2. Genes of this operon encode for the isoenzyme pMMO2 (Yimga *et al.*, 2003, Baani & Liesack, 2008, Dunfield *et al*., 2002). *pmoA*2 sequences of this second isoenzyme posses only 68,5% identity and 83,0% identity to the first *pmoA* at the amino acid level. In *Methylocystis* strain SC2 it could be shown that the methane monooxygenase encoded by *pmoCAB*2 is responsible for the oxidation of methane at atmospheric concentrations (Baani & Liesack, 2008, Ricke *et al*., 2004). Divergent copies of the *pmo* operon have also been found in strains of *Verrucomicrobia* and *Crenothrix* (Dunfield *et al*., 2007, Stoecker *et al*., 2006). Very recently a second isoenzyme could be detected in same type Ia and type II methanotrophs: pXMO (Tavormina *et al.*, 2011, Vorobev *et al.*, 2014). pxmA sequences, coding a subunit of the pXMO, are only distantly related to characterized *pmoA* sequences (53% identical and 73% similar on the amino acid level). Additionally, the operon structure shows an unusual non-canonically gene order *pxmABC* (Tavormina *et al*., 2011).

The pMMO of methanotrophic bacteria is a member of a diverse enzyme family: the copper containing membrane-bound monooxygenases (CuMMOs). Monooxygenases in general are enzymes that catalyze the insertion of one oxygen atom, derived from molecular oxygen,

into many organic substrates (van Berkel *et al.*, 2006). The bacterial CuMMOs, that require copper ions for hydroxylation of their substrates, were thought to be restricted to methanotrophic bacteria and ammonium oxidizing bacteria (AOB) for a long time. The pMMO and the ammonium monooxygenase (AMO) are evolutionary related enzymes that share many characteristics like subunit composition, metal component, inhibition profile and operon structure (Holmes *et al*., 1995). While the pMMO is relatively substrate specific and only able to oxidize methane and short-chained alkanes and alkenes (Burrows *et al.*, 1984, Trotsenko & Murrell, 2008), the AMO has a wide substrate spectrum including several apolar compounds such as carbon monoxide and some hydrocarbons (Hooper *et al.*, 1997). The AMO is also able to oxidize methane but does not play a significant role in global methane oxidation (Bender & Conrad, 1994, Bodelier & Frenzel, 1999, Bosse *et al.*, 1993, Jiang & Bakken, 1999). A third member of the CuMMOs has been found in strains of *Nocardia* and *Mycobacterium*: pBMO (Hamamura *et al.*, 1999, Hamamura *et al.*, 2001, Sayavedra-Soto *et al.*, 2011). The butane monooxygenase is a new branch in the family of CuMMOs and shows that there is no restriction of the CuMMOs to MOB and AOB. Additionally, sequences of CuMMOs have been found that could be linked to ethane and ethylene degrading (Nakamura *et al*. BAH22833, BAH22839; Redmond *et al.*, 2010, Suzuki *et al.*, 2012).

1.3 Hydrocarbons and hydrocarbon degrading bacteria

Alkanes and alkenes are exclusively formed by carbon and hydrogen atoms that can be linear, cyclic or branched. Small hydrocarbons up to a length of four carbon atoms are gaseous at ambient temperatures while larger molecules are liquid or solid. Significant sources of short chained hydrocarbons are seeps and vents from natural gas and oil

deposits. Natural gases contain methane (70-99%), 1-10% ethane and other gaseous hydrocarbons (Cooley *et al.*, 2009, Shennan, 2006). Although the major part of the short chained alkanes and alkenes is created by geochemical processes, microorganisms, marine algae, insects and plants provide hydrocarbons in most soil and water environments, too (Cooley *et al*., 2009, Giebler *et al.*, 2013). They are produced as moisture barriers, as reserve materials and pheromones (Nie *et al.*, 2014, van Beilen & Funhoff, 2007). Anaerobic decomposition in soil sediments, sewage sludge and anaerobic digesters result in gases consisting of methane (50-60%), CO_2 (40%) and a minor concentration (up to 1%) of non-methane volatile organic compounds also containing small hydrocarbons (Shennan, 2006, Tassi *et al.*, 2009). Studies on microbial hydrocarbon degradation started about a century ago (Söhngen, 1913). The research focused mainly on topics related to oil production and the use of bacteria and yeasts to convert oil components and solve oil-pollution problems (van Beilen & Funhoff, 2007). Though the apolar hydrocarbons are very inert and need much energy to be activated, many organisms metabolize alkanes and alkenes. Microorganisms including bacteria, yeasts and fungi involved in the degradation were identified during the last century (Labinger & Bercaw, 2002, Rojo, 2009b, van Beilen & Funhoff, 2007, Shennan, 2006). Most of the bacterial strains that grow on hydrocarbons are heterotrophic and use other carbon sources as growth substrate in addition (Harayama *et al.*, 2004, Margesin *et al.*, 2003, Rojo, 2009b). Many hydrocarbon degrading bacteria can utilize a wide range of alkanes and alkenes for they contain multiple alkane hydroxylases with overlapping substrate ranges (Kotani *et al.*, 2003, Sabirova *et al.*, 2006, van Beilen & Funhoff, 2007, van Beilen *et al.*, 2003). The predominat group of bacteria that can grow on hydrocarbons is the so called CMNR group; Gram-positive bacteria belonging to the genera *Corynebacterium*,

Mycobacterium, *Nocardia*, and *Rhodococcus* (Shennan, 2006, Hamamura *et al*., 2001). Gram-negative representatives of alkane degraders belong to the genera *Pseudomonas*, *Acinetobacter*, *Alcaligenes* and *Burkholderia* (Shennan, 2006). Beside the heterotrophic bacteria, that prefer other grows substrates to alkanes, some bacteria seem to be highly specialized to grow on hydrocarbons (e.g. *Alcanivorax*, *Thalassolituus*) (Rojo, 2009, Sabirova *et al*., 2006, Brakstad & Lodeng, 2005).

The first step of the aerobic degradation of gaseous hydrocarbons is the initial oxidation catalyzed by a monooxygenase. Alkanes are oxidized to the primary or secondary alcohols and further converted to aldehydes or ketones respectively. Alkenes are oxidized by adding an oxygen atom across the olefin bond forming an epoxyalkane, a highly reactive and toxic product that is immediately metabolized (Shennan, 2006, Wentzel *et al.*, 2007, Kotani *et al.*, 2006). Different enzyme classes are involved in the oxidation of hydrocarbons. Most alkane oxygenases have a wide substrate range. The methane monooxygenases sMMO and pMMO play a key role in the degradation of methane, the shortest hydrocarbon. Enzymes that are related to the sMMO are involved in the oxidation of gaseous alkanes. A butane monooxygenase (BMO) similar to the sMMO hydroxylates C_2-C_9 alkanes in *Pseudomonas butanovora* (Sluis *et al.*, 2002, Dubbels *et al.*, 2007). A BMO with properties of the sMMO was found in *Mycobacterium vaccae* OB5 (Hamamura *et al*., 1999). *Gordonia sp*. TY-5, *Mycobacterium sp*. TY-6 and *Pseudonocardia sp*. TY-7 possess a propane monooxygenase similar to the sMMO, oxidizing propane at the terminal or subterminal position (Kotani *et al*., 2006, Kotani *et al.*, 2007). Butane monooxygenases similar to the pMMO were found in *Nocardia* and *Mycobacterium*: pBMO (Hamamura *et al*., 1999, Hamamura *et al*., 2001,

Sayavedra-Soto *et al*., 2011). Another class of enzymes involved in hydrocarbon degradation are *alk* hydroxylases, integral-membrane non haem diiron monooxygenases that oxidize alkanes at the terminal position. The *alkB* gene is coding the trans-membrane alkane monooxygenase of the alk enzyme system and is used as a marker gene to detect and study alkane degraders (van Beilen *et al.*, 2001, Bertrand *et al.*, 2005). AlkB homologues show high sequence diversity. They have been found in Gram-positive and Gram-negative microorganism, including strains of the genera *Acinetobacter*, *Alcanivorax*, *Burkholderia*, *Mycobacterium*, *Pseudomonas* and *Rhodococcus* (Smits *et al.*, 1999, Smits *et al.*, 2002, Marin *et al.*, 2003, van Beilen *et al.*, 2004, Kuhn *et al.*, 2009). Most AlkB hydroxylase homologous are involved in the oxidation of C_5-C_{16} alkanes. However, the *Pseudomonas putina* GPo1 AlkB oxidizes propane and butane as well. Hence, the AlkB hydroxylases may play an important role in the oxidation of gaseous alkanes. An oxidation of methane and ethane by AlkB could not be shown (van Beilen *et al.*, 2005). Another group alkane hydroxylases are the Cytochrome P450 enzymes that are involved in the degradation of C_5-C_{16} alkanes. Those enzymes are ubiquitous among bacteria (e.g. strains of the genera *Acinetobacter*, *Mycobacteria*, *Rhodococcus*) and yeasts that are involved in the degradation of alkanes in some environments (van Beilen *et al*., 2005, Sekine *et al.*, 2006, Funhoff *et al.*, 2006, Schmitz *et al.*, 2000, Lida *et al.*, 2000). Several other alkane hydroxylases including Cu^{2+}-dependent alkane hydroxylases and flavin-binding monooxygenases were found but they are specialized in the oxidation of long-chained alkanes and do not play a role in the oxidation of gaseous alkanes (Tani *et al.*, 2001, Throne-Holst *et al.*, 2007, Feng *et al.*, 2007). Under anaerobic conditions bacteria use nitrate, sulfate or ferric iron instead of oxygen as electron acceptor to degrade hydrocarbons (Aeckersberg *et al.*, 1991, Ehrenreich *et al.*, 2000, Rueter *et al.*,

1994, Seeliger *et al.*, 1998). Though the growth of anaerobic alkane degraders is very slow, they play an important role in the degradation of hydrocarbons in the environment. The degradation of the short chained alkanes propane and butane could be shown by a strain of the Desulfosarcina/Desulfococcus cluster (Kniemeyer *et al.*, 2007). Strains of other genera (e.g. *Azoracus*, *Rhodocyclus*, *Desulfobacterium* and *Desulfovibrio*) are involved in the degradation of longer alkanes (C_6-C_{20}) (Aeckersberg *et al*., 1991, Ehrenreich *et al*., 2000, Rueter *et al*., 1994).

1.4 Aims of this study

The particulate methane monooxygenase (pMMO), the key enzyme of the aerobic methane oxidation, is a member of the Cu-containing membrane-bound monooxygenases (CuMMO), a family of widespread and diverse enzymes. Methane oxidizing bacteria (MOB) possessing the pMMO have been studied intensively within the last years. The existence of multiple copies of the pMMO (isoenzymes) within the genome of MOBs is known and analyzed for quite some time. However, a new pMMO isoenzyme (pXMO) has been found recently in several MOB that is only distantly related to characterized pMMOs. Research on the distribution of the pXMO among MOB just started. The physiological function of this enzyme is still unknown. Furthermore, sequences of CuMMO isoenzymes (iCuMMOs), distantly related to pMMO and ammonium monooxygenase (AMO) sequences, were found in several phylogenetical studies in different habitats. The distribution of the iCuMMO sequences and possible substrates of the corresponding iCuMMO enzymes are unknown.

This PhD thesis focused on iCuMMOs to get more insights into their distribution and ecological functions and to extend the view on the CuMMOs which was until now mostly restricted to pMMOs and AMOs.

Chapter 2 An unexpected diversity of copper containing membrane-bound monooxygenases: new pmoA-*like sequences retrieved from aquatic environments and pure cultures*

Here we analyzed different environmental habitats and methanotrophic pure cultures for the occurrence of CuMMO sequences that are only distantly related to known pMMOs. Newly generated primers targeting selected environmental CuMMO groups (iCuMMOs) were used to study the environmental distribution and the possible physiological function of the pXMO in *Methylobacter luteus* 53v in an incubation experiment.

Chapter 3 Magnetic capture of iCuMMO sequences: A prove of concept

Culture independent PCR based methods are powerful tools to study methanotrophic communities in diverse habitats. Here, we established a method to avoid the use of specific primers that are essential for the success of the PCR dependent methods.

Chapter 4 Monooxygenases involved in the degradation of short chained gaseous hydrocarbons in a rice field soil

Bacterial monooxygenases are widespread in nature and are able to oxidize a variety of different substrates. In this chapter we analyzed if Cu-containing monooxygenases and AlkB hydroxylases are involved in the oxidation of alkanes and alkenes in rice filed soils and which organisms are the key players in the hydrocarbon degradation.

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2 An unexpected diversity of Cu-containing membrane-bound monooxygenases: new *pmoA*-like sequences retrieved from aquatic environments and pure cultures.

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2.1 Abstract

An isoenzyme of a Cu-containing membrane-bound methane monooxygenase (CuMMO) that is only distantly related to known CuMMO sequences has been recently found in several alpha- and gammaproteobacterial methanotrophs. Classical primer sets targeting the *pmoA* gene (encoding a subunit of the CuMMO) discriminate against these sequences. Here, we designed new reverse primers targeting in total four deep branching groups of CuMMO: the gammaproteobacterial isoenzyme and three related environmental clusters. We studied the occurrence of these *pmoA*-like sequences in nature and in methanotrophic pure cultures. Pyrosequencing results show that they are widespread and highly abundant in rice field soils and lake sediments. We furthermore observed habitat-specific distribution patterns. In pure cultures the isoenzyme seems to be restricted to strains of the type Ia genera *Methylomonas*, *Methylobacter* and *Methylosarcina*. A phylogenetic comparison of *pmoA*, *pxmA* and 16S rRNA genes of these strains indicates that the *pxmA* evolved vertically within the type Ia methanotrophs. Incubation studies of *Methylobacter luteus* with different

substrates let us suggest that the physiological role of the CuMMO isoenzyme is the oxidation of methane rather than ammonium or ethane.

2.2 Introduction

Cu-containing membrane-bound monooxygenases (Coleman *et al.*, 2011) comprise two enzymes catalyzing key reactions in global carbon and nitrogen cycles: methane and ammonia monooxygenases. Methane monooxygenases are the key enzymes in methane oxidizing bacteria that help to mitigate methane emissions (Frenzel, 2000, Conrad, 2009, Reeburgh, 1997) which is, next to carbon dioxide, the most important greenhouse gas (Intergovernmental Panel on Climate Change, 2007) . Methanotrophs interfere with the global methane cycle by acting as a biofilter in high-methane environments like wetlands and landfills (Reim *et al.*, 2012, Henneberger *et al.*, 2012, Conrad & Frenzel, 2002), or as a sink to atmospheric methane in upland soils (Conrad, 1996, Bender & Conrad, 1992). Methanotrophs have the unique ability to use methane as their sole carbon and energy source (Trotsenko & Murrell, 2008), but some were recently found to exploit also simple organic substrates (Belova *et al.*, 2011, Dedysh *et al.*, 2005, Theisen *et al.*, 2005). The majority of cultivated methanotrophs are proteobacteria. Among these canonical methane oxidizers, two types are distinguished: type I and type II (Whittenb.R *et al.*, 1970). This historically grown differentiation corresponds well to molecular phylogenies (Lüke & Frenzel, 2011). Type I methanotrophs are gammaproteobacteria and further divided into type Ia (e.g. *Methylobacter, Methylovolum, Methyloglobulus, Methylomicrobium*, *Methylomarinum*, *Methylomonas*, *Methylosarcina,* and *Methylosoma*) and type Ib (e.g. *Methylocaldum*,

Methylococcus, Methylogaea and *Methylothermus / Methylohalobius*). Type Ic was also defined as a separate group represented by one cultivated ammonium oxidizer, *Nitrosococcus oceani*, and putative MMOs (JR2/JR3, USC-gamma, OPU1; Lüke & Frenzel, 2011, Horz *et al.*, 2005, Knief *et al.*, 2003, Hayashi *et al*., 2007). The alphaproteobacterial type II methanotrophs includes the genera *Methylocystis*, *Methylosinus, Methylocapsa, Methylocella and Methyloferula*. More recently, the spectrum has widened: the filamentous gammaproteobacterial *Crenothrix* and *Clonothrix* were found to be methanotrophs (Stoecker *et al.*, 2006, Vigliotta *et al.*, 2007), methane oxidizing *Verrucomicrobia* were isolated from extreme and moderate environments (Pol *et al.*, 2007, Islam *et al.*, 2008, Dunfield *et al.*, 2007, Sharp *et al.*, 2014, van Teeseling *et al.*, 2014), and the nitrite-reducing and O_2-generating Ca. *Methylomirabilis oxyfera* was characterized (Ettwig *et al.*, 2010). Nearly all methanotrophic bacteria possess a particulate methane monooxygenase (pMMO), while some have an additional Fe-containing soluble MMO (sMMO). The latter is the only MMO in *Methylocella* and *Methyloferula* (Dedysh *et al*., 2004, Dedysh *et al*., 2000, Dunfield *et al*., 2003, Vorobev *et al*., 2011). Besides its main substrate, the pMMO may also oxidize alternative substrates like ammonia, short chained alkanes, and haloalkanes (Elliott *et al.*, 1997, Semrau, 2011, Burrows *et al.*, 1984, Bedard & Knowles, 1989). The genes for the three subunits of pMMO are organized as *pmoCAB*. They can occur in near sequence-identical or divergent copies within the genome. One example of a sequence-divergent pMMO copy is the pMMO2 of *Methylocystis* SC2 that has different methane oxidation kinetics providing a selective advantage at low methane concentrations (Baani & Liesack, 2008). More recently, another pMMO isoenzyme (referred to as pXMO) organized as *pxmABC* has been found in

type Ia and type II methanotrophs (Tavormina *et al.*, 2011, Svenning *et al.*, 2011, Vorobev *et al*., 2014).

The *pmoA* gene encodes for the beta-subunit of the pMMO. It is highly conserved and often used in environmental studies to detect and characterize methanotrophs. The homologous *pxmA* sequences form a deep-branching separate lineage (M84_P105) that clusters phylogenetical between characterized *pmoA* sequences and the *amoA* sequences of ammonia oxidizers (Lüke & Frenzel, 2011, Dumont *et al.*, 2014). M84_P105 is related to some other environmental sequence clusters (e.g., RA21, TUSC, AOB_rel; (Dumont *et al*., 2014, Lüke & Frenzel, 2011)). For pragmatic reasons, this group of clusters will be named **i**ntermediate **c**opper containing **m**embrane-bound **m**ono**o**xygenases (iCuMMO) and the term *pxmA* will be used as synonym for the gene encoding the beta-subunit of an iCuMMO. Substrate spectrum and specificity of the respective proteins are unknown.

The *pmoA*-targeting standard primer set covers a wide range of the methanotrophic diversity (Holmes *et al.*, 1995). Besides *pmoA* sequences the primer co amplifies genes encoding for similar CuMMOs like the *amoA* of betaproteobacterial ammonium oxidizers and, to a minor degree, also *pxmA* sequences may be co-amplified, in addition. Using a modified primer, sequences belonging to the M84_P105 cluster were identified as the *pxmA* of a type Ia methanotroph (Tavormina *et al*., 2011). Unfortunately, the chosen primers had some mismatches against the environmental sequences falling into this cluster. While the preferred substrate of this isoenzyme in type Ia and type II methanotrophs is still unknown, other iCuMMO sequences cluster near to sequences of putative ethane monooxygenases

(Redmond *et al.*, 2010). Hence, one may speculate that the respective proteins' substrates are short-chained alkanes, too.

Here, we designed specific primers targeting the iCuMMO groups M84_P105, RA21, AOB_rel and TUSC (Lüke & Frenzel, 2011), respectively. Studying the occurrence of these clusters in aquatic environments, we applied pyrosequencing to samples from three rice fields and two lake sediments. In addition, we tested pure cultures for presence of iCuMMO encoding genes. We retrieved *pxmA* sequences from *Methylobacter*, *Methylosarcina* and *Methylomonas*, and studied the transcription of the respective gene in *Methylobacter luteus* 53v under methane, ethane, both substrates together, and ammonia, respectively. Population growth approximated by copy number of *pxmA* were followed by competitive PCR (Reim *et al*., 2012) and compared to number of transcripts.

2.2 Material and Methods

Field sites and samples

Soil samples were taken from fields in Italy and China. The Italian paddy field is situated in the lowlands of river Po (Vercelli: N 45°20'; W 8°25') and managed by the CRA-Agricultural Research Council, Rice Research Unit, in Vercelli. This field had been planted to wetland rice for more than a century (Lüke *et al.*, 2010). Soil parameters and agricultural practices have been described elsewhere (Holzapfelpschorn & Seiler, 1986, Kruger *et al.*, 2001). The Chinese paddy fields are situated near Cixi, province Zhejiang. One field is under permanent agriculture since 50 years (China50: N 30°11.066'; E 121°21.351'), the other since 2000 years

(China2000: N 30°05.455’; E 121°26.738’). Soil parameters and history have been described elsewhere (Ho *et al.*, 2011, Kolbl *et al.*, 2014, Cheng *et al.*, 2009). Samples were taken from the plow layer before fields were flooded in spring 2009. Upon arrival in Germany, the samples (approximately 500 kg per site) were homogenized manually. In short, pots were planted with rice and kept in a greenhouse according to agricultural practice and climate, respectively. Details have been described elsewhere (Roth *et al.*, 2013). Samples were taken from pots planted to local rice varieties, *qian you yi hao* (Chinese soils) and KORAL (Italian soil). For a comparison of all three sites see also Lüke *et al.*(2014).

Lake sediments were sampled from Lake Constance (Germany) and Lake Neusiedl (Austria). The sample from Lake Constance (N 47°43.382’; E 9°10.751’) was taken from organic rich sediment in the shallow littoral next to a small reed bed. The sample from Lake Neusiedl (N 47°55.951'; E 16°45.397') was taken from a ditch through the reed belt.

Total DNA and RNA were extracted from freeze-dried soils as described elsewhere (Reim *et al*., 2012). For amplification of *pxmA* sequences total DNA was extracted from the three paddy fields and the lake sediments. The primers A189f, M84_P105r, TUSCr, RA21_2r and AOB_relr were modified (adapters and barcodes were attached) and the PCR was performed as described before (Lüke *et al*., 2014). 454 amplicon sequencing was performed by GATC (Konstanz, Germany). The pyrosequencing data were evaluated with the ARB software package (Ludwig *et al.*, 2004). Sequences containing insertions or deletion resulting in a shift of the reading frame were excluded manually. For further phylogenetic analysis only sequences with a read length of at least 130 amino acids were used.

Pure cultures: Nucleic acid extraction and PCR

From pure cultures 0.5 mL were suspended in phosphate buffer (pH8) and TNS. Cells were disrupted by bead beating. DNA and RNA were purified by phenol-chloroform-isoamyl alcohol and chloroform-isoamyl-alcohol extraction. Nucleic acids were precipitated with PEG, washed with ethanol (70%), and eluted in EB buffer. Samples used for transcriptional analysis were digested with RQI DNase (Promega, Madison, WI, USA) following the manufactures protocol. RNA was purified with the RNeasy® Plus mini Kit (Qiagen, Hilden, Germany).

pmoA genes were amplified using the forward primer A189f and the reverse primer A682r (Table 1; (Holmes *et al*., 1995)). For amplification of *pxmA* sequences four reverse primers were designed based on an alignment of publicly available sequences clustering within the iCuMMO groups. The novel reverse primers were designed to target the clusters M84_P105, RA21, TUSC, and AOB_rel (Table 1). The PCR reactions were performed as described before (Lüke *et al*., 2010).

16s rRNA genes were amplified using the primers Eu9/27f (5'-GAG TTT GAT C(AC)T GGC TCA G-3') (Lane, 1991) and Eu1492r (5'-ACG G(CT)T ACC TTG TTA CGA CCT-3') (Weisburg *et al*., 1991). The composition of the PCR Mix corresponded to that used for the *pmoA* and *pxmA* PCR. The PCR program started with an initial denaturing step at 94 °C for 5 min, followed by 30 cycles of denaturing (1 min at 94 °C), annealing (1 min at 53 °C) and elongation (1 min at 72 °C). A final elongation step was carried out at 72 °C for 10 min. PCR products were analyzed by 1 % agarose gel electrophoresis and visualized by with GelRed® Nucleic Acid Stain (Biotium, Hayward, CA, USA).

Table 1: Primers targeting *pmoA* and *pxmA*. The start positions of a primer are given for a *pmoA* alignment without gaps in which the forward primer A189f is set to position 189.

Target	Name	Position	Sequence	Reference
pmoA, general	A189f	189	5'- GGN GAC TGG GAC TTC TGG -3'	(37)
pmoA, general	A682r	685	5'- GAA SGC NGA GAA GAA SGC -3'	(37)
pmoA, general	mb661r	661	5'- CCG GMG CAA CGT CYT TAC C -3'	(74)
pxmA	pxmA634r	634	5'- CCA RAA RTC CCA RTC NCC -3'	(34)
M84_P105	M84_P105r	589	5'-GCG GAT GTA TTG RAA NCC-3'	This study
RA21	RA21r	619	5'-GAT GAT NCG CAR ATA TTC-3'	This study
TUSC	TUSCr	623	5'-GCT CGA TGA TGC GGA TGT-3'	This study
AOB_rel	AOB_relr	619	5'- GAT GAT NCG GAT RTA YTC-3'	This study

Pure cultures: cloning, sequencing and sequence analysis

Bacterial pure cultures used in this study are listed in Table 3. The cultures were cultivated under 10% methane in air in NMS or AMS (Whittenbury *et al*., 1970) at 25°C and 250 rpm in the dark.

Gene libraries of *pmoA*, *pxmA* and the 16S rRNA gene were constructed using pure culture nucleic acid extracts. PCR amplicons were ligated into pGEM-T vector plasmids (Promega, Mannheim, Germany) and transformed into *Escherichia coli* JM109 competent cells (Promega, Mannheim, Germany) according to the manufacturer's instruction. Plasmid DNA was sequenced with the ABI prism BigDye terminator cycle Ready Reaction Kit with AmpliTaq polymerases (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction using primers M13 rev-29 (5'-CAG GAA ACA GCT ATG ACC-3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG-3'). Sequencing was carried out in both directions. Sequences were assembled and vectors sequences were cut off using the SeqMan software

(DNA-Star software package, Lasergene, Madison, WI, USA). Phylogenetic analysis were done in ARB ver. 5.2 (Ludwig *et al*., 2004) and the online RAxML platform (Stamatakis *et al.*, 2008, Stamatakis, 2006). Sequences used for pure culture analysis contained at least 142 (*pxmA*) or 140 (*pmoA*) amino acids, and 697 nucleotides (16S rRNA), respectively.

For the construction of phylogenetic trees *pmoA*, *pxmA* and *amoA* sequences from public databases and an existing database (Lüke & Frenzel, 2011) were used additionally. 16S rRNA sequence analyses were based on the SILVA 108 database, release September 2011 (Quast *et al.*, 2013).

Incubations

For pure culture incubation 30 mL of an ammonium mineral salt medium (AMS) were inoculated with 1.5 mL of a *Methylobacter luteus* 53v pre-culture. The cultures were incubated with air and 2% methane (A, C) or 2% ethane (B). To set up (C), 2% ethane was added when methane was nearly consumed. The cultures were incubated at 25°C and 200 rpm in the dark. Substrate consumption was followed by gas chromatography.

Competitive t-RFLP

A competitive PCR (cPCR) was performed as described before (Reim *et al*., 2012). DNA standards were generated by PCR of rice field soil DNA (China2000) using the primers A189f and Inner-rev-M84_P105_2_Mbacter (5'-GAT GAT GCG CAG RTA TTC CGA CCG GTT GGT GGA ACA TG-3') (Reim *et al*., 2012). RNA standards for quantifying the transcriptional level of *pxmA* were generated by PCR using the primers A189f_T7 (5'-AAT ACG ACT CAC TAT AGG GGG NGA CTG GGA CTT CTG G-3') and Inner-rev-M84_P105_2_Mbacter.

2.3 Results

Environmental iCuMMO sequences

To study the occurrence of *pxmA* in different environments, samples were analyzed by deep sequencing with primers targeting the iCuMMO groups M84_P105, RA_21, TUSC and AOB_rel (Table 1). The basis for the phylogenetic analysis was an extensive database of *pmoA*, *pxmA* and *amoA* sequences from pure cultures and various environments (Lüke & Frenzel, 2011). The deep sequencing analysis resulted in about 21.400 high-quality *pxmA* and *pmoA* sequences (Figure 1, Table 2).

Table 2: Phylogenetic distribution of sequences retrieved with the four new reverse primers depicting the percentage of sequences clustering within a specific lineage of *pmoA/pxmA*. Sequences retrieved with primer 682r from Vercelli, China50, and China2000 are shown for comparison (15).

Reverse primer	Phylogenetic assignment									Total number
	M84_P105	RA21	TUSC	AOB_rel	Type Ia	Type Ib	Type II	FG	Other*	
M84_P105r	**99.74**	0.03	0.07	0.00	0.00	0.00	0.12	0.01	0.04	7575
RA21r	80.52	**17.42**	0.00	0.01	0.00	0.00	0.00	2.00	0.05	7766
TUSCr	1.04	0.00	**47.39**	39.24	0.13	0.13	11.73	0.07	0.26	1534
AOB_relr	0.14	0.06	31.32	**35.83**	0.14	19.64	10.78	0.57	1.50	4878
682r	0.03	0.09	0.9	7.33	0.26	33.03	26.05	0	32.28	3439

* for the A682r primer, others include mainly the amoA gene of ammonia oxidizing betaproteobacteria.

Virtually all sequences retrieved with the reverse primer M84_P105r fell into the M84_P105 cluster, suggesting that this primer is indeed very specific (Table 2). Sequences of this group showed a wide distribution throughout all environments, being found in all rice field soils and the lake sediments. Within the M84_P105 group, *pxmA* sequences formed the subgroups G1-G5 (Figure 2). Independent on treeing algorithm (neighbor joining or maximum likelyhood method) or subset of sequences used in analysis, sequences of each group always clustered together. All subgroups contained environmental sequences.

Subgroup 1 includes in addition *pxmA* sequences of *Methylobacter tundripaludum* SV96, *Methylobacter marinus* A45, *Methylobacter luteus* 53v, *Methylomonas methanica* S1, *Methylomonas spec.* VMn. Group 2 contains *pxmA* sequences of *Methylosarcina quisquilarum* AML-D4, *Methylosarcina fibrata* AML-C10 and *Methylomicrobium album* BG8. Subgroup 3 is represented by *pxmA* sequences of a Methyloglobulus morosus strain belonging to the type Ia methanotrophs (Deutzmann *et al.*, 2014). Subgroup 5 includes *pxmA* sequneces of the type II methanotrophs *Methylocystis* sp. SB2 and *Methylocystis rosea*. Subgroups G1 and G3 were mainly represented by sequences retrieved from lake sediments whereas G2, G4 and G5 contain mainly of sequences amplified from rise soils.

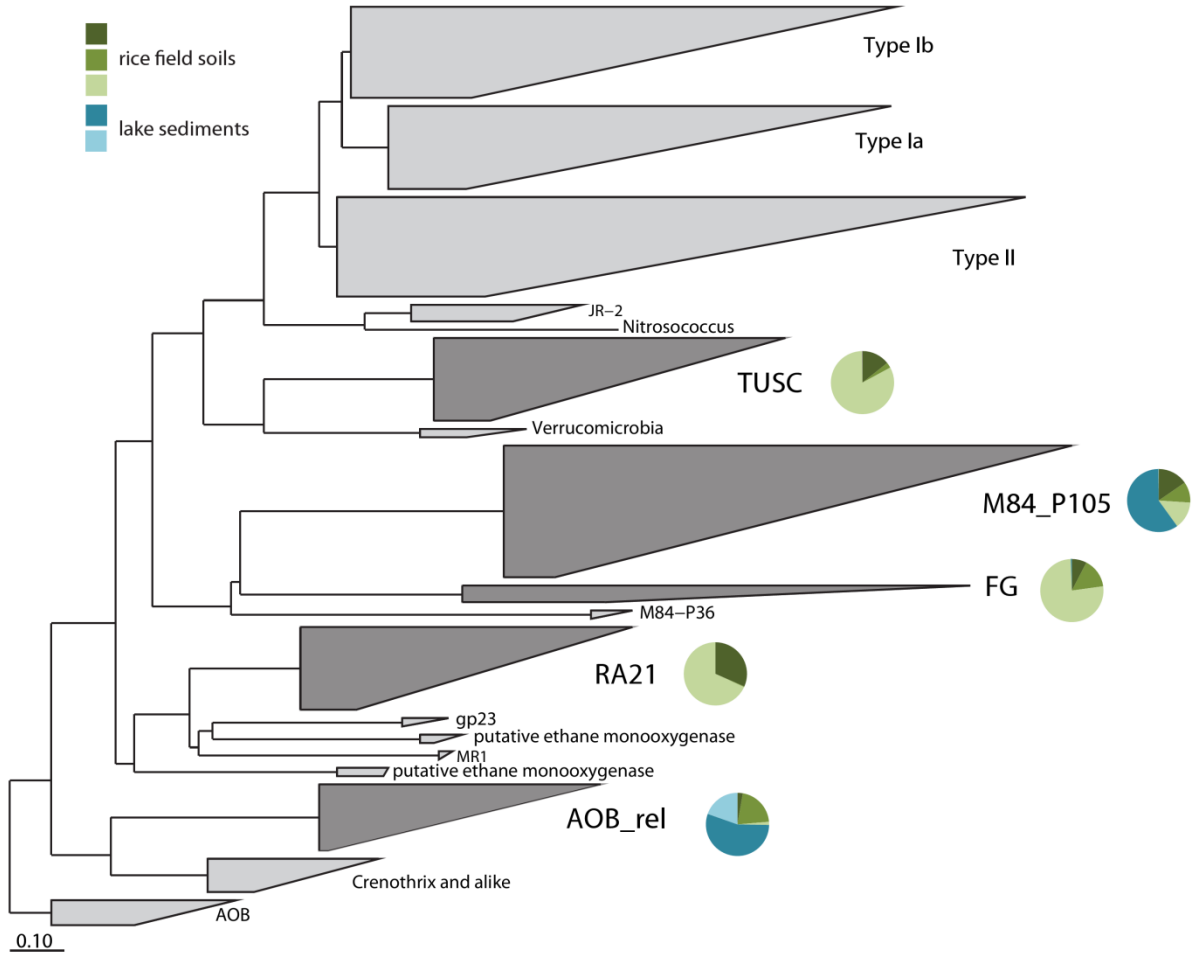

Figure 1: Phylogeny of iCuMMO sequences. Neighbor joining tree of partial *pmoA* and related sequences, based on 135 deduced amino acids. The tree combines sequences retrieved by pyrosequencing using newly designed primers with *pmoA* and *pxmA* sequences from public databases. The iCuMMO groups TUSC, RA21, M84_P105, AOB_rel, and FG are marked in dark grey. Environments are color-coded: dark green, Vercelli; green, China50; light green, China2000; dark blue, Lake Constance; light blue, Lake Neusiedl. The exact numbers of sequences per environment are given in Table 2.

Sequences that cluster within the iCuMMO groups RA_21 and TUSC were almost exclusively detected in the rice field soils. Most of the sequences affiliated to the RA_21 group were detected in Vercelli and China50, and most of the sequences affiliated to the TUSC group in Vercelli and China2000. The AOB_rel group was dominated by lake sediment sequences and sequences amplified from China2000. Next to the great percentage of sequences that cluster

within the already known environmental iCuMMO groups, we detected few sequences that fell outside the iCuMMO clusters. In most cases only few sequences clustered together outside the known groups. We did not indicate these sequences as new groups. An exception was the new group **FG** (Freshwater sediment Group) that contains about 200 *pxmA* sequences amplified from rice field soils.

***pxmA* in pure cultures**

Using the newly designed primers, representative pure cultures of type Ia, type Ib and type II methanotrophs were tested for the occurrence of *pxmA* genes (Table 3). *pxmA* genes could be retrieved from type Ia methanotrophs *Methylomonas spec* VMn, *Methylobacter luteus* 53v, *Methylosarcina fibrata* AML-C10 and *Methylosarcina quisquilarum* AML-D4. No *pxmA* genes could be detected in type Ib methanotroph *Methylococcus capsulatus* Texas and *Methylocaldum* E10a, in type II methanotroph *Methylosinus trichosporium* OB3b, *Methylosinus trichosporium* I4/1 and *Methylocystis heyeri* H2(T), and type Ia methanotroph *Methylomicrobium alcaliphilum* 2OZ. In contrast to the study of Tavormina and colleagues (Tavormina et al., 2011), *pxmA* could also be detected in strains of the genus *Methylosarcina*. All *pxmA* sequences clustered within the M84_P105 group. The phylogeny of *pmoA*, *pxmA* and 16S rRNA sequences of type Ia methanotrophs was compared using Maximum-Likelihood trees of nine *pmoA*, eight *pxmA* and ten 16S rRNA gene sequences (Figure 3). In addition, a pair-wise comparison of sequence similarities between partial *pxmA, pmoA* and 16S rRNA gene sequences was performed. The relationship of similarities of *pmoA* and *pxmA* was linear for genes (R=0.77) and deduced proteins (R=0.76). Also, there was a linear relationship between similarities of 16S rRNA genes and *pxmA* genes (R=0.74) or

proteins (R=0.63), and between 16S rRNA genes and *pmoA* genes (R=0.81) or proteins (R=0.83). All correlation coefficients are highly significant ($p < 0.01$).

Table 3: Occurrence of *pxmA* in type Ia MOB pure cultures. The source gives either the DSMZ strain identification or the reference from which sequences were extracted for comparative phylogenetic analysis (Figure 3). +/-: presence/absence of the respective gene; NA: sequence not available; *: sequenced in this work.

Taxonominc name	Strain	Source	pxmA	pmoA	16S rRNA
Methylomonas methanica	S1	1	+	+	+
Methylomonas sp.	VMn	2	+*	+*	+*
Methylobacter luteus	53v	NCIMB 11914	+*	+*	+*
Methylobacter tundripaludum	SV96	DSM 17260	+	+	+
Methylobacter marinus	A45	1	+	NA	+
Methylosarcina quisquilarum	AML-D4	DSM 13737	+*	+*	+*
Methylosarcina fibrata	AML-C10	DSM13736	+*	+*	+*
Methylosarcina lacus	LW14	1	-	+	+
Methylomicrobium album	BG8	1	+	+	+
Methylomicrobium kenyense		1	-	+	+
Methylomicrobium alcaliphilum	2 Z	DSM 19304	- *	+*	+*

1: Tavormina *et al*. 2011 (34). 2: *Methylomonas sp*. VMn is from the culture collection at the MPI for Terrestrial Microbiology in Marburg. The original source is not documented. The culture has a 16S rRNA-sequence similarity of 98% to *Methylomonas methanica* (AF150806,1) and other *Methylomonas sp*. strains like *Methylomonas sp*. LW16.

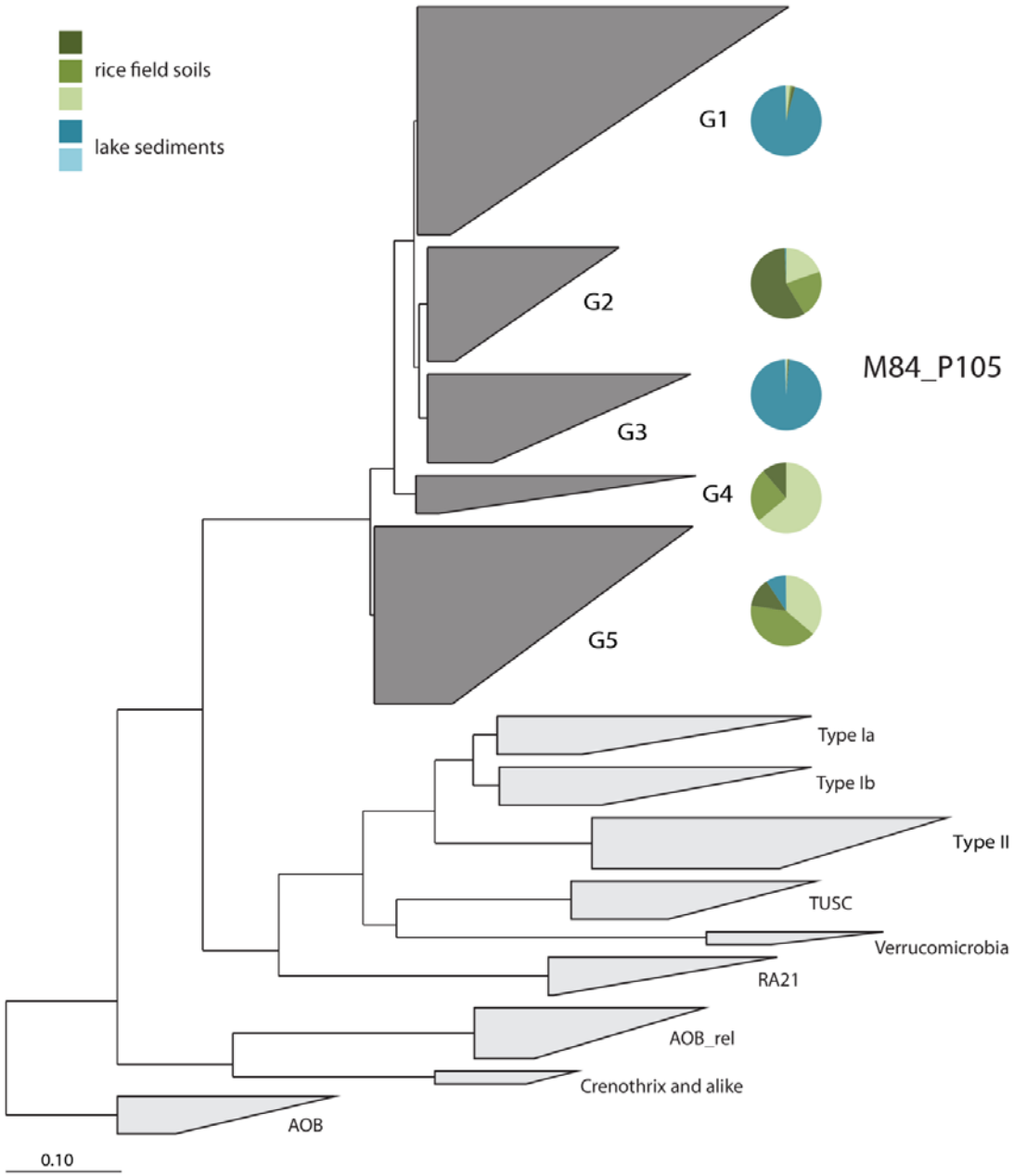

Figure 2. Phylogenetic and ecological differentiations within the M84_P105 cluster. Neighbor joining tree of partial *pmoA* and related sequences as in Figure 1. In addition to environmental sequences, group 1 contains *pxmA* sequences from *Methylobacter tundripaludum*, *Methylobacter marinus*, *Methylobacter luteus*, *Methylomonas methanica* and *Methylomonas spec*. Group 2 contains environmental sequences and *pxmA* sequence of *Methylosarcina quisquilarum*, *Methylosarcina fibrata* and *Methylomicrobium album*. Groups 3 contains environmental sequences and pxmA sequences from *Methyloglobulus morosus*. Group 4 contain only environmental sequences. Group 5 contain environmental *pxmA* sequences and sequences of type II methanotrophs *Methylocystis* sp. SB2 and *Methylocystis rosea*. Environments are color-coded: dark green, Vercelli; green, China50; light green, China2000; dark blue, Lake Constance; light blue, Lake Neusiedl. The exact numbers of sequences per environment are given in Table S1.

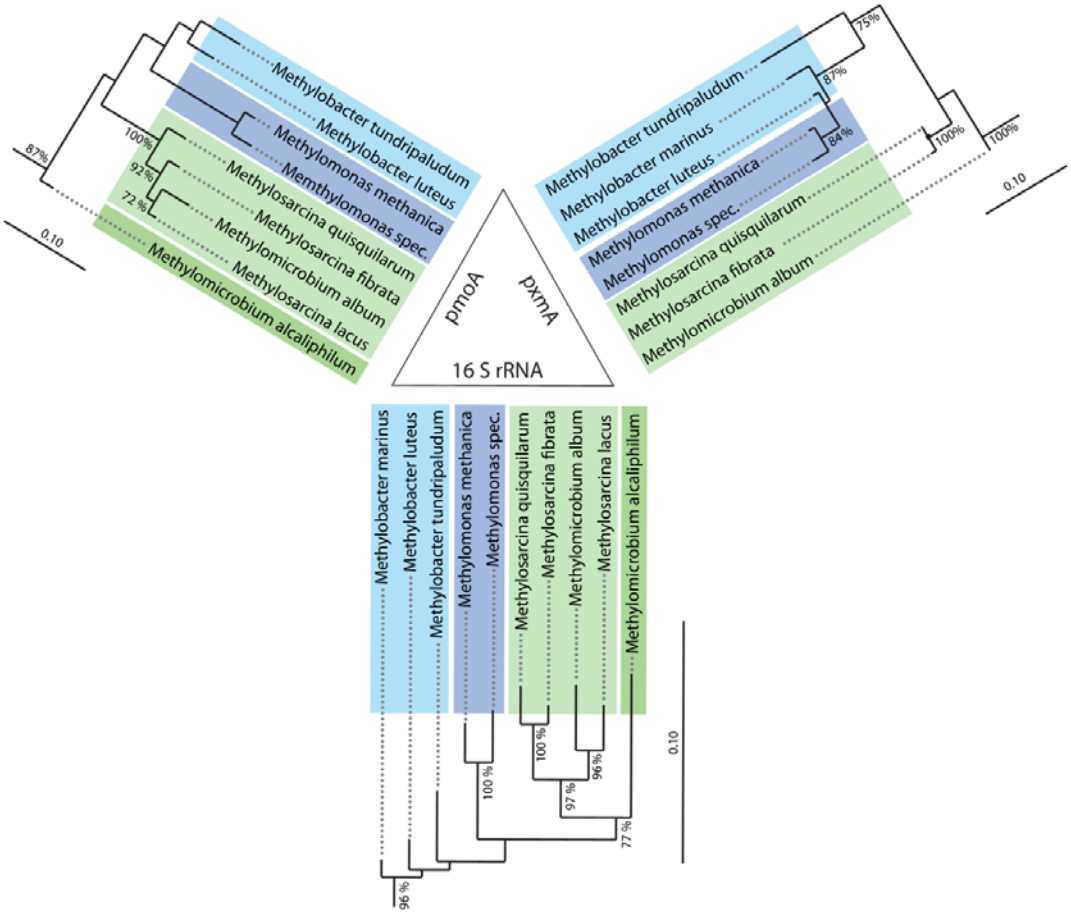

Figure 3. Maximum likelihood trees of corresponding *pmoA* (A; 140 amino acids), *pxmA* (B; 142 amino acids), and 16S rRNA sequences (C; 697 nucleotides) of type Ia methanotrophic bacteria. Sequences retrieved in this study are marked by an asterisk.

Transcription of *pxmA*

As the physiological function of pXMO is still unknown, an incubation study with different substrates was performed: methane, ethane, and a combination of methane and ethane (Figure 4). Substrate usage, population growth and transcriptional *pxmA* levels were studied exemplarily in *Methylobacter luteus* 53v. Methane was rapidly oxidized in all setups. The methane concentrations decreased from 700 µM to 17µM within three days of incubation,

and from 680-750 µM to 120-130 µM within four days of incubation. The incubation with ethane in air alone showed initially a minor decrease of ethane, but stayed constant afterwards (data not shown). In the experiment with methane and ethane (Figure 4), the latter was added after 55.5 h, when methane was nearly used up. Ethane oxidation started immediately and stopped at a threshold concentration of 390-420 µM (n=2). *pxmA* transcripts could be detected after 45.5 hours of incubation. The highest number of transcripts was measured after 53h ($6.46 \cdot 10^4$ copies/µL). The copy number decreased when methane oxidation stopped and before ethane was added after 77 h. No *pxmA* transcripts could be detected afterwards

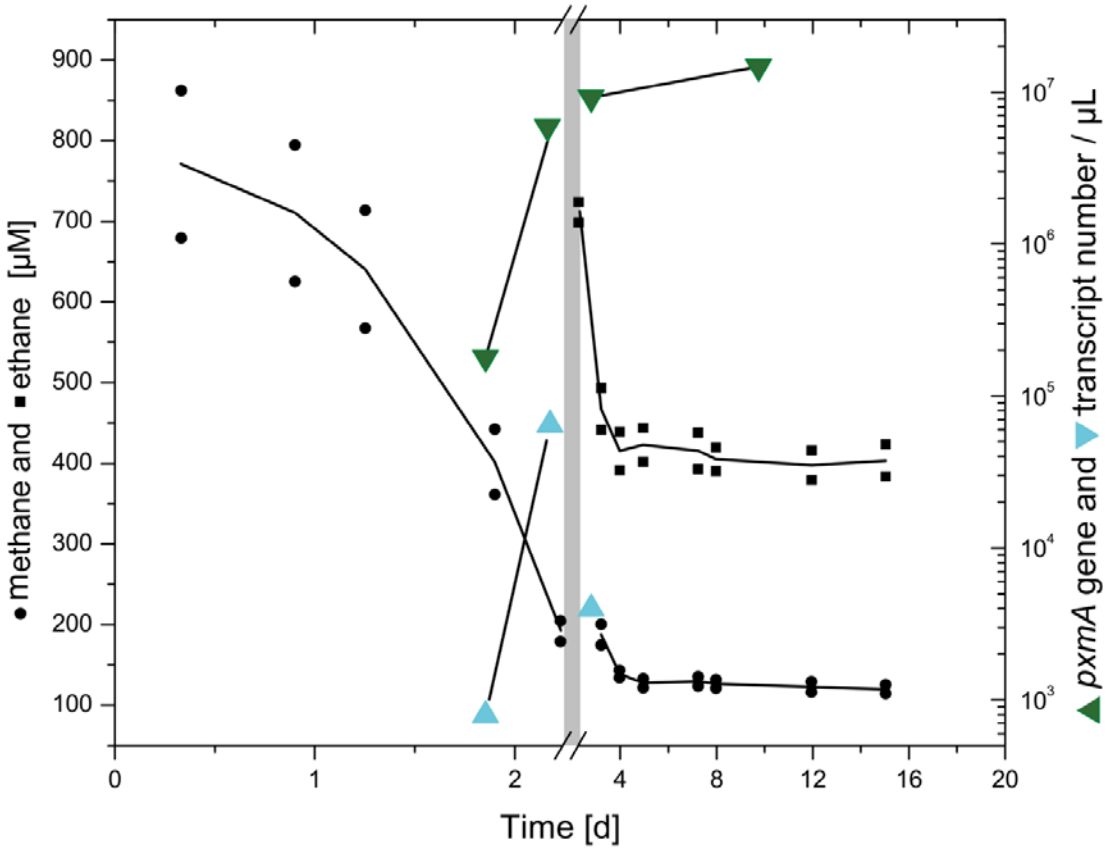

Figure 4. Substrate consumption, *pxmA* gene and transcript copy number of *Methylobacter luteus* (NCIMB 11914) with methane and ethane as substrates (Setup C).

2.4 Discussion

The diversity of *pmoA* sequences in rice field soils and other environments has been analyzed in a variety of studies. Next generation amplicon sequencing of *pmoA* has furthermore indicated that the methanotrophic diversity seemed to be well covered (Lüke & Frenzel, 2011). In contrast, *pxmA* sequences that cluster within the intermediate Cu-containing membrane-bound monooxygenase groups (iCuMMO) are not well studied so far. Only few sequences of these groups were available which lead to the conclusion that these sequences might be rare in nature. However, the available iCuMMO sequences were retrieved from many different environments like forest-, volcanic-, arctic- and dessert soils, as well as paddy soils, landfill cover soils, freshwater lakes and others (Angel & Conrad, 2009, Dorr *et al.*, 2010, Holmes *et al.*, 1999, Jia *et al.*, 2007, King & Nanba, 2008, Knief *et al.*, 2005, Pacheco-Oliver *et al.*, 2002, Reay *et al.*, 2001, Singh *et al.*, 2009). Hence, these sequences are widely distributed in nature though they seem to be not very numerous. This fact could be due to the use of the classical primer pair A189f/A682r (Holmes *et al*., 1995) that has the potential to amplify a wide range of *pxmA* sequences but is known to discriminate against several environmental clusters. Also quite often used is the primer par A189f/mb661 (Costello & Lidstrom, 1999) that discriminates even stronger against non-canonical *pxmAs*. The reverse primer A682r and the primer pxmA634r that were used to amplify the *pxmA* in another study (Tavormina *et al*., 2011) showed several mismatches to sequences of the iCuMMOs. With the newly designed reverse primers targeting the iCuMMO groups we could detected about 21.000 *pxmA* sequences. The modification of the classical primer sets resulted in a totally different distribution of sequences within the *pmoA* tree and threw a new light on the to-date only little observed iCuMMOs. Contrary to what was previously

assumed sequences of the iCuMMO are very numerous and widely distributed in freshwater sediments and rice field soils. Despite this wide dispersal, environment-related distribution patterns could be observed. While the groups TUSC, RA21 and FG are mainly represented by rice field soil sequences, the AOB_rel and the M84_P105 groups seem to be mixed groups of rice field soil and lake sediment sequences (Figure 1). However, a closer look at the distribution of the sequences within the mixed cluster M84_P105 showed that sequences of different habitats tended to cluster in different subgroups (Figure 2). Analyses of more environments will show, if this division is also true for other habitats.

Four of these subgroups contain *pxmA* sequences of cultivated type Ia and type II methanotrophs. The *pxmA* gene of *Methylobacter* and *Methylomonas* species cluster within the subgroup G1. However, the *pxmA* of the phsychrophilic *Methylobacter tundripaludum* cluster separately from other G1 sequences, but together with rice field soil sequences. Thus, the *pxmA* of *Methylobacter tundripaludum* is present in freshwater environments of the temperate zones, while its *pmoA* has mainly retrieved from the Arctic. The subgroup 2 contains *pxmA* sequences of two *Methylosarcina* strains and of *Methylomicrobium album*. It has been shown that 16S rRNA and *pmoA* sequences of *Methylomicrobium album* cluster closely to sequences of *Methylosarcina* (Wise *et al.*, 2001, Kalyuzhnaya *et al.*, 2008, this study) yet apart from other *Methylomicrobia* species. Thus, *Methylomicrobium album* is not representative for its genus and a re-classification has already been proposed (Kalyuzhnaya *et al*., 2008).The *pxmA* could not be detected in *Methylomicrobium alcaliphilum* and *Methylomicrobium kenyense* (Tavormina *et al*., 2011). These are both halo(alkali)philic methanotrophs that were isolated from highly alkaline soda lakes (Khmelenina *et al.*, 1997,

Sorokin *et al.*, 2000). Neutrophilic *Methylomicrobium* strains like *Methylomicrobium pelagicum* (Sieburth *et al.*, 1987) or *Methylomicrobium japanense* (Fuse *et al.*, 1998) still need to be tested for presence of the *pxmA* gene. Subgroup 3 contains primarily environmental sequences retrieved from Lake Constance. Additionally, *pxmA* sequences of a newly isolated type Ia methanotroph *Methyloglobulus morosus* which was isolated from this lake clustered within this subgroup (Deutzmann et al., 2014). While the subgroups 1, 2 and 3 are represented by type Ia methanotrophs, subgroups 5 contains *pxmA* sequences of type II methanotrophs.

A ternary diagram constructed from phylogenetic inference of *pmoA*, *pxmA* and 16S rRNA of type Ia methanotrophic gene sequences showed a good agreement between tree structures (Figure 2). In addition, a pair-wise comparison of DNA and protein sequence similarities of *pxmA* versus *pmoA*, *pxmA* versus 16SrRNA and *pmoA* versus 16S rRNA revealed a significant correlation. No evidence of a recent horizontal transfer of the *pxmA* gene was found in type I methanotrophic bacteria. It rather seemed that the *pxmA* gene was acquired early in evolution and developed further in parallel to the *pmoA* and the 16S rRNA genes. It remains unanswered when this happened. It is assumed that the ability to oxidize methane was achieved by the alphaproteobacteria just once in their evolution (Tamas *et al.*, 2014). If this is also true for the gammaproteobacteria it is likely that the *pxm* operon was acquired before the radiation of the type I methanotrophs started. The *pxmA* in type I methanotrophs has been found until now only in strains of type Ia methanotrophs, no in type Ib methanotrophs. In a sense this is surprising, because we could show that the *pxmA* is widely distributed in freshwater environments, where type Ib methanotrophs dominate. But no

pxmA sequences could be retrieved from the type Ib strains *Methylococcus capsulatus* Texas and *Methylocaldum sp.* However, most type Ib sequences from freshwater habitats cluster within phylogenetic groups that are not represented by any cultured representative (Lüke & Frenzel, 2011). Either the type Ib methanotrophs do not possess the *pxmA* or we just cannot yet link a *pxmA* to its corresponding type Ib *pmoA* due to the lack of representative pure cultures. Because of that, it is difficult to guess if the *pxmA* was acquired before or after the division of the type Ia und type Ib methanotrophs.

The physiological role of the different iCuMMOs is unknown until now. They are distantly related to methane and ammonium monooxygenases and to marine sequences from putative ethane degraders (Coleman *et al*., 2011, Redmond *et al*., 2010, Suzuki *et al.*, 2012, Sayavedra-Soto *et al.*, 2011). It seemed likely that iCuMMOs could also be involved in the degradation of these substrates. It was suggested that the pXMO isoenzyme in type Ia methanotrophs plays a role in the ammonium oxidation (Tavormina *et al*., 2011) but we could not see any evidence that ammonium was used as a substrate for the iCuMMO in this study (no growth on AMS medium without methane addition; data not shown). Furthermore, incubation experiments of *Methylobacter luteus* 53v do not indicate that ethane is a possible substrate. The culture grew well on methane but showed no growth on ethane. Additionally, the transcription of the *pxmA* could only be detected when methane was available as a substrate. However, this transcription was very low. It has been shown earlier that pMMO isoenzymes in alphaproteobacteria and Verrucomicrobia are expressed at different methane or oxygen concentrations (Baani & Liesack, 2008, Khadem *et al.*, 2012). Thus, it could be possible that the *pxm* operon encodes for a methane monooxygenase with

an alternative enzyme kinetic that provide a selective advantage under changing environmental conditions.

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3 Magnetic capture of iCuMMO sequences: A prove of concept

3.1 Abstract

Culture independent methods are good molecular tools to study the distribution and diversity of methanotrophic communities in different environments. Most of these methods are PCR based techniques that rely on sequence specific primers. In environmental studies, when little or no sequence information of the target organisms is available, the generation of appropriate primers is difficult. Here, we adapted the magnetic hybridization capture method (MHC) to detect Cu-containing membrane-bound monooxygenases (CuMMOs) in environments and to avoid the use of specific primers. Exemplarily, a group of sequences clustering into the environmental iCuMMO group FG were chosen to develop and evaluate the MHC. Biotinylated capture probes targeting sequences of the FG group were generated. The probes were used to capture FG sequences out of a complex mix of environmental sequences. First results were successful: FG sequences could be detected and concentrated by MHC. Following improvements of this method could make the MHC a powerful method to study under represented organisms in different habitats.

3.2 Introduction

Aerobic methane oxidation is performed by methane oxidizing bacteria (MOB), a specialized widespread group of organisms that can be found in many terrestrial and aquatic soils. The key enzyme of the aerobic methane oxidizing pathway of most MOB is the particulate methane monooxygenase (pMMO) that belongs to the great family of Cu-containing membrane-bound monooxygenases (CuMMOs). A subunit of the pMMO is encoded by the *pmoA* gene. This gene is highly conserved, and often used as a functional marker gene to identify methanotrophs.

Common tools to detect and study methanotrophic communities in nature are culture independent methods like PCR based techniques. To obtain preferably significant results using these techniques sequence specific primers are crucial. Primer mismatches prevent binding to target sequences and lead to weak or no amplification results. Several primer sets targeting the *pmoA* gene have been established to cover the methanotrophic diversity. The classical primer set is A189f / A682r (Holmes *et al.*, 1995). It has been used frequently in many environmental studies. However, this primerset has disadvantages in specificity. Besides *pmoA* sequences, sequences of the *amoA* of betaproteobacterial ammonium oxidizers and, to a minor degree, other *pxmA* sequences (genes of the environmental intermediate CuMMOs; iCuMMOs) are often co-amplified. Another drawback of this primerset is the poor coverage of sequences of environmental *pxmA* sequences (Bourne *et al.*, 2001). To overcome the problems of specificity and coverage several alternative primers were designed to amplify *pmoA* genes, e.g. mb661 (Costello & Lidstrom, 1999), A650r

(Bourne *et al*., 2001), A621r (Tuomivirta *et al.*, 2009), and nmb650r (Shrestha *et al.*, 2010). The reverse primer mb661 was designed to specifically amplify *pmoA* sequences and exclude *amoA* and *pxmA* sequences. A650r lacks a good coverage of the *pmoA* sequences but covers *pxmA* of environmental groups. A621r mainly amplifies sequences of type II methanotrophs and is especially used when A682r yields no products in amplification of those sequences. Though it was tried frequently to find a universal *pmoA* primer, none is available that covers the *pmoA* as well as the *pxmA* sequences of the iCuMMO groups.

A current example of the importance of the use of specific primers is the detection of the pMMO isoenzyme (pXMO) in MOB (Hainbuch *et al*., in prep., Tavormina *et al.*, 2011). Methanotrophic pure cultures have been studied for a long time (e.g. Bowman *et al.*, 1993, Whittenbury, 1984, Wise *et al.*, 2001). But the pXMO in MOB could be detected not until the reverse primer of the classical primer set was exchanged by an alternative reverse primer. Though the classical primer set A189f/A682r have the potential to amplify *pxmA* sequences, several primer mismatches lead to a dramatically under-representation of those sequences in pure culture and environmental studies. Additionally, A189f/A682r discriminates against *pxmA* sequences that cluster in several environmental iCuMMO groups (RA21, TUSC, ABO-rel, FG). Only few sequences of these groups were published, but the design of new reverse primers moved the iCuMMO groups to the fore. Their sequences are very numerous and widely distributed in nature and of more ecological importance than believed so far.

Culture independent PCR based methodologies are powerful tools to study methanotrophic bacteria in the environments. However, specific primers are essential for the success of PCR dependent methods. Important for the design of specific primers are appropriate template

sequences. In environmental studies, when little or no sequence information of the target organisms is available to design suitable primers, PCR techniques become a great challenge. The environmental iCuMMO groups, for example, are not represented by pure cultures. Additionally, only partial *pxmA* sequences with a length of about 450 bp, derived from next generation sequencing, are available as templates for primer design. This makes the design of specific primers a difficult or insoluble task, especially if the group of target organisms is only represented by few sequences. A method that is independent of specific primers is the magnetic hybridization capture (MHC). This method works in principle in the following way: A biotinylated oligonucleotide hybridization probe is designed that is specific for the target sequence. The probe is incubated with a heterogeneous mix of nucleic acids to allow it to anneal to the corresponding target sequence. The nucleotide mix containing the probe-target hybridization is afterwards incubated with streptavidin-coated magnetic beads. The streptavidin binds to the biotin of the probes. A magnetic force is than aplied to the probe-target-magnetic bead complex which makes it possible to concentrate and separate this complex from non-target DNA by several washing steps. The use of MHC provides several advantages compared to PCR techniques. While it is advantageous for primer design to have long template sequences (for primers border the PCR amplicon), short template sequences are sufficient for capture probe design. The probe binds within the target DNA fragments, which makes it possible to capture fragments ranging in length of a few hundred bases to many megabases in size. Another advantage is that hybridization methods are less prone for sequence mismatches than PCR primers. This makes them very useful for environmental sequence enrichments where only little sequence information is available. A great diversity of sequences could be captured with a single probe.

So far, the MHC technique is mainly constricted to clinical studies and pure culture researches. Here, the method should be applied to environmental researches. As target sequences for MHC a subgroup of the newly found iCuMMO Freshwater Group (FG_N3) was chosen exemplarily. Few sequences with a length of about 450 bp of this group are available. The main focus of this study is to adapt MHC for a routinely use in analyzing bacterial diversity in nature. By studying the iCuMMO Freshwater Group in particular, the aim is to capture long target sequences by MHC. These long sequences could deliver important information for the study of phylogenetic relationships and could give an insight into the gene structure of the iCuMMO operons. Studies by Tarvormina *et al.* (2011) showed that pXMO operons have a gene order, which differs from known pMMO operons. The question, if this gene order is unique for this group, or if alternative operon structures are widespread among the environmental iCuMMO clusters, could be resolved by the use of MHC techniques. In addition, sequence information of the operons of the iCuMMO Freshwater Group (FG_N3) and other environmental groups could help to design new gene specific primers for downstream analyses like qPCR. Several reverse primers have been designed targeting sequences of iCuMMO groups, whereas the forward primer A189f was used for a long time in many studies. This primer seems to match most of the methanotrophic *pmoA* sequences, but we do not know, if this is true for the sequences of the environmental groups. New sequence specific primers based on captured sequences could help to evaluate the use of different primers.

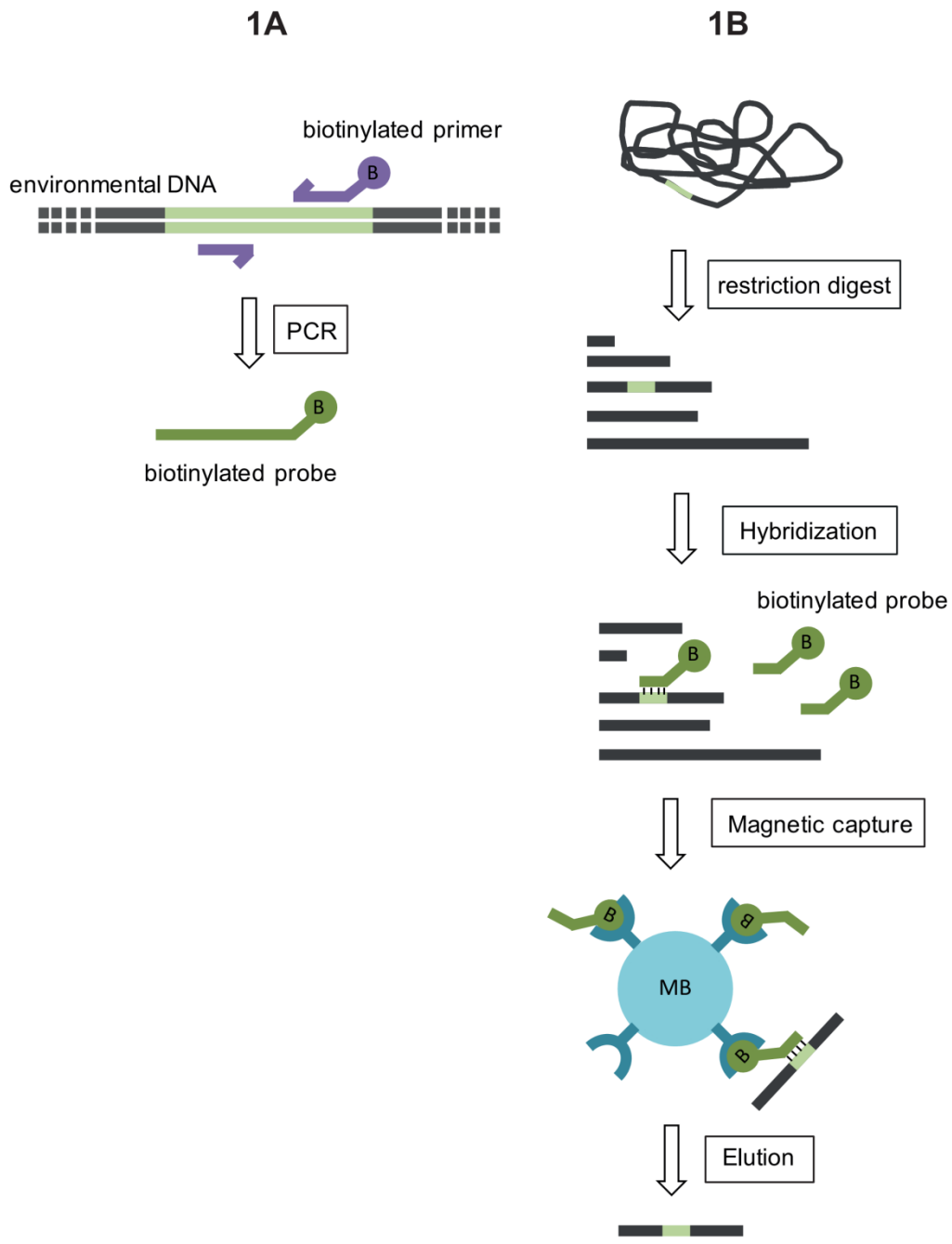

Figure 1: Illustration of the principle of the probe design (1A) and the magnetic capture (1B).

3.3 Material and Methods

Field sites, samples and adapter

Soil samples were collected from Chinese paddy fields near Cixi, province Zhejiang, that have been under permanent agriculture for the past 2000 years (N 30°05.455'; E 121°26.738'). Soil parameters and history have been described elsewhere (Cheng *et al.*, 2009, Ho *et al.*, 2011, Kolbl *et al*., 2014). Total DNA was extracted from freeze-dried soils as described before (Reim *et al.*, 2012). Adapters and their corresponding primers are listed in Table 1. All adaptors were modified to contain a restriction site for the restriction enzymes EcoRI and KpnI.

Table 1: Adapter and corresponding adapter primers. Highlighted in grey: modified adapter restriction sites.

Adapter	Sequence	Reference
GW. Adp_mod	GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGTACCGAATTCATGT	Clontech
SWA-f_mod	CGCAG GCTGGCAGTCTC TTTAGGGTTACACGATTGCTTGGTACCGAATTCATGT	Tsuchiya *et al*., 2009
ADP2_mod	CTAATACG ACTCACTATAGGGCTCGAGCGGCCGGGCAGGTGGTACCGAATTCATGT	Thole *et al.*, 2009
Adapter primer	**Sequence**	**Reference**
GW. Adp_mod-AP1	GTAATACGACTCACTATAGGGC	Clontech
GW. Adp_mod-AP2	ACTATAGGGCACGCGTGGT	Clontech
SWA-f_mod-AP1	CGCAG GCTGGCAGTCTCTTTAG	Tsuchiya *et al*., 2009
SWA-f_mod-AP2	CTCTTTAGGGTTACACGATTGCTT	Tsuchiya *et al*., 2009
ADP2_mod-AP1	GGATCCTAATACGACTCACTATAGGGC	Thole *et al*., 2009
ADP2_mod-AP2	TATAGGGCTCGAGCGGC	Thole *et al*., 2009

Restriction digest and ligation

Extracted environmental DNA (2 µg) and adapter DNA (2 µg) were digested with KpnI or EcoRI (Fermentas, St. Leon-Rot, Germany) at 37 °C for 3h. Digested environmental DNA was purified afterwards using the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Digested adapter DNA was purified with Post-Reaction Clean-Up Spin Columns (Sigma-Aldrich, Munich, Germany) following the manufacturer's protocol.

Ligation of digested environmental DNA and adapter DNA was performed using T4 DNA Ligase (Promega, Madison, Wisc., USA). 500ng environmental DNA and 250 ng adapter DNA were mixed with 5 U T4 DNA Ligase and 5 µL Ligase 10x Buffer, and filled up to 50 µL with molecular grade water (Sigma). Ligation was carried out at 4 °C over night.

Probe design

Primers to amplify the magnetic capture probe were designed based on an alignment of 35 sequences clustering in the subgroup 3 of the Freshwater Group (FG_N3) (Hainbuch *et al*. in revision). PCR reactions with newly designed primers FG_new3for (5'-GTGGCGATTTCCTACTGGAGC-3') or FG_new3for_biot (5'-GTGGCGATTTCCTACTGGAGC-3' containing 5': Biotin-TEG) and FG_new3rev (5'-TGTCCAGTCGAGGATGATCCC-3') were performed. 200ng template DNA (China 2000) was mixed with 0.8 U of Taq Polymerase (Invitrogen, Darmstadt, Germany), 5 µl 10X PCR buffer without Mg^{2+} (Invitrogen, Darmstadt, Germany), 2 µL MgCl (50mM) (Invitrogen, Darmstadt, Germany), 1.5 µL dNTP mix (10mM each), 0.02 mg bovine serum albumine (Roche), 5% (v/v) DMSO, 30 pmol of each

primer and molecular-grade water (Sigma- Aldrich, Munich, Germany) in a total volume of 50 µl. The PCR program started with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturizing (30 sec at 94°C), annealing (30 sec at 54 °C) and elongation (30 sec at 72 °C). A final elongation step was carried out at 72 °C for 10 min. PCR products were analyzed by 1 % agarose gel electrophoresis, visualized with GelRed® Nucleic Acid Stain (Biotium, Hayward, CA, USA) and purified via gel extraction using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Cloning and sequencing of the capture probe was performed as described before (Chapter 2).

Hybridization and magnetic capture

500 ng environmental DNA or adaptor ligated environmental DNA and 500 ng biotinylated probe were incubated for 5 min at 95°C and mixed afterwards with a preheated (65°C) hybridization mixture containing 0.3 µL 10% SDS, 0.6 µL Denhard's reagent (50x) (Sigma-Aldrich), 8.4 µL SSC 20x (Invitrogen, Darmstadt, Germany) and molecular-grade water (Sigma- Aldrich, Munich, Germany) to a final volume of 25 µL. The hybridization mix was incubated for 24 h at 65 °C shaking.

For magnetic capture 5 µL of resuspended Dynabeads® M-280 Streptavidin (Dynabeads® kilobase BINDER™ kit, Invitrogen Darmstadt, Germany) were transferred into a 1.5 mL tube an placed on a magnet (MagneSphere® Technology Magnetic Separation Stand, Promega, Madison, Wisc., USA). After removing the supernatant, Dynabeads were washed with 25 µL Binding solution und finally resuspended in 25 µL Binding solution (Dynabeads® kilobase BINDER™ kit, Invitrogen Darmstadt, Germany). The hybridization mix (room temperature)

was carefully mixed with the Dynabeads and incubated at room temperature for 3 h on an overhead shaker. The hybridization/Dynabead mix was washed twice with 50 µL washing solution (Dynabeads® kilobase BINDER™ kit, Invitrogen Darmstadt, Germany), once with molecular-grade water (Sigma- Aldrich, Munich, Germany) and finally resuspended in 25 µL molecular-grade water (Sigma- Aldrich, Munich, Germany). To dissolve the environmental DNA from the captures biotinylated probes, the hybridization/Dynabead mix was denaturized for 3 min at 96°C and placed on the magnet. The supernatant containing the environmental DNA was collected.

The supernatant was analyzed by PCR with the primer pair A189f (Holmes *et al.,* 1995) and RA21r (Chapter 2). The reverse primer RA21r was chosen because sequences of the iCuMMO Freshwater Group (FG_N3) were amplified with this reverse primer in an earlier next generation sequencing analysis (Hainbuch *et al*., in revision). The PCR mastermix, PCR, cloning and sequencing were performed as described before (Chapter: probe design).

Amplification strategies

Whole genome amplification was performed using the REPLI-g® Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Adapter PCR was performed as described before (Chapter: probe design) using the adapter primer (Table 1) or combinations of adaptor primer and the gene specific primers A189f, A682r (Holmes *et al*., 1995), RA21r (Chapter 2), FG_new3for and FG_new3rev (this study). Additional PCR reactions were performed with the LongRange PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

3.4 Results

Capture probe design

Biotinylated probes were designed to target the subgroup FG_N3 of the recently discovered Freshwater Group (FG). Capture probes should be specific for the FG_N3 group and should feature a good coverage. Therefore, the probes were generated by PCR amplification with FG_N3 group specific primers. Rice field soil DNA (China 2000) was used as a template DNA, for sequences of the FG_N3 group were found in this environment in an earlier study (Hainbuch *et al*., in revision). The specific primers were designed on the basis of available FG_N3 sequences (Hainbuch *et al*., in revision). The PCR amplification to generate the probes resulted in 157 bp sequences. Those were cloned, sequenced and phylogenetically analyzed. Probe sequences of two suitable clones (Probe_FGN3_1 and Probe_FGN3_2) clustered within the FG_N3 group (Figure 2, 3). Both were very similar and were used to generate the biotinylated probe by PCR with the FG_N3 group specific primers with a biotin labeled forward primer.

Figure 2: Alignment of probe sequences Probe_FGN3_1 and Probe_FGN3_2 (black) with sequences of the Freshwater Group subgroup FG_N3 (blue). Nucleotide position refers to the forward primer A189.

Magnetic Capture

The aim of the MHC in this study is to capture FG_N3 sequences out of complex mix of environmental sequences. As basic material for MHC environmental DNA (China2000) was chosen and was restricted with the enzymes EcoRI or KpnI to get fragments of a suitable length for capture. Both enzymes belong to the class of restriction enzymes that possess a 6 base pair recognition site. The occurrence of restriction sites in genomes can be calculated by the mathematical formula $[1/4^{(N)}]$, whit N = number of nucleotides present in the recognition site (Ji & Braam, 2010). Theoretically, EcoRI and KpnI should cleave genomes every 4096 base pairs. Preferable, the chosen restriction enzymes should not have a recognition site within the *pxmA* sequence and the operon of the iCuMMO FG_N3 groups. However, it is not possible to predict the cleavage of the restriction enzymes within the iCuMMO Freshwater Group (FG_N3) sequences. But to get an insight into the distribution of the cleavage sites within iCuMMO sequences several pMMO operons, including the operons of *Methylomicrobium japanense* (AB253367), *Methylosinus trichos*porium (MTU31650),

Methylocaldum sp. T-025 (AB275418), *Methylococcus capsulatus* Bath (AE017282) and *Methylocystis strain* SC2 (BX649604), were analyzed as a substitute for iCuMMO operons. Though gene sequences of the pMMO and iCuMMO operons are only distantly related the pMMOs may give an insight into the distribution of the restriction sites of the chosen restriction enzymes. None pMMO operon contained one of the restriction sites, so the enzymes EcoRI or KpnI were chosen for this study.

After cleavage, DNA fragments were hybridized with the biotinylated probe and captured with magnetic beads (Figure 1). The captured fragments were eluated and analyzed by PCR with primer pair A189 /RA21r for sequences clustering into the Freshwater Group FG_N3. Amplicons of the test PCR were cloned and sequenced. In total eight clones were analyzed. One sequence could not be related to any iCuMMO group. Six sequences clustered within the M84_P105 group that is related to the Freshwater Group. One sequence clustered within the FG_N3 group (Figure 3).

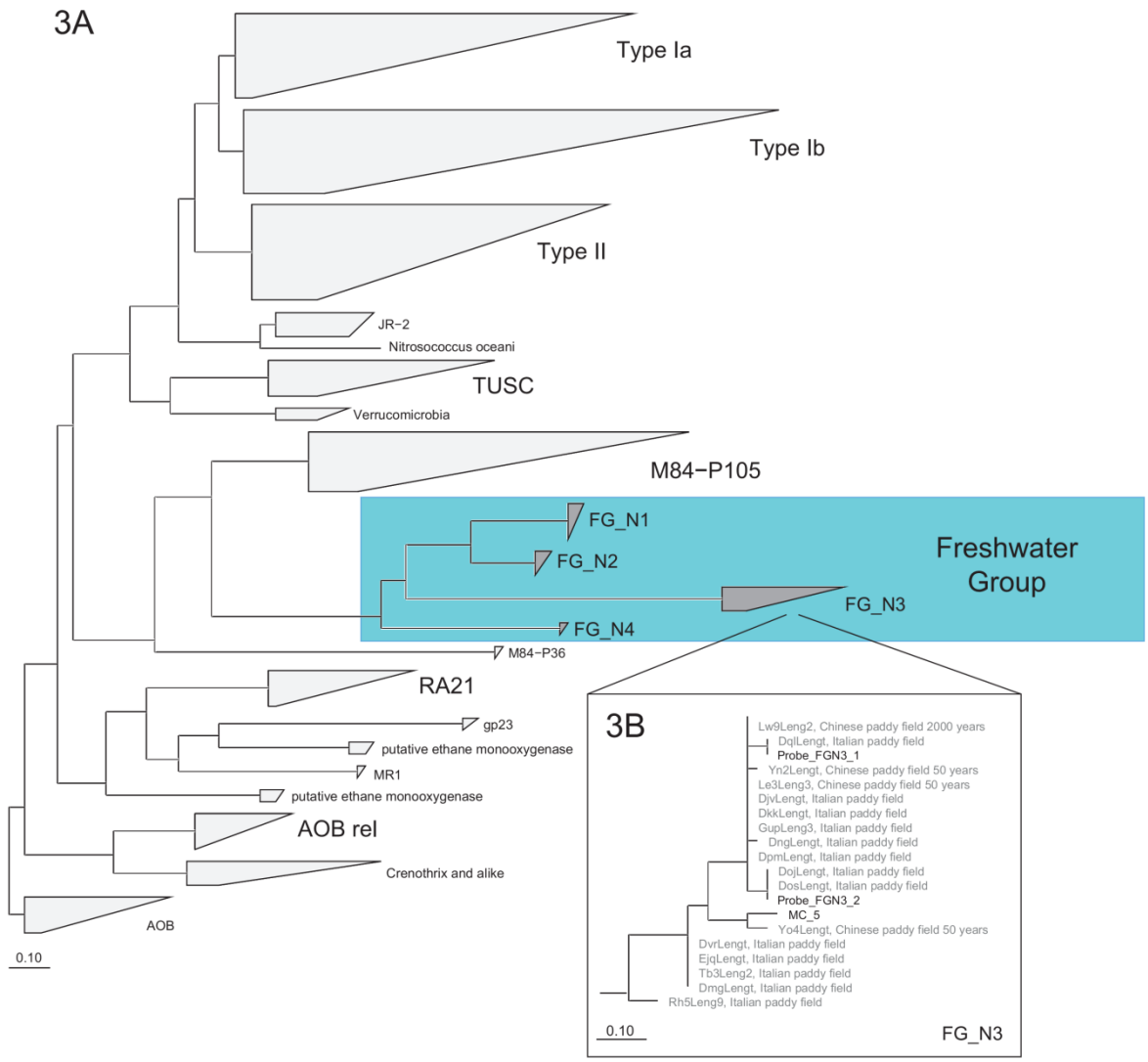

Figure 3: Phylogeny of Freshwater Group sequences. Neighbor joining tree of partial *pmoA and pxmA* sequences, based on 135 deduced amino acids. 3A: The tree combines sequences retrieved by a prior pyrosequencing analysis with *pmoA* and *pxmA* sequences from public databases. The Freshwater Group is highlighted in blue. 3B: Close up of the Freshwater subgroup FG_N3 with sequences of the capture probes Probe_FGN3_1 and Probe_FGN3_2 and the sequence retrieved from magnetic capture MC_5.

Amplification strategies

The sequencing of the first MHC sample showed that the method of the magnetic capture is generally working and that sequences of the FG_N3 group were among the captured sequences. Hence, the method is suitable for the use in environmental studies. However, the nucleic concentrations of the captured and eluated fragment after MHC (MHC output) is relatively low (about 5ng /µL). For downstream analysis like next generation sequencing these concentration are not sufficient. Hence, two methods were tested to increase the DNA concentration of the MHC output.

Multiple displacement amplification (MDA)

MDA plays an important role in genomic research, when the amount of DNA is limited. ϕ29 DNA polymerase and random primers are used to amplify micrograms of DNA from samples that contain only very little starting material (down to 1ng). Here, we used MDA to amplify the MHC output. In principle, the amplification worked. MDAs resulted in samples that contained 350 ng/µL DNA (total amount: 17.5 ng). Fragment size ranged from about 1 kb to > 10kb. The DNA amplified by the ϕ29 DNA polymerase is single stranded (ssDNA). For further analyses by next generation sequencing a second strand synthesis of the ssDNA to double stranded DNA (dsDNA) needed to be performed. Here, this synthesis by further PCR amplification with random hexamers failed.

Adaptor Ligation-Based PCR

Generally, this method includes the digest of an adapter and an uncharacterized DNA sequence by restriction enzymes, the ligation of the adaptor and the uncharacterized DNA,

and the PCR amplification by adaptor specific primers. Here, three adaptors with their corresponding adaptor primers were tested. After ligation, PCR reactions with different primer-combinations were tested for optimal amplification results of the uncharacterized DNA (MHC output): (1) forward and reverse adaptor primer, (2) a combination of adaptor primer and gene specific primer and (3) gene specific primers. Amplicons could only be achieved from setups with specific primers. PCR reactions with adaptor primer or a combination of adaptor primer and specific primer failed.

3.5 Discussion

Magnetic beads and target specific oligonucleotide capture probes were used to isolate and purify nucleic acids in several studies (Lund *et al.*, 1988, Mangiapan *et al.*, 1996, Millar *et al.*, 1995, Muir *et al.*, 1993, Olsvik *et al.*, 1994, Parham *et al.*, 2007, Rodriguez *et al.*, 2012, Gnirke *et al.*, 2009). Although the use of this techniques is mainly constricted to clinical studies and pure culture researches so far, the methods hold a great potential for environmental studies with their complex metagenomes, low copy number sequences, and the presence of possible inhibitors that impact downstream analyses (Carpenter *et al.*, 2013, Jacobsen, 1995).

To prove the concept of magnetic hybridization capture (MHC) a group of sequences (FG_N3) of a prior deep sequencing analysis (Hainbuch *et al*. in revision) was chosen. Those sequences were co-amplified in the previous study by the primer pair A189/RA21r. The percentage of amplified FG_N3 sequences in previous study was 0.4%. FG_N3 sequences

were exemplarily determined as target DNA for MHC. The aim was to enrich the target DNA and to capture preferably long target sequences for operon analyses. First MHC tests were successful. The analysis of the MHC output showed an increase of the fraction of FG_N3 sequences from 0.4 % (precious study) to 12.5 % after MHC. These first results showed that the target enrichment worked effective. We were able to capture and enrich the target sequences out of a complex mixture of environmental sequences without the use of PCR and gene specific primers.

MHC may provide lots of advantages, but some technical challenges need to be solved before this method can be used routinely in environmental studies. Beside some minor challenges like probe specificity that could be improved for example by the use of RNA probes, the major inconvenience of MHC is the low MHC output concentration. The nucleic acid concentrations after MHC range from 3-5 µg/µL. A PCR amplification step of the MHC output is unavoidable to provide a sufficient amount of DNA for NGS methods. Multiple displacement methods (MDM) seem to be a good choice. The advantage of MDMs is the little amount of starting material that is required and the ability of the ϕ29 DNA polymerase to amplify long sequences (Alsmadi *et al.*, 2009). However, a disadvantage of this method is the production of ssDNA. In this study, the second strand synthesis required for further downstream analysis failed. A problem might have been that MDM sequences were too long or branched for a proper second strand synthesis. An alternative for MDMs could be ligation mediated PCR methods (LM-PCR). LM-PCR include PCR methods like inverse PCR (Ochman *et al.*, 1988, Tsaftaris *et al.*, 2010), vectorette PCR (Arnold & Hodgson, 1991, Hengen, 1995) and cassette PCR (Isegawa *et al.*, 1992, Kilstrup & Kristiansen, 2000, Padegimas & Reichert, 1998,

Rishi *et al.*, 2004, Siebert *et al.*, 1995) that are commonly used for genome walking. These methods have in common that uncharacterized DNA sequences can be detected by the use of flanking DNA with known sequences (e.g. adaptors). Here, we choose an adaptor PCR method that includes the digestion of environmental and adaptor DNA by restriction enzymes, the ligation of both, and the amplification of the ligated product with adaptor. Unfortunately, no convincing results could be obtained by the following adaptor PCR, so far.

A critical step in LM-PCR methods is the choice of proper restriction enzymes for DNA cleavage. Though the cleavage frequency can be calculated theoretically, the actual fragment sizes of the digested environmental DNA cannot be predicted, when sequence information of the target organisms are unavailable. An inappropriate restriction enzyme could, for example, lead to fragments that are too long for downstream analysis like PCR amplification. So, a focus in the optimizing of the LM-PCR should be put on the selecting of proper restriction enzymes. Since no operon or genome information are available for the environmental iCuMMO groups, genomes of characterized MOB and ammonium oxidizing bacteria could be used to evaluate different restriction enzymes, alternatively. Whole genome DNA of pure cultures should be digested to predict the fragment sizes after digestion. Restriction fragments should range in the size of about 3 kb to allow the following amplification by long range PCR. Fragments of contemplable enzymes should afterwards be analyzed for the occurrence of target sequences like *pmoA, pxmA* and *amoA* sequences. This evaluation could help to choose a restriction enzyme for the MHC of environmental iCuMMO sequences, but a final assessment could only be made when the restriction enzyme is directly used in LM-PCR with environmental DNA. Another critical point that appears

during MHC is the capture of unspecific DNA fragment due to universal adaptors. LM-PCR adaptors are ligated to environmental DNA before MHC. During enrichment by MHC, adaptor sequences can hybridize to each other, which may lead to long fragment chains and unspecific capture of nontarget DNA fragments. This could be avoided by the use of blocking oligonucleotides, whose sequences correspond to the sequence of the respective adaptor. Other common problems of LM-PCR methods are low ligation efficiencies and primer mispriming, which lead to low specificity and efficiency. Several improved protocols were developed to overcome these problems (Padegimas & Reichert, 1998, Thanh *et al.*, 2012).

Though the MHC and the following LM-PCR need to be improved to obtain optimal results for downstream analyses like NGS, it could be shown that these techniques are suitable for environmental researches. MHC can provide totally new insights into the gene sequences and operon structures of the iCuMMO groups and may help in general to get deeper insights into the extremely rich world of yet uncultivated organisms.

3.6 References

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4 Monooxygenases involved in the degradation of short chained gaseous hydrocarbons in a rice field soil

4.1 Abstract

The great enzyme family of bacterial monooxygenases contains several enzyme systems able to initiate the degradation of short chained alkanes by oxidation. Members of this family are the Cu-containing membrane bound monooxygenases (CuMMOs). They are known oxidize methane (pMMO), ammonium (AMO) and short chained alkanes (BMO). The group of the environmental intermediate CuMMOs (iCuMMO) seems to be of great importance in nature, too. Due to a sequences relationship of the (iCuMMO) to potential alkane degraders it is speculated that they could be also involved in alkane oxidation. Another enzyme system, the alkB hydroxylase, is often present in heterotrophic bacteria and is involved in the alkane degradation in many environments. Here, we analyzed the distribution and activity of monooxygenases in rice field soils incubated with short chained alkanes (methane, ethane, propane and butane). The bacterial community structure at several time points of the incubation was analyzed by T-RFLP fingerprinting and deep sequencing analysis. We observed that type II methanotrophs are the predominat member of the methanotrophic community in methane, ethane and propane long time incubations and that they are active, transcribing the *pmoA* and *pmoA2*. The iCuMMO of the groups RA21, AOB_rel and M84_P105 seems to play no role in the degradation of alkanes in rice field soils. Bacterial strains possessing an AlkB homologue contributed to a great part of the bacterial community

in ethane, propane and butane incubation. But no transcription of the alkB could be shown leading to the suggestion that the alk system is not involved in alkane degradation in rice fields too.

4.2 Introduction

Alkanes and alkenes are exclusively formed by carbon and hydrogen atoms. Small hydrocarbons up to a length of four carbon atoms are gaseous at ambient temperatures. Though these apolar molecules are very inert and need much energy to be activated, many organisms metabolize alkanes and alkenes (Labinger & Bercaw, 2002, Rojo, 2009, van Beilen & Funhoff, 2007). Significant sources of short chained hydrocarbons are seeps and vents from natural gas and oil deposits. Hydrocarbons from these sources contain methane (70-99%), 1-10% ethane and higher gaseous hydrocarbons (Cooley *et al*., 2009, Shennan, 2006). The major part of the short chained alkanes and alkenes is created by geochemical processes. But prokaryotes, marine algae, insects and plants also produce hydrocarbons in most soil and water environments (Cooley *et al*., 2009, Giebler *et al*., 2013). They are produced as moisture barriers, as storage materials and pheromones (Nie *et al*., 2014, van Beilen & Funhoff, 2007). Anaerobic decomposition in soils, sediments, sewage sludge and anaerobic digesters result in gases consisting of methane (50-60%), CO_2 (40%) and up to 1% of non-methane volatile organic compounds (also containing hydrocarbons) (Shennan, 2006, Tassi *et al*., 2009).

Short chained alkanes and alkenes are metabolized by a variety of microorganisms. The smallest hydrocarbon, methane, is utilized by the methane oxidizing bacteria (MOB) as a sole

carbon and energy source. Some of the MOB can additionally use multicarbon compounds such as acetate, pyruvate, succinate, malate and ethanol (Semrau *et al*., 2011, Trotsenko & Murrell, 2008, Belova *et al*., 2011, Dedysh *et al*., 2005, Theisen *et al*., 2005). The key enzyme of the methane metabolism is the methane monooxygenase (MMO) that initializes the aerobe methane oxidation (Frenzel, 2000, Conrad, 2009). Two forms of the MMO can be distinguished in methane oxidizing bacteria (MOB): the particulate methane monooxygenase (pMMO), present in most MOB, and the soluble methane monooxygenase (sMMO). Both enzymes can catalyze the oxidation, respectively, the co oxidation of several alkanes in addition to methane.

Other short chained gaseous alkanes (ethane, propane and butane) and alkenes (ethylene, propylene and butylene) are used by many different types of microbes including bacteria, yeast and fungi (van Beilen *et al*., 2003, Shennan, 2006). Most of the bacterial strains that are able to oxidize hydrocarbons are heterotrophic bacteria (Harayama *et al*., 2004, Margesin *et al*., 2003, Rojo, 2009). The predominat group of typical soil bacteria that can grow on hydrocarbons is the so called CMNR group; Gram-positive bacteria belonging to the genera *Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Rhodococcus*. In enrichment experiments with gaseous alkanes, most isolates belong to members of the CMNR groups. They may be key-players in alkane degradation (Shennan, 2006, Hamamura *et al*., 2001). Gram-negative representatives of alkane degraders belong to the genera *Pseudomonas*, *Acinetobacter*, *Alcaligenes* and *Burkholderia* (Shennan, 2006). Beside the heterotrophic bacteria, that prefer other grows substrates to alkanes, some bacteria seem to be highly

specialized to grow on hydrocarbons (e.g. *Alcanivorax*, *Thalassolituus*) (Rojo, 2009, Sabirova *et al*., 2006, Brakstad & Lodeng, 2005).

Many alkane degrading bacteria contain multiple alkane hydroxylases with overlapping substrate ranges (Kotani *et al*., 2003, Sabirova *et al*., 2006, van Beilen & Funhoff, 2007, van Beilen *et al*., 2003). Several enzyme systems are known to be involved in short chained hydrocarbon oxidation. One family of alkane hydrocarbon oxidizing enzymes are the soluble di-iron and membrane bound copper containing monooxygenases. They are phylogenetically related to the sMMO and pMMO of methanotrophic bacteria. Pseudomonas butanovora possess a non-heme butane monooxygenase (BMO) similar to the sMMO that oxidizes C_2 to C_9 alkanes (Doughty *et al*., 2006). A propane monooxygenase (PMO) similar to sMMO was found in Gordonia sp. TY5 (Kotani *et al*., 2003). Copper containing monooxygenases similar to pMMO were found in *Nocardioides* CF8 and *Mycobacterium* (Coleman *et al*., 2011, Sayavedra-Soto *et al*., 2011). The integral membrane bound non-heme di-iron alkane hydroxylase (alk system) is another member of the alkane oxidizing enzymes (van Beilen & Funhoff, 2007). Most membrane bound di-iron alkane hydroxylases oxidize alkanes longer than C_{10}. However, it was shown that AlkB in Pseudomonas putida GPo1 oxidizes the short chained alkanes propane and butane (Johnson & Hyman, 2006). Other alkane oxidation systems like Cytochrome P450 alkane hydroxylases, Cu^{2+}-dependent alkane hydroxylases and flavin-binding monooxygenases are known but they are specialized for oxidation of long-chained alkanes and do not play a role in the oxidation of gaseous alkanes (van Beilen *et al*., 2005, Sekine *et al.*, 2006, Funhoff *et al.*, 2006, Schmitz *et al.*, 2000, Lida *et al.*, 2000, Tani *et al.*, 2001, Throne-Holst *et al.*, 2007, Feng *et al.*, 2007).

In rice field soils and aquatic habitats many sequences were recently retrieved from only distantly related pMMO sequences (Hainbuch *et al*., in revision). They cluster together with previously found sequences from various environments forming groups (iCuMMOs) without pure culture representatives that are phylogenetically located between *pmoA* and *amoA* sequences (Angel & Conrad, 2009, Holmes *et al.*, 1999, Jia *et al.*, 2007, Knief *et al.*, 2005, Dorr *et al.*, 2010, King & Nanba, 2008, Pacheco-Oliver *et al.*, 2002, Reay *et al.*, 2001). Substrates and physiological functions of the corresponding enzymes could not be resolved until now. However, a phylogenetic relationship of these sequences to potential ethane degraders suggests short chained hydrocarbons as potential substrates (Redmond *et al.*, 2010). Hence we hypothesize that iCuMMOs might play a role in the degradation of small hydrocarbons in nature. Here, we incubated Chinese rice field soil slurries with short chained gaseous alkanes and alkenes (methane, ethane, ethylene, propane, propylene, butane and butylenes). The substrate consumption was followed by Gas Chromatography (GC) analysis with time. The structure of the microbial communities was analyzed at various time points during incubation on DNA and mRNA level by T-RFLP and deep sequencing with primers targeting the *pmoA*, *pxmA*, *alkB* and 16S rRNA gene.

4.3 Material and Methods

Field sites and Incubation

Soil samples were collected from Chinese paddy fields near Cixi, province Zhejiang, that have been under permanent agriculture for the past 2000 years (N 30°05.455'; E 121°26.738'). Soil parameters and history have been described elsewhere (Cheng *et al.*, 2009, Ho *et al.*, 2011, Kolbl *et al*., 2014).

Soil slurries were prepared by mixing 10 mL of dematerialized water with 2g of freeze dried rice field soil. They were incubated with air and 10 % of alkanes (methane, ethane, propane or butane) or alkenes (ethylene, propylene or butylene). A first experimental setup was incubated at 25°C, a second setup at 20°C, 25°C or 37°C and 200 rpm in the dark. Alkane, alkene and oxygen consumption was followed by gas chromatography. The soil slurries were regularly flushed with fresh air to replenish oxygen. Afterwards alkanes and alkenes were reestablished by injection. To gain an overview of the hydrocarbon degradation the hydrocarbon consumption was illustrated in an overview graph. Hydrocarbon consumption was calculated in µM over time (Figure 1 and 2). The slurries were incubated for 17 and 41 days (methane incubation), 129, 168, 223, 262 and 348 days (ethane and propane incubation) and 168, 223, 313 and 348 days (butane incubation). The slurries incubated with the alkenes ethylene, propylene and butylenes were incubated for 337 days. After incubation aliquots of 2 mL soil slurry were shock frozen in liquid nitrogen and stored for further analysis at – 80 °C.

Nucleic acid extraction

Total DNA and RNA were extracted from thawed soil slurry samples as described elsewhere (Hainbuch *et al*., in revision). RNA was prepared using the RQ1 RNase-free DNase (Promega, Madison, Wisc., USA) followed by purification with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

T-RFLP Analyses

The *pmoA* genes were amplified using the primers A189f (5'- GGN GAC TGG GAC TTC TGG -3') and A682r (5'- GAA SGC NGA GAA GAA SGC -3') (Holmes *et al.*, 1995). For *pxmA* sequence amplification forward primer A189f and reverse primers M84_P105r (5'-GCG GAT GTA TTG RAA NCC-3'), AOB_relr (5'- GAT GAT NCG GAT RTA YTC-3') or RA21r (5'-GAT GAT NCG CAR ATA TTC-3') (Hainbuch *et al*., in revision) were used. *alkB* genes were amplified using the primers AlkBf (5'-AAY CAN GCN CAY GAR CTN GGN CAY AA -3') and AlkBr (5'- GCR TGR TGR TCN GAR TGN CGY TG -3') (Kloos *et al.*, 2006). The 16S rRNA primers used in this study were 343Fmod (5'- TAC GGG WGG CWG CA -3') and 748Rmod (5'- GGG TMT CTA ATC CBK TT -3') (Kohler *et al.*, 2012).

For T-RFLP analyses all forward primers were FAM-labeled. PCR amplifications at different time points of the alkane incubation with primers targeting *pmoA*, *pxmA*, *alkB* and 16S rRNA genes were performed as described before (Lüke *et al.*, 2014). For T-RFLP analyses of mRNA samples cDNA was generated using the SuperScript® III Reverse Transcriptase (Invitrogen, Darmstadt, Germany). Two µL of purified total RNA was mixed with 0.5 pmol random hexamers (Invitrogen), 10 pmol of each dNTP (Promega) and molecular-grade water (Sigma-Aldrich, Munich, Germany) in a total volume of 11.5 µL. The mix was denaturized for 5 min

at 60°C and hold at 25°C afterwards. 4 µL of the first strand buffer, 0.1 µmol DTT, 0.01 mg bovine serum albumin (Roche), 1 U SUPERase In™ RNase Inhibitor (Ambion, Austin, Tex., USA), 200 U SuperScript ™ III RT and molecular-grade water (Sigma-Aldrich) in a total volume of 7.5 µL were mixed to the RNA mix. The first strand synthesis was performed at 50°C for 60 min. The reaction was inactivated at 70°C for 15 min. The following PCR reaction was performed as described above. PCR products were analyzed on a 1% agarose gel and extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen).

The *pmoA*, *pxmA* and *alkB* PCR products (100 ng) were digested with the FastDigest MspI enzyme (Fermentas, St. Leon-Rot, Germany) at 37°C for 5 min. The bacterial 16S rRNA gene PCR product was digested with FastDigest Cfr13I (Fermentas, St. Leon-Rot, Germany) at 37°C for 5 min. After digestion the samples were purified with Post-Reaction Clean-Up Spin Columns (Sigma-Aldrich) according to the manufacturer's instructions. Two µL of the samples were mixed with 11 µL Hi-Di Formamide (Applied Biosystems, Foster City, Calif., USA) and 0.2 µl of an internal DNA fragment length standard (MapMarker 1000, 50−1,000 bp, x-rhodamine, Eurogentec, Ougree, Belgium). After denaturation for 2 min at 94°C the restriction fragments of the samples were separated and detected with capillary electrophoresis and an automatic sequencer 3130 Genetic Analyzer, Applied Biosystems; 30 min at 15 kV and 9 µA) and the t-RF fragments analyzed with GeneMapper Version 4.0 (Applied Biosystems).

Deep Sequencing Analysis

For 454 amplicon sequencing the primers A189f, A682r, M84_P105r, RA21r, AOB_relr, AlkBf, AlkBr, 343Fmod and 748Rmod were modified and the PCR was performed as described

before (Lüke *et al*., 2014). The sequencing was performed at GATC (Konstanz, Germany). The pyrosequencing data of *pmoA* and *pxmA* samples were evaluated with the ARB software package (Ludwig *et al.*, 2004). Sequences containing insertion or deletion resulting in a shift of the reading frame were excluded manually. For further phylogenetic analysis only sequences with a read length of at least 130 amino acids were used. Pyrosequencing data of the 16S rRNA gene samples were aligned using the online SINA alignment service (Pruesse *et al.*, 2012) and were further analyzed by sequence analyses with the ARB software using the SILVA 108 database, release September 2011(Quast *et al.*, 2013). *alkB* sequences were analyzed using BLAST and MEGAN (Altschul *et al*., 1990, Huson *et al*., 2011).

4.4 Results

Alkane and alkene Uptake

Methane consumption started after 6 days of incubation and increased rapidly in all replicates. Altogether, about 690 µM of methane were consumed within 17 days, 2200 µM after 41 days (Figure 1A). Ethane consumption started after a lag phase of 35 to 160 days (Figure 1B). 410 µM of ethane was consumed after 130 days of incubation, 940 and 1970 µM after 348 days. Propane oxidation started after 90 to 179 days (Figure 1C). 500 to 650 µM of propane were consumed after 131 to 348 days. The longest lag phase could be detected for butane (Figure 1D). After 200 days the butane consumption started. In total, about 170 to 360 µM was consumed after 348 days. At the beginning of the alkane and alkene incubation oxygen was consumed rapidly without a detectable decrease of the alkane and alkene

concentrations in all samples. The oxygen concentrations decreased to 5 – 10 % after 25 days and down to 2-5 % after 80 days of incubation. During this time alkane oxidation already started in several samples but stopped again due to limited oxygen concentrations. Hence, after 80 days soil slurries were flushed with fresh air. The oxygen concentrations were monitored afterwards. When oxygen was available all alkanes were consumed continuously. Ethane and propane were oxidized relatively rapid after the lag phase, while butane seemed to be oxidized slower. No alkene consumption could be detected within 348 days of incubation.

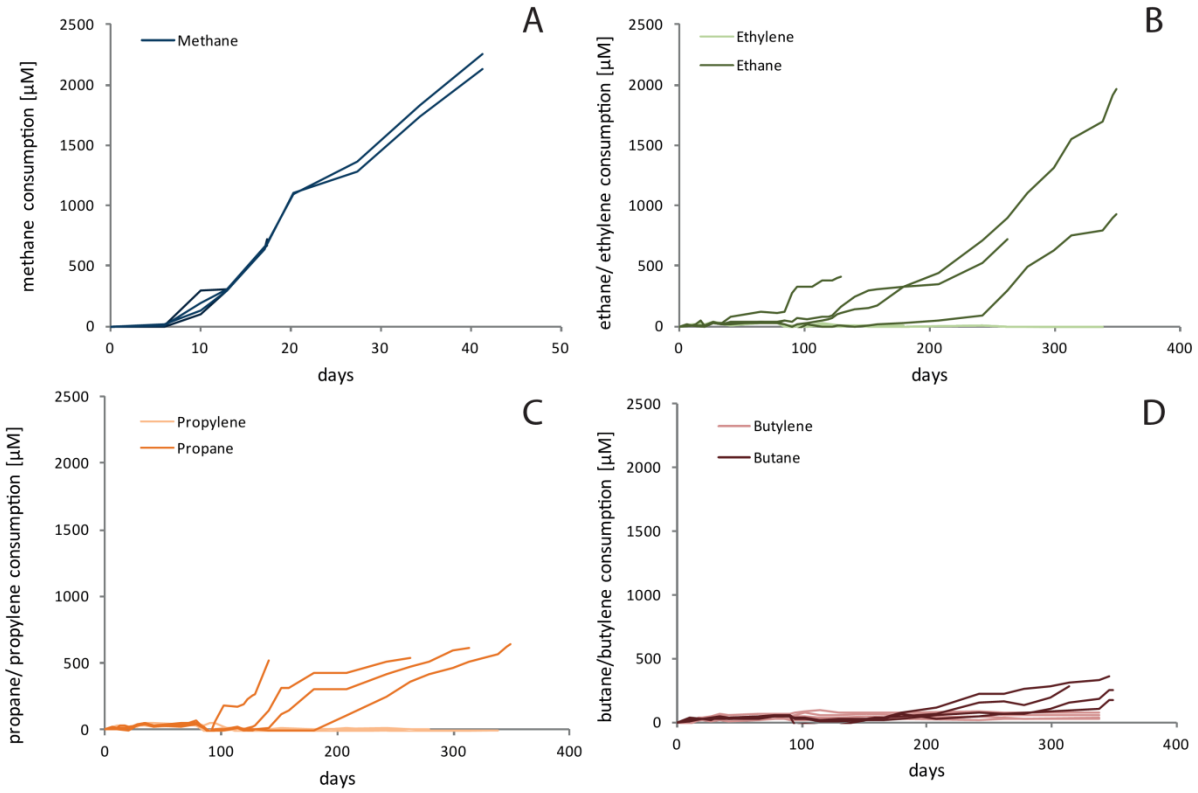

Figure 1: Alkane and alkene consumption over incubation time. A: methane incubations (17 and 41 days). B: ethane (129, 262, 348 days) and ethylene (348 days) incubation. C: propane (129, 262, 313 and 348 days) and propylene (348 days) incubation. D: butane (313, 348 days) and butylene (348 days) incubation

In a second experiment the effect of different temperatures on oxidation of ethane, propane, and butane, respectively, was tested (Figure: 2). At 25°C, ethane consumption started immediately in three out of four replicates. Propane consumption started after 50 to 75 days and butane consumption after 50 to 116 days of incubation. At 30°C, ethane and propane consumption started after 50 to 150 days, butane consumption after 116 to 154 days. At 37°C ethane oxidation started after a lag phase of 50 days in three replicates. No oxidation could be detected in propane and butane incubations at 37°C.

To compare the average consumption of the alkanes at different temperatures the time point 154 days was chosen. This time point lies at the end of the incubation experiment and includes measurements of all 4 replicates. In average 219.9 µM ethane, 294.0 µM propane and 312.6 µM butane were consumed after 154 days at 25 °C. Compared to the first incubation experiment (Figure 1) the average consumption of all alkanes lie approximately in the same range. Differences between the first and the second set up might be due to the oxygen limitation in the first experiment. In the second set up the oxygen concentration was monitored during the entire incubation. The average ethane consumption after 154 days at 30°C was 626.5 µM. The consumption nearly tripled compared to the incubation at 25°C. Propane consumptions after 154 days were nearly similar at 25°C (294.0 µM) and 30°C (275.5 µM). In contrast the consumption of butane was much lower at 30°C (64.5 µM) compared to the incubation at 25°C. At 37°C only ethane was oxidized. The total consumption was 286.5 µM, which lies in the range of ethane that was consumed at 25°C. No propane or butane was oxidized at 37°C.

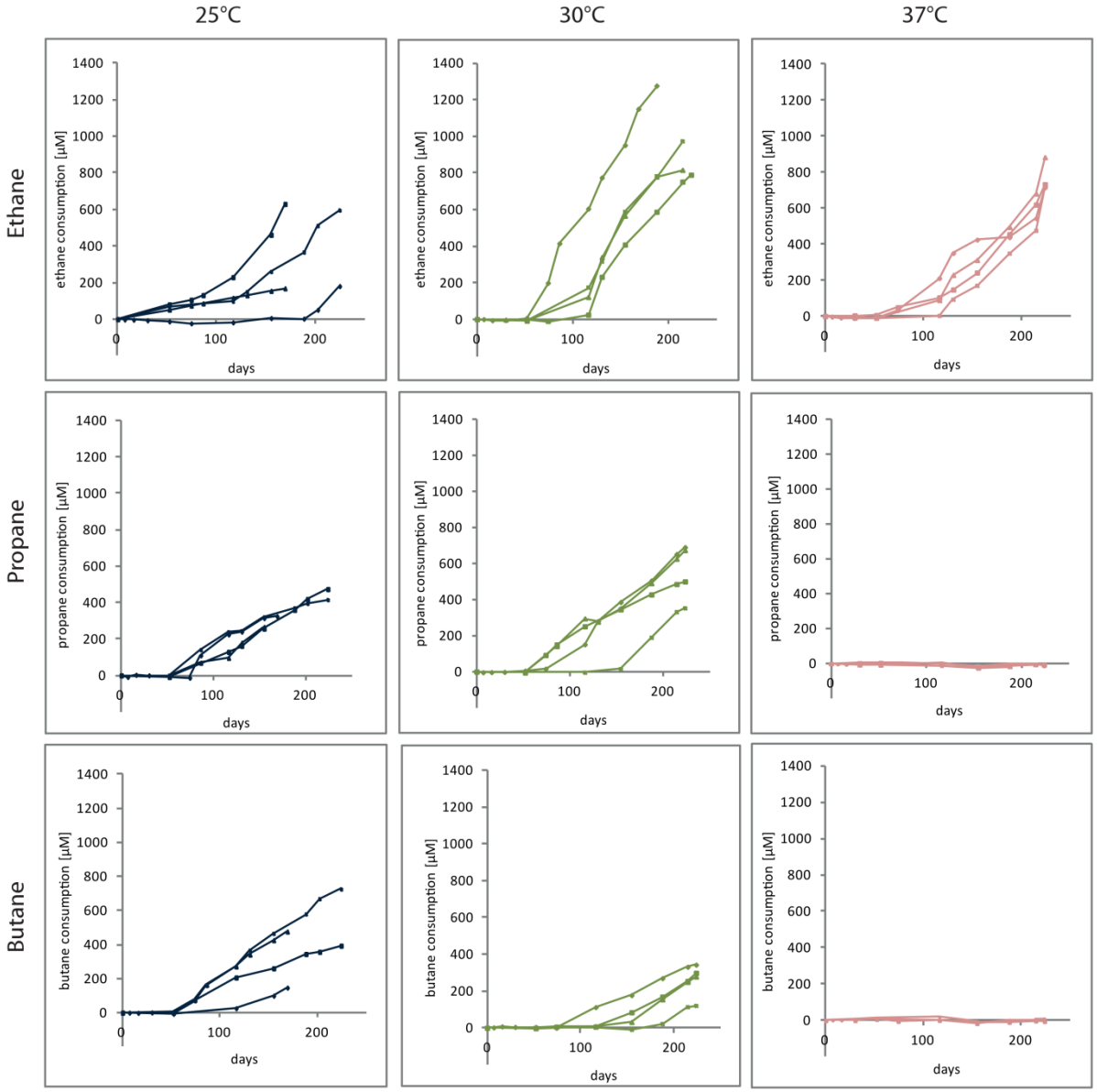

Figure 2: Alkane and alkene consumption over incubation time at different incubation temperatures. The first row shows the results of the ethane incubation, the middle row the propane incubations and the lowest row the butane incubations. Graphs in blue: alkane incubations at 25 °C incubation temperature, in green: incubations at 30 °C, in red: incubations at 37°C.

T-RFLP Analyses

To study the changes of the bacterial community composition in methane, ethane, propane and butane incubations over time, T-RFLP analyses were performed. Primers were used to target the 16S rRNA gene and the *pmoA*, *pxmA* and *alkB* genes. Additionally, *pmoA* transcripts were analyzed by T-RFLP.

T-RFLP analyses of the 16S rRNA gene resulted in very diverse T-RFLP patterns (data not shown). In methane incubations three fragments were dominant after 17 days of incubation: 117bp, 125bp and 146bp. After 41 days the fragment 146bp dominated. This fragment was also prominent in ethane incubations though other fragments appeared and disappeared during incubation (e.g. 135bp, 438 bp). The fragment 125bp that was also prominent in methane incubations after 168 days. Towards the end of the incubation the T-RFLP pattern became more diverse. Several new fragments appeared (e.g. 55bp, 60bp, 156bp). T-RFLP pattern of the propane and butane incubations were even more diverse. Though the known fragments from methane and ethane incubation (126bp, 135 bp, 147bp and 156bp) also appear in propane and butane incubations lots of other fragments, sometimes only in small percentages, came up during the course of the experiment. The high quantity of fragments made it difficult to evaluate the 16S rRNA gene T-RFLP. Additionally, TRFs could not be assigned to corresponding OTUs on the basis of the 16S rRNA gene sequences retrieved from pyrosequencing analysis (see below: Pyrosequencing Analysis). However, the T-RFLP analyses showed that the methane incubation was mainly dominated by a few OTUs that also appeared in the incubation with the other alkanes.

The T-RFLP analysis of the *alkB* gene revealed three prominent fragments of the size 128bp, 137bp and 230bp (data not shown). Especially, the propane and butane incubations were mainly dominated by those three fragments. Methane and ethane incubations showed a wider range of fragments (e.g. 66bp, 100bp and 196bp), though the three fragments 128bp, 137bp and 230bp were also present. Until now, no clone library is available to evaluate the *alkB* fragments and assign them to microorganisms. However, the T-RFLP results lead to the assumption that the diversity of organisms possessing the *alkB* is higher in methane and ethane grown samples than in propane and butane grown samples.

Figure 3 shows the summary of the results of the *pmoA* T-RFLP analyses. The most prominent fragments in most samples had the length of 245bp or 352bp. OTUs were assigned based on clones and pyrosequencing analyses (Lüke *et al.*, 2011, Lüke & Frenzel, 2011, Yimga *et al.*, 2003). The fragment 245bp can be assigned to the *pmoA* of type II methanotrophs *Methylocystis* and *Methylosinus*. The other dominant fragment of the size 352bp can be either assigned to the *pmoA* of type I methanotrophs or the *pmoA*2 of *Methylocystis* and *Methylosinus*. Fragment 536bp is assigned to type I methanotrophs.

The fragment pattern from methane grown samples stayed relative constant after 17d and 41d of incubation. The samples seem to be dominated by type II methanotrophs and type I methanotrophs. In ethane incubations the relative composition of fragments changed over time. The high relative abundance of the fragments 245bp (*Methylocystis* and *Methylosinus)* and 352bp (Type I methanotrophs or isoenzyme *pmoA*2 of type II methanotrophs) seem to decrease in favor of the abundance of the fragments 236bp, 448bp, 509bp and 536bp (Type I methanotrophs). The fragments 236bp, 448bp and 509bp could not be related to any known

methanotroph so far. T-RFLP results for propane incubations could only be achieved at 129d and 168d. PCR amplifications at later time points of the propane incubation (262d and 313d) failed. No amplification results could be obtained in butane grown samples at any incubation point suggesting that the concentration of the target sequences were low.

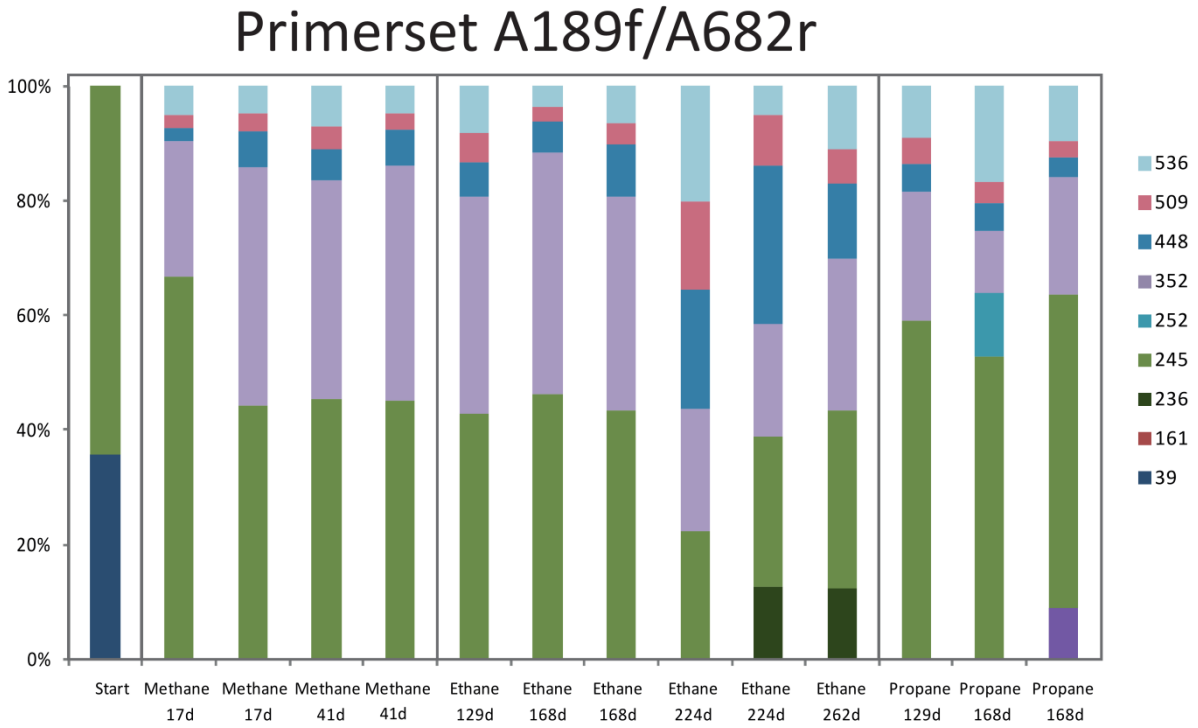

Figure 3: Overview of the relative composition of T-RFLP results retrieved with *pmoA* targeting primer set A189f/A682r at different time points of alkane incubations.

To analyze *pxmA* sequences three T-RFLP setups were performed with the primer sets A189f/M84_P105r, A189f/RA21r and A189f/AOB_relr, targeting three iCuMMO groups. Amplifications with the primers A189f/RA21r resulted in no PCR product, suggesting organisms containing sequences clustering into the environmental RA21 group do not play a role in alkane degradation in rice field soils. The T-RFLP pattern achieved with the primerset

A189f/AOB_relr in samples grown with methane or ethane was dominated by two fragments: 245bp and 350bp (data not shown). The fragment pattern was very similar to the pattern resulted in amplification with the *pmoA* primer set A189f/A682r. Previous deep sequencing studies (Hainbuch *et al*., in prep.) showed that the primer pair A189f/AOB_relr is not specific to sequences of the iCuMMO group AOB_rel but can also amplify sequences belonging to other iCuMMO groups and *pmoA* sequences of type Ib and type II methanotrophs. On the basis of previous deep sequencing studies (Chapter 1) the fragments 245bp and 350bp could not be assigned to the AOB_rel group. So, it is likely that these fragments do not represent sequences of the iCuMMO group AOB_rel but co amplified sequences of type II methanotrophs. Fragment 245bp can be assigned to the *pmoA* of *Methylocystis* and *Methylosinus* and 352bp the *pmoA2* that is present in several type II methanotrophs or type I methanotrophs.

Figure 4 shows an overview of the T-RFLP results of *pxmA* sequences obtained with the primerset A189f/M84_P105r. The fragment patterns from methane grown samples were similar after 17 days and 41 days. Based on pyrosequencing results (Hainbuch *et al*., in revision) the dominant fragment 33bp could be related to sequences of the iCuMMO group M84_P105 though this fragment is not exclusively restricted to the M84_P105 groups but could represent type I and II methanotrophs and other iCuMMO groups, too. The other three fragments 225bp, 374bp and 426bp could also be assigned to M84_P105 sequences but additionally to sequences of type I methanotrophs and *pmoA*2 sequences of type II methanotrophs. In ethane grown samples the fragment diversity increased. The most prominent new fragment 350bp could not be assigned to any known M84_P105 sequences

but to type I methanotrophs or *pmoA*2 sequences. T-RFLP results of propane grown samples could be obtained from incubation after 129 and 168 days of incubation. As in the T-RFLP results of the *pmoA* (figure 3) no PCR product could be amplified at later time point of the propane incubation suggesting low template concentrations or no templates in those samples. The fragment pattern of the propane incubation is similar to the pattern of the methane incubation with the prominent fragments 33bp, 225bp, 374bp and 436bp though some less abundant additional fragment appeared.

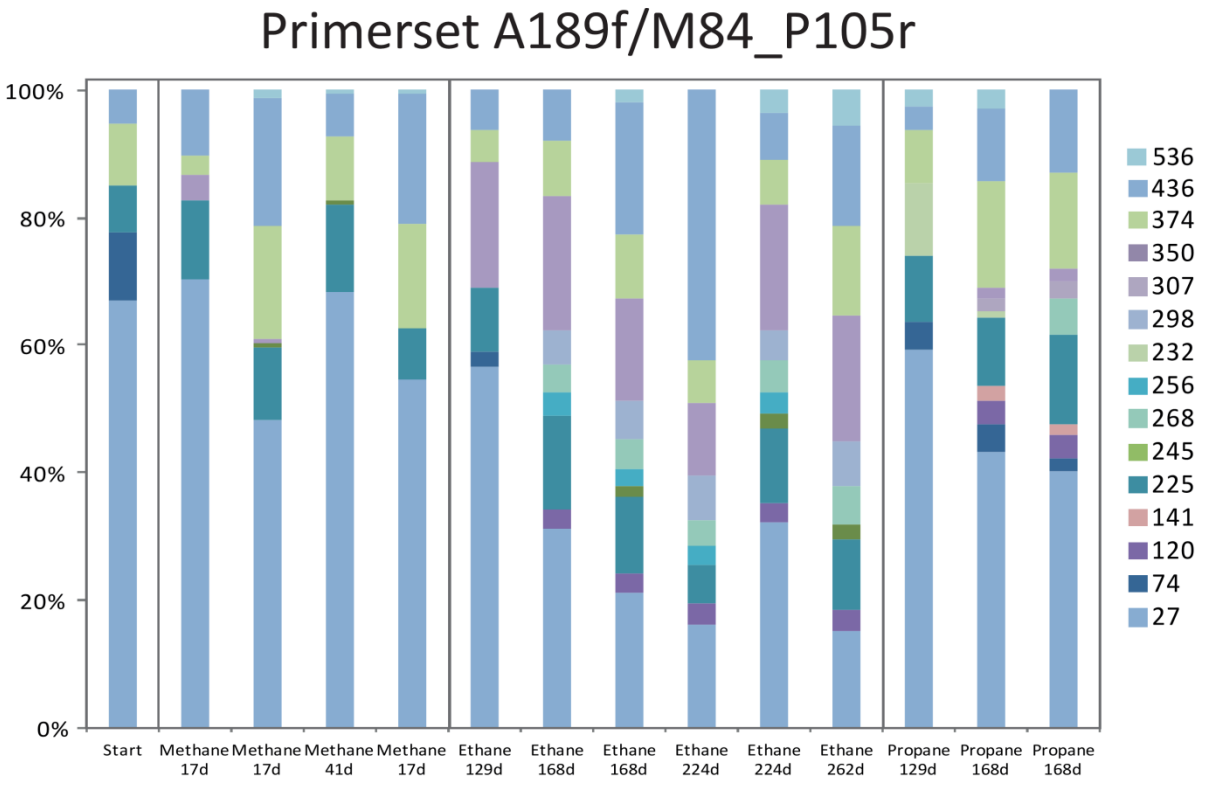

Figure 4: Overview of the relative composition of T-RFLP results retrieved with the primer set A189f/M84_P105r at different time points of alkane incubations.

Transcriptional T-RFLP analyses were performed for *pmoA*, *pxmA* and *alkB* sequences with the same primer sets that were used on DNA level (A189f/A682r, A189f/M84_P105r,

A189f/RA21r, A189f/AOB_relr and AlkBf/AlkBr) in all alkane incubations at different time points. mRNA transcripts could be detected exclusively with the *pmoA* targeting A189f/A682r primers. Neither the use of the primers A189f/M84_P105r, A189f/RA21r, A189f/AOB_relr targeting the sequences of the iCuMMO groups nor the use of the primers targeting *alkB* sequence resulted in amplicons. Results of the mRNA T-RFLP amplified with *pmoA* targeting primers are shown in figure 5. The most prominent fragments on mRNA basis (245bp and 352bp) accord to the most frequent fragments that could be detected on DNA level using the A189f/A682r primer pair. The fragment pattern in samples grown with methane stayed constant at day 17 and 41. The same fragment pattern could be detected in ethane incubations after 168 and 224 days. In propane grown samples the transcription was low at day 168.

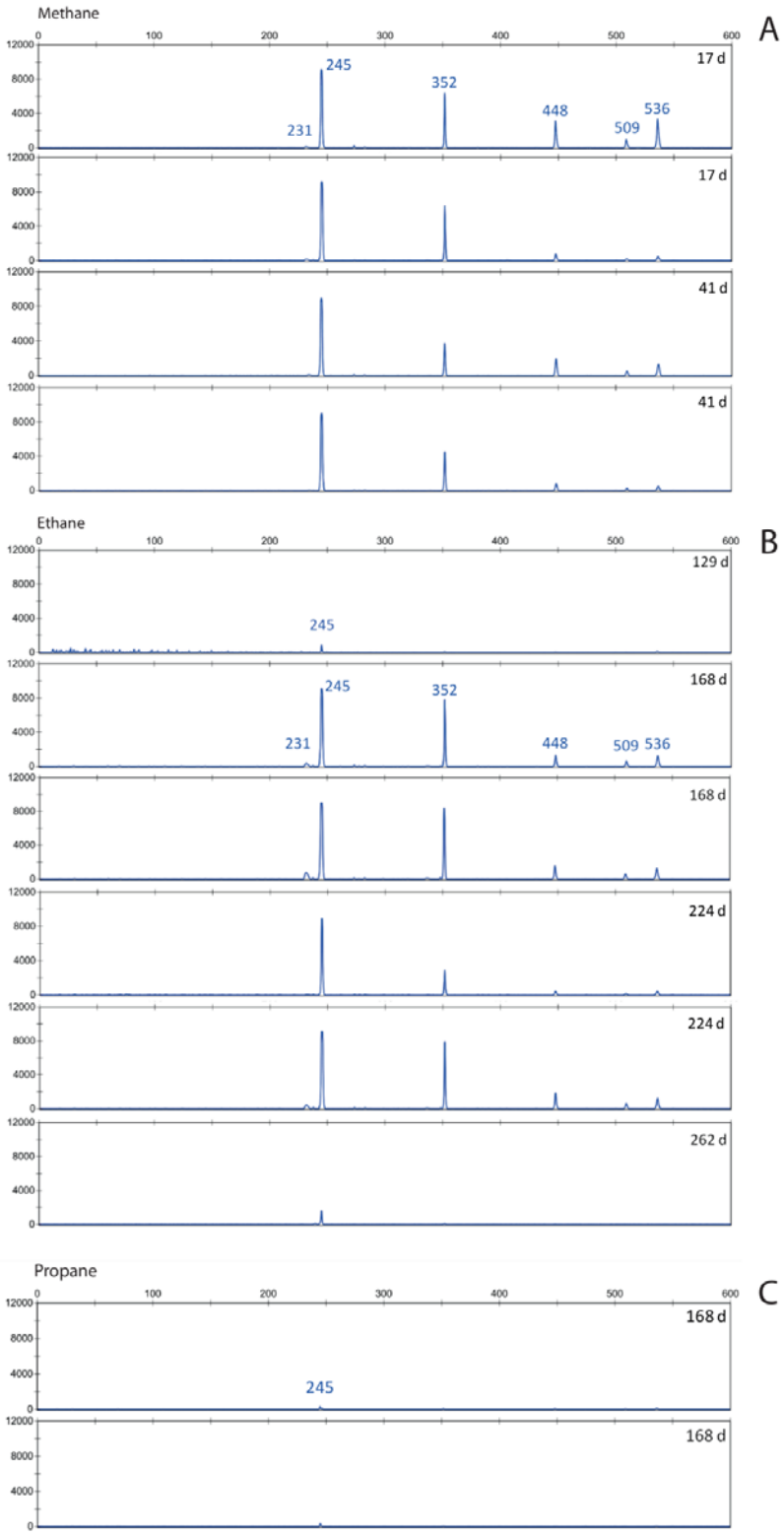

Figure 5: Overview of the relative composition of T-RFLP results retrieved from transcriptional analyses with the primer set A189f/M84_P105r at different time points of alkane incubations. A: methane incubation (17 and 41 days). B: ethane incubation (129, 168, 224 and 262 days). C: propane incubation (168 days). Number in blue: fragment sizes in bp.

Deep sequencing analysis

T-RFLP analyses gave an overall impression of the temporal change in the bacterial community composition in methane, ethane, propane and butane incubations. Yet, deep sequencing analysis declares for much finer insights in the composition. Primers targeting the *pmoA* (A189f/A682r), the *pxmA* of the M84_P105 group (A189f/M84_P105r), the *alkB* (AlkBf/AlkB) and the 16S rRNA gene (343Fmod/748Rmod) were used for deep sequencing analysis. The analysis was performed at a late time point of the alkane incubation. It resulted in about 58500 sequences. After first quality checks 13853 sequences of appropriate length (*pmoA*, *pxmA and* 16S rRNA gene: >400 bp, AlkB: >100 bp) remained for further quality verification. After sequence aligning in total 9971 high quality sequences remained for phylogenetic analysis.

Figure 6 shows sequences retrieved by deep sequencing with the primer pairs A189f/A682r and A189f/M84_P105r. Most sequences amplified by the A189f/M84_P105r primerset clustered within the G5 subgroup of the iCuMMO M84_P105 in all alkane incubations. A small portion of sequences (3-5%) clustered into the *pmoA2* group of the type II methanotrophs. 0.2 % of sequences of the ethane incubation clustered into the G4 subgroup of the iCuMMO M84_P105. The *pmoA* analysis was dominated by sequences clustering within groups of type II methanotrophs. At incubation start type II sequences contributed to 76.4 % to the total amount of sequences. 22.2 % clustered into the group of ammonium oxidizers (AOB) and 1.4% into the group of the Type Ib methanotrophs. The relative proportion of type II sequences increased in methane incubation to 90.1 % and to

100.0 % in ethane incubation. No sequences could be obtained from propane incubation with the *pmoA* primerset.

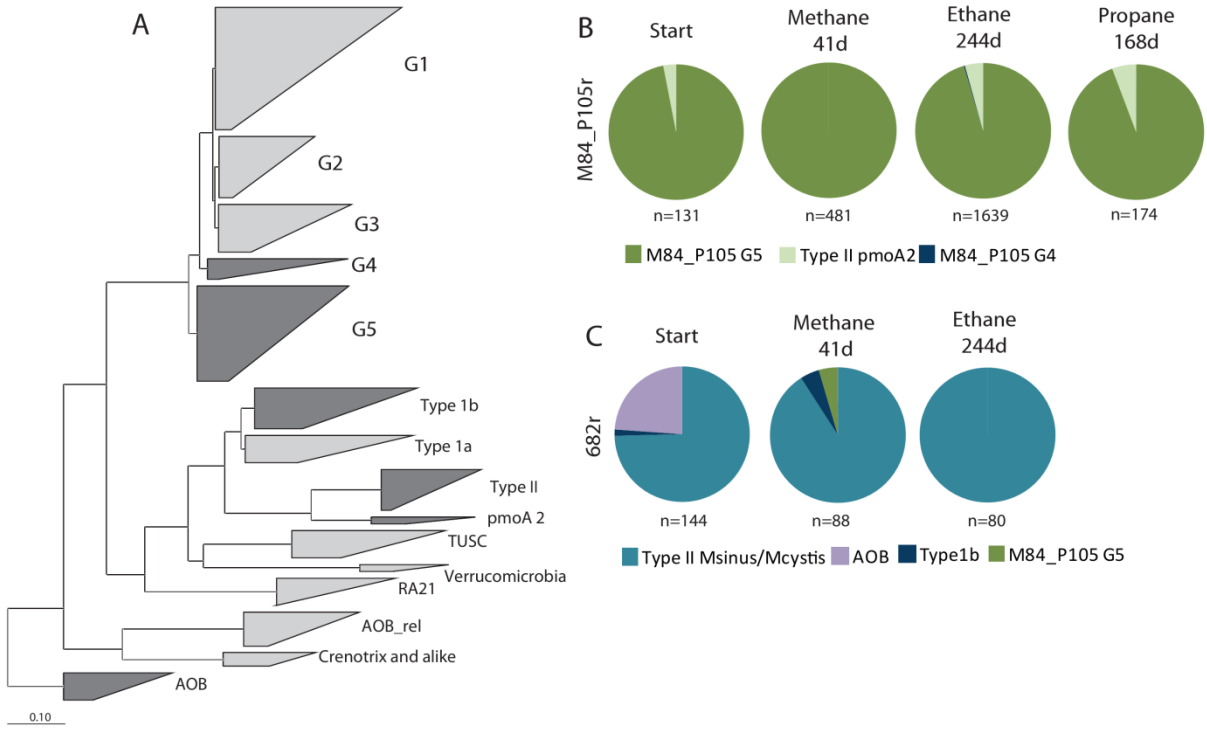

Figure 6: Deep sequencing analysis targeting the *pmoA* and the *pxmA*. A: Phylogenetic tree containing *pmoA* and *pxmA* sequences. Highlighted in dark grey: phylogenetic groups that contain sequences of the deep sequencing analysis. B: diagrams of sequences retrieved with the primer set A189f/A682r targeting the *pmoA*. Small numbers: numbers of sequences retrieved. C: diagrams of sequences retrieved with the primer set A189f/M84_P105r targeting the *pxmA*. Small numbers: numbers of sequences retrieved.

Deep sequencing analyses of the *alkB* gene resulted in 1740 sequences of which 1353 sequences could be assigned to the domain of the bacteria by the metagenome analyzer MEGAN5 (Huson *et al.*, 2011). While the percentage of sequences that could be assigned was low in the start and methane grown samples (18.1 % and 13.3 %) the percentage

increased in ethane (88.0%), propane (90.0%) and butane (97.0%) samples. Additionally, the percentage of bacterial samples that could be assigned to bacterial orders increased with the length of the alkanes (Figure 7). In samples taken before incubation start the greatest part (23.3%) of bacterial sequences that could be assigned to a bacterial order belonged to the Actinomycetales. The relative proportion of Actinomycetales sequences increases in ethane (41.5 %), propane (74.8%) and butane grown samples (71.5%). Most of the sequences clustering into this group in propane and butane grown samples could be related to sequences of *Nocardiaceae*. However, for methane grown samples not more than 30 sequences could be retrieved from which only 4 could be assigned suggesting a low target concentration in those samples.

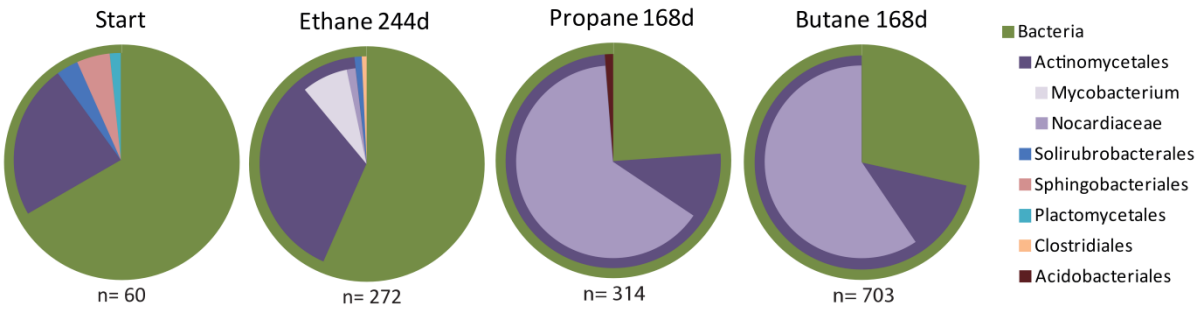

Figure 7: Deep sequencing analysis of the *alkB* gene. Numbers below the chart: total number of sequences that could be assigned to bacteria.

The results of the deep sequencing analysis with bacterial 16S rRNA primers are summarized in figure 8. The bacterial community in the starting material was dominated by sequences of species belonging to the class of Actinobacteria, Fimicutes and betaproteobacteria. After 41 days of methane incubation the relative composition of the bacterial community changed.

The samples grown with methane were dominated by two bacterial groups: the order of Clostridiales and the family *Methylocystaceae*. Ethane, propane and butane grown samples were more diverse, though the community composition of alkane incubations looked very similar. Among the alkane incubations the main differences are primarily founded by the relative distributions of the bacterial orders. The proportion of the proteobacterial TH18 cluster increased from few percentages in the starting material to 10 – 28 % in ethane, propane and butane incubations. The relative proportion of betaproteobacteria decreased while the alphaproteobacteria increased compared to the starting material. Though the proportion of the alphaproteobacteria is similar in ethane, propane and butane grown samples, the composition differs. In ethane grown samples sequences of the family *Methylocystaceae* contribute to the portion of alphaproteobacteria sequences. But *Methylocystaceae* sequences are infrequent in propane grown samples and not detectable in samples grown with butane. The class of Actinobacteria that is very diverse in the staring material is mainly dominated by the orders Frankiales and Corynebacteriales in ethane, propane and butane grown samples. Sequences belonging to the phylum of the Firmicutes are present in all samples. They are most dominant in samples grown with methane but also present in ethane, propane and butane grown samples, though the relative proportion of sequences of the order Bacillales increase compared to the order of Clostridiales. The gammaproteobacterial Chromatiales only appear in butane grown samples.

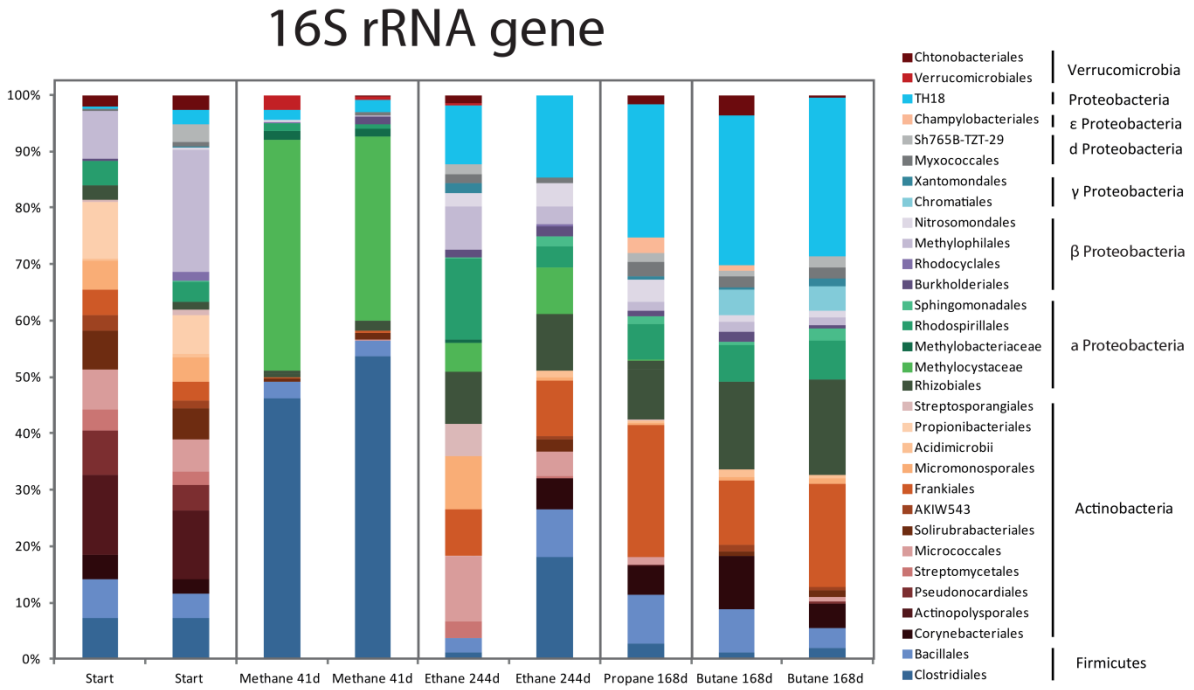

Figure 8: Deep sequencing analysis based on the bacterial 16S rRNA gene.

4.5 Discussion

Gaseous hydrocarbons are ubiquitous in nature. They are part of natural gases formed by geochemical processes or produced by microorganisms, marine algae, insects and plants as moisture barriers, reserve materials and pheromones in most soil and water environments (Cooley *et al.*, 2009, Shennan, 2006, Giebler *et al.*, 2013, Nie *et al.*, 2014, van Beilen & Funhoff, 2007). In this study the bacterial community involved in the degradation of short chained gaseous hydrocarbons in a rice field soil was analyzed. Thereby, a special focus was set on the methanotrophic community, on microorganisms possessing methane monooxygenase isoenzymes belonging to the iCuMMO groups and on bacteria utilizing the alk enzyme system to degrade alkanes and alkenes.

Methanotrophic bacteria involved in alkane degradation

In this study, the bacterial communities were analyzed by two methods: T-RFLP and deep sequencing. T-RFLP is a robust and reproducible fingerprinting method that has been used in a variety of ecological studies to analyze methanotrophic communities (Lüke *et al*., 2011, Horz *et al.*, 2001). T-RFLP results indicate that a great part of the methanotrophic community in methane, ethane and propane incubations persist of species of the type II methanotrophic genera *Methylocystis* and *Methylosinus*. Fingerprinting methods like T-RFLP are sometimes limited in phylogenetic resolution and differentiation. Also in this study, several T-RFs could not or not clearly be affiliated to one specific methanotrophic group. A dominant T-RF (352bp), for example, represents both the pMMO of type I methanotrophs and the pMMO2 isoenzyme of type II methanotrophs. However, the supposed dominance of type II methanotrophs based on T-RFLP data could be verified by deep sequencing analysis both on *pmoA* and 16S rRNA level.

The deep sequencing analysis of *pmoA* and the bacterial 16S rRNA gene provided a large dataset of high-quality sequences and gave a deep insight into the methanotrophic community: Most *pmoA* gene sequences could be assigned to type II methanotrophs (start: 76.4 %, methane: 90.1 %, ethane: 100%, propane: no results). Additionally, almost all 16S rRNA sequences that could be assigned to methanotrophic bacteria belonged to the alphaproteobacterial family *Methylocystaceae* that contains amongst others the genera *Methylocystis* and *Methylosinus.*

The alphaproteobacterial type II methanotrophs are ubiquitous in most environments. Due to their ability of stress tolerance they are thought to be very stable and able to form

ubiquitous seed banks in soil environments (Eller *et al.*, 2005, Krause *et al.*, 2012, Ho & Frenzel, 2012, Graham *et al.*, 1993). However, despite their wide distribution active type II methanotrophic communities could only be shown in few studies. The activity of methanotrophs has been monitored in a variety of environmental studies by transcriptional gene analysis or stable isotope probing (SIP). Both methods seem to be largely congruent and seem to illustrate the activity of the methanotrophs in a similar dimension (Henneberger *et al.*, 2014). In most studies based on gene transcript analyses, *pmoA* transcripts of type I methanotrophs dominated while transcripts of type II methanotrophs could not or only be retrieved in low levels (Bodrossy *et al.*, 2006, Krause *et al.*, 2010, Krause *et al*., 2012, Liebner *et al.*, 2009). The active methanotrophic community detected by SIP experiments in varies habitats was dominated by type I methanotrophs, too (Qiu *et al.*, 2008, Dumont *et al.*, 2011, Graef *et al.*, 2011). In this study the transcription of *pmoA* was taken as a proxy for activity and was analyzed by T-RFLP (Figure 5). Transcripts of the *pmoA* could be detected in methane, ethane and propane samples and could be related to the *pmoA* and *pmoA*2 of type II methanotrophs.

In methane grown samples active methanotrophs were detected after 17 and 41 days of incubation. It was shown in previous studies that type II methanotrophs became active after long term incubations of 30 days though the transcription level was low (Krause *et al*., 2012). Another study shows a dominance of active type II methanotrophs after 40 days of incubation after biomass destruction (Ho *et al*., 2011).In ethane and propane incubations the relative proportion of methanotrophic bacteria seems to be rather low. 6.6 % and 0.02 % of the bacterial 16S rRNA sequences retrieved by deep sequencing could be assigned to

methanotrophic bacteria. However, the proportion increased compared to the starting samples (no sequences of methanotrophic bacteria could be detected). Transcripts of *pmoA* and *pmoA*2 of type II methanotrophs could be detected in ethane grown samples and in very low transcripts levels in propane incubations, too. The II methanotrophs seem to play a dominant role within the methanotrophic community in long time methane incubation in environments where short alkanes like ethane and, to a minor part, propane are available as substrate. However, longer alkanes like butane do not seem to affect bacterial methanotrophic communities.

It has been believed for a long time that methanotrophic bacteria are obligate and that they could grow exclusively on methane, methanol and some other C_1 compounds (Bowman, 2006). But it is known that at least some methanotrophs grow on substrates with carbon-carbon bounds like acetate, pyruvate, succinate, malate and ethanol (Dedysh *et al.*, 2005, Dunfield *et al.*, 2010, Belova *et al.*, 2011, Im *et al.*, 2011). Facultative type II methanotrophs could to be involved in the degradation of alkanes in this study, too. A transcription of *pmoA* and *pxmA* of type II methanotrophs was monitored. But it seems unlikely that the corresponding pMMOs are involved in the alkane oxidation. pMMOs have a narrow substrate range. They are known to be able to co-oxidize several other hydrocarbons like ethane (e.g. Chapter 2) but these substrates are usually not assimilated and used as a growth substrate by methanotrophs possessing the pMMO. It could be possible that the soluble methane monooxygenase (sMMO) is involved in alkane degradation by the type II methanotrophs. The sMMO has a wider substrate range than the pMMO. But further studies

need to confirm this assumption because the analysis of the sMMO was not a in the focus of this study.

However, until now no methanotrophic bacterium could be detected that grow on ethane or propane as a carbon source. It could be possible that type II methanotrophs in this study do not directly grow on short alkanes. Methanotrophs have been found in close interactions with other organisms such as plants and algae (Kip *et al.*, 2010, van der Ha *et al.*, 2011) or other bacteria (Modin *et al.*, 2007, van der Ha *et al.*, 2013, Hrsak & Begonja, 2000, Iguchi *et al.*, 2011). Besides abiotic factors like oxygen, nutrients, moisture and temperature that affect methanotrophic activity (Conrad, 2007, Hanson & Hanson, 1996, Krause *et al*., 2012, Reim *et al.*, 2012) other bacteria interact with methanotrophs significantly. While providing carbon compounds derived from methane, methanotrophs benefit in return from other bacteria by the remove of toxic waste product from methane oxidation, the providing of essential metabolites and the creation of a stable environment with ideal gas composition (Dumont *et al*., 2011, Murase & Frenzel, 2007, Petersen & Dubilier, 2009, Hanson & Hanson, 1996, Iguchi *et al*., 2011, Stock *et al.*, 2013, Ho *et al.*, 2014). To date, several bacteria, including strains of *Xanthobacter* (Lidstromoconnor *et al.*, 1983, Uchiyama *et al.*, 1992), *Hyphomicrobia* (Bowman, 2006), *Rhizobium* (Iguchi *et al*., 2011) and *Spingopyxis* (Jeong *et al.*, 2014) have been found to life in close relationship with methanotrophs or stimulate their growth. These bacteria belong to orders that are known to include bacterial families that are able to oxidize short chained alkanes like ethane and propane. In a bacterial consortium, the heterotrophic bacteria could provide metabolic products like ethanol that may be used as growth substrate by facultative methanotrophs like type II *Methylocystis* strains (Im *et al*.,

2011). In this study, for example, 16S rRNA sequences in ethane and propane incubation could be related to *Xanthobacter* strains. Hence, consortia might be possible. However, those assumptions are speculative and cannot be verified by the results of this study. A stable isotope study with labeled 13C alkanes might give the yet unproven insights into the degradation pathways of short gaseous alkanes in rice fields and could indicate the role of the methanotrophic bacteria within this pathway.

iCuMMOs seem to play no role in the degradation of alkanes and alkenes in rice field soils. The iCuMMO groups RA21, AOB_rel and M84_P105 were analyzed by T-RFLP in methane, ethane, propane and butane samples. No amplification products could be found with primers targeting the AOB_rel and the RA21. Sequences of the iCuMMO group M84_P105 could be detected by T-RFLP and pyrosequencing. Most M84_P105 sequences clustered into the subgroup 5 that contains *pxmA* sequences of two *Methylocystis* strains: *Methylocystis* sp. SB2 and *Methylocystis rosea*. Few sequences clustered within the *pmoA*2 group of the type II methanotrophs. But no transcripts could be amplified suggesting that the pXMO is not involved in the degradation of alkanes and alkene, too. Enzymes of the three analyzed iCuMMO groups seem to have other physiological functions than the oxidation of alkanes. They might be involved in the methane oxidation like the pMMO2 of the type II methanotrophs and may provide selective advantages in environments with fluctuating nutrient availabilities (e.g. low oxygen concentrations). However, the physiological functions could not be resolved until now.

Alkane degrading bacteria

Compared to researches on methanotrophic bacteria, the interest in microorganisms able to metabolize short chained gaseous alkanes has been rather low for a long time. However, new prospects in biotechnological fields like biotransformation and remedial treatments have lead to an extended interest in alkane degrading bacteria (ADB). Lots of studies that focus on alkane degrading bacteria in nature have been obtained in oil contaminated and marine environments, few in soil environments (Sutton *et al.*, 2013, Brakstad & Lodeng, 2005, Sabirova *et al.*, 2006, Wang *et al.*, 2010, Redmond & Valentine, 2012, Giebler *et al*., 2013, Schulz *et al.*, 2012). To detect ADB in rice field soils and to study their abundance and diversity, the marker gene *alkB,* coding the trans-membrane alkane monooxygenase of the Alk enzyme system, has been used (Kuhn *et al.*, 2009, Perez-de-Mora *et al.*, 2011, Wang *et al*., 2010). ADB were analyzed by *alkB* T-RFLP and pyrosequencing as well as 16S rRNA pyrosequencing. The evaluation of T-RFLP results was utterly impossible due to a lack of an appropriate clone library and the resulting inability to assigned T-RFs to ADB, but pyrosequencing results gave an insight into the community composition. ADB virtually played no role in rice field samples incubated with methane. This can be explained by the fact that bacteria possessing the alk enzyme system or other alkane degrading systems like bacterial P450 oxygenases or propane and butane monooxygenases are usually not able to oxidize methane (van Beilen & Funhoff, 2005).

Most sequences retrieved by *alkB* pyrosequencing from ethane, propane and butane grown samples could be assigned to the genera *Mycobacterium* and *Nocardia* that belong to the Actinomycetales (Figure 7), a divers order of the Gram- positive Actinobacteria.

Mycobacterium seemed to be dominant in ethane, *Nocardia* in propane and butane incubations. 16S rRNA pyrosequencing analyses confirmed these results. *Mycobacterium* and *Nocardia* are members of the common alkane degrading CNMR group. In rice field soils the members of the CNMR group do not seem to be very dominant (start: 3.4 %, ethane: 2.7%, propane: 5.1 %, butane: 6.8%). Other bacterial groups were more frequent and increased during alkane incubations. The order Rhizobiales with the families *Bradyrhizobiaceae*, *Xanthobacteraceae*, *Hyphomicrobiaceae* and the environmental cluster DUNssu371 contributed to 9.2 % (ethane), 10.2% (propane) and 16.1 % (butane) to the total bacterial community. The order Rhizobiales is known to contain species that possess the alk enzyme system and that could therefore be involved in the degradation of short chained alkanes (Ouattara *et al.*, 2003, Maier *et al.*, 1978, Malik & Claus, 1979). Bacillales sequences belonging to the order of Firmicutes contribute to 5.3 % (ethane), 8.4 % (propane) and 5.5 % (butane) to the bacterial community. The alk enzyme could be found in species of this order, too (Tourova *et al.*, 2008). Rhizobiales and Bacillales species could be detected in this study only by 16S rRNA analysis. On the basis of the *alkB* marker gene no sequences could be found that could be related to these bacterial orders. A possible explanation for the discrepancy between 16S rRNA and *alkB* could be a detection limitation of *alkB* sequences by the used primerset. The *alkB* gene sequences are very diverse among the ADB. This diversity could hardly be covered by a single primer set. In this study, a primer set was chosen that amplifies a great part of sequences of the *alkB*-containing bacterial community. But it seems to lack *alkB* sequences of Rhizobiales and Bacillales species (Jurelevicius *et al.*, 2013, Kloos *et al*., 2006). To overcome the problem of sequence coverage a use of several *alkB* primer sets covering different *alkB*-containing bacterial groups should be recommended

for further analyses of these genes. Although the relative proportion of *alkB* containing bacteria like *Mycobacterium*, *Nocardia,* Rhizobiales and Bacillales increased in alkane incubations over time, no *alkB* transcripts could be detected. An explanation could be that bacteria possessing the alk enzyme system are involved in alkane degradation but use other enzyme complexes to oxidize alkanes. Many hydrocarbon degrading bacteria are known to contain multiple alkane hydroxylases with overlapping substrate ranges (Kotani *et al.*, 2003, Sabirova *et al*., 2006, van Beilen & Funhoff, 2007, van Beilen *et al.*, 2003). Mycobacterium strains, for example, are known to contain six different monooxygenases including alkB, Cytochrome P450 and propane and butane monooxygenases (Coleman *et al.*, 2011). It could be possible that not the alk enzyme system but another monooxygenase is involved in the degradation of short chained alkanes in rice fields.

Beside species of the orders Corynebacteriales, Rhizobiales and Bacillales that are known to be involved in degradation of alkanes, species of other bacterial orders are also dominant in ethane, propane and butane incubations, too (Figure 8). Surprisingly, in spite the fact that the alkane grown samples were incubated aerobically, a great portion of sequences retrieved by 16S rRNA deep sequencing could be assigned to the bacterial order of the *Clostridiales*, a group of mainly anaerobe Gram-positive bacteria. One may speculate that these bacteria could have grown in anoxic micro aggregates beside the aerobically growing methanotrophs. During incubation the alkanes were oxidized rapidly and oxygen concentrations sank low (concentrations of about 5%) which could have favored the growth of the anaerobic Clostridiales and other anaerobic bacteria in anaerobic microstructures.

A great part of sequences clustered within the order of the Frankiales (start: 3.7%, ethane: 8.8%, propane: 23.1 %, butane: 14.7 %). Most of the sequences clustering within this order could be assigned to the family *Sporichtyaceae,* facultative anaerobe chemoorganotrophic bacteria that grow on complex hydrocarbons like humic acids (Williams *et al.*, 1989, Suzuki *et al.*, 1999). Alkane degradation is not described for the *Sporichtyaceae.* The order of Rhodospirillales is mainly represented by sequences assigned to environmental clusters (I-10 and DA111) related to sequences of the family Rhodospirillaceae (Ethane: 8.8%, Propane: 6.2 %, Butane: 6.6 %). The Rhodospirillaceae are bacteria that are able to photo assimilate organic compounds under anaerobic conditions and are able of chemotrophic growths under aerobic conditions. Another group of sequences that contributed to a great part of the bacterial community clustered within the proteobacterial environmental cluster TH18 (start: 1.5 %, ethane: 12.3 %, propane: 23.2%, butane: 27.3%). Sequences of this cluster has been found in a variety of environments (Elshahed *et al.*, 2008, Forget *et al.*, 2010, Isenbarger *et al.*, 2008, Lesaulnier *et al.*, 2008, Riviere *et al.*, 2009, Santelli *et al.*, 2008, Zhang *et al.*, 2010). But so far no representative pure cultures and thereby no information about physiology of the corresponding organisms are available. It could be possible that TA18 proteobacteria are involved in alkane degradation.

Alkene degraders

In this study a focus was set on the analysis of the degradation of alkanes in a rice field soil and the analysis of the involved bacterial community. But additional incubations were performed with alkenes as substrates: Ethylene, propylene and butylene. Alkenes are oxidized by monooxygenases forming epoxy-alkanes, highly reactive products that are toxic

to cells. But several microorganisms are able to rapidly eliminate those intermediate products and are thereby able to grow on alkenes. Common species that metabolize short chained alkenes belong to the genera *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Pseudomonas* and *Xanthobacter* (Debont, 1976, Furuhashi *et al.*, 1981, Habetscrutzen *et al.*, 1984, Saeki *et al.*, 1999, Vanginkel *et al.*, 1987, Verce *et al.*, 2000, Vlieg *et al.*, 1998, Shennan, 2006). Alkane incubation showed that members of some of these genera are present in rice field soils and that they were able to be enriched by alkane incubations. However, no oxidation of alkenes could be detected during about 350 days of incubation. Though the genera containing alkene degrading bacteria are present, the alkene degraders of those groups are absent or not active in rice field soils.

Summery and conclusion

Among the methanotrophic bacteria the type II methanotrophs are dominant in long term methane incubations as well as in ethane and propane incubations. It could be shown that the community is active and not only present in a dormant stadium. Methanotrophs seem to be involved in ethane and propane degradation in rice field soils, whereat it can be speculated if they use alkanes directly or use metabolic substrates provided by heterotrophic bacteria. As a part of a consortium the methanotrophs may play an important role in the degradation of short chained alkanes in rice field soils. Important heterotrophic bacteria that might be involved in alkane degradation belong to orders of the Corynebacteriales, Rhizobiales and Bacillales. If those bacteria use the alk enzyme system or other alkane degrading enzymes systems like Cytochrome P450 or propane or butane

monooxygenases could not be clearly explained certainly. The oxidation of hydrocarbons in rice field soil and in general may be more important than previously assumed. Further analysis (e.g. SIP studies) could help to get a better understanding of the processes and microorganisms involved in alkane degradation and could help to show another aspect of role of methanotrophs in this environment that seems to be not only restricted to methane oxidation.

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5 General discussion and outlook

Methanotrophic bacteria have been studied for over a century. The first bacterium with the ability to utilize methane as a sole carbon and energy source has been already isolated in 1906, followed by many other isolates (Söhngen, 1906, Whittenb.R *et al.*, 1970, Tavormina *et al.*, 2011, Vorobev *et al.*, 2014). Intense studies on methanotrophs in the following years gave deep insights into their biochemistry, diversity and environmental distribution and helped to discover and understand their complex ecological function of methanotrophs in nature. Especially, culture independent methods, like next generation sequencing, provided enormous datasets and extended the knowledge that we have about methanotrophic bacteria. The enzyme that stands in the focus of the research on methanotrophs is the methane monooxygenase (pMMO) a particulate Cu-containing membrane-bound monooxygenase (CuMMO).

In this PhD thesis the main focus lies on the study of CuMMO that are only distantly related to the well characterized pMMO: the intermediate CuMMOs (iCuMMOs). Chapter 2 focuses on the occurrence of iCuMMO genes in nature and methanotrophic pure cultures and possible physiological functions of the corresponding enzyme. New reverse primers targeting *pxmA* subunits of deep branching environmental iCuMMO groups (M84_P105, AOB_rel, TUSC and RA21) were designed for environmental pyrosequencing analysis of rice

field soils and lake sediments and for pure culture screening. An incubation study was performed to resolve the physiological function of the group M84_P105 iCuMMOs. Chapter 3 reviews the development of a primer independent method (magnetic hybridization capture, MHC) for detection of iCuMMO sequences in nature. In Chapter 4 the participation of hydrocarbon monooxygenases and iCuMMOs in alkane and alkene oxidation in nature was analyzed. T-RFLP and next generation sequencing was used to detect the diversity of monooxygenase enzymes involved in the hydrocarbon degradation in rice field soils.

The CuMMOs are a diverse enzyme family. Several bacterial phyla including proteobacteria, Verrucomicrobia, Actinobacteria and the candidate division NC10 are known to possess CuMMOs. The physiological functions of CuMMOs range from the oxidation of methane (pMMO) and ammonium (AMO) to the oxidation of short chained hydrocarbons (e.g. pBMO). In several environmental studies on the field of microbial ecology iCuMMOs sequences were detected by co-amplification (Angel & Conrad, 2009, Holmes *et al.*, 1999, Jia *et al.*, 2007, Knief *et al.*, 2005, Dorr *et al.*, 2010, King & Nanba, 2008, Pacheco-Oliver *et al.*, 2002, Reay *et al.*, 2001). In this thesis the wide distribution of the iCuMMOs in rice field soils and aquatic habitats could be shown (Chapter 1). The iCuMMOs might not be rare in nature as assumed so far but may play an important role in the global cycle of elements. The physiological role of the analyzed iCuMMO groups remains still unknown. A relationship of iCuMMO sequences to sequences of alkane degraders led us suggest that the iCuMMO might be involved in hydrocarbon oxidation (Nakamura *et al*. BAH22833, BAH22839; Redmond *et al.*, 2010, Suzuki *et al.*, 2012) , but no indication could be found that short

alkanes are the main substrates of the iCuMMO (Chapter 2 and 4). However, it could be possible that hydrocarbons are co-substrates of the iCuMMOs that provide electrons for energy yielding mechanisms. Microorganisms possessing the iCuMMO might not grow on hydrocarbons as sole substrates but may benefit from hydrocarbon degradation while growing on other substrates like methane. It could be shown that the growth of methanotrophic bacteria could be stimulated by the co-oxidation of ethane in addition to methane in previous studies (e.g. Malashenko *et al.*, 2000). Another possible role of the iCuMMO could be substrate oxidation under limiting conditions (e.g. methane of oxygen). An alternative function of the pMMO in methanotrophic bacteria was demonstrated earlier in alphaproteobacteria: The isoenzyme pMMO2 is expressed at low methane concentrations (Baani & Liesack, 2008). Another pMMO isoenzyme was found in verrucomicrobial methanotrophs that was expressed under low oxygen concentrations (Khadem *et al.*, 2012). The iCuMMOs could be enzymes that are active under substrate limiting conditions and may therefore provide a selective advantage in certain environments.

Culture independent methods like next generation sequencing provided new insights in the distribution of iCuMMO sequences in rice field soils and aquatic habitats. But these methods cannot provide information about the microorganisms possessing the corresponding proteins or their physiological function. It is not possible to draw a conclusion from the phylogenetic relationship of iCuMMO sequences to pMMO and AMO sequences to a similar substrate range of these enzymes. Studies on bacterial pure cultures are necessary to get explicit insights into the physiological functions of the iCuMMOs. Unfortunately, most iCuMMO groups cannot be related to known microorganism. An exception is the iCuMMO

group M84_P105. Sequences clustering into the M84_P105 group were identified as sequences of the pXMO isoenzyme of alpha- and gammaproteobacteria (Chapter 1, Tavormina *et al*., 2011, Vorobev *et al*., 2014). To study the function of the pXMO, genetic manipulations of methanotrophic strains containing the isoenzyme may be beneficial. In previous studies, knock-out mutants of type II methanotrophs were established to study the function of the pMMO2 isoenzyme. pMMO2 mutants were generated by sequence-specific fusion PCR (Baani & Liesack, 2008). *pmo* genes were deleted by replacing them with an antibiotic marker gene. Comparative incubation studies of mutants and wild type strains revealed a growth of mutants, which only possessed the functional pMMO2 isoenzyme, at low methane concentrations for several months. However, the generation of knock-out mutants is a slow process taking fairly long time. Recently, new genetic systems to establish knock-out mutants in methanotrophic bacteria were published (Puri *et al.*, 2015). They may help to simplify the creation of mutants for further analysis of substrate specificity of the pXMO and other iCuMMOs.

The occurrence of multiple copies of genes coding the pMMO in genomes of methanotrophic bacteria has been reported in several studies (Baani & Liesack, 2008, Dunfield *et al.*, 2002, Op den Camp *et al.*, 2009, Ricke *et al.*, 2004, Stoecker *et al.*, 2006, Stolyar *et al.*, 1999). The pXMO of alpha- and gamma-proteobacteria was first described by Tavormina *et al*. (2011). The operon structure of the pXMO is non-canonically with the gene order *pxmABC.* It varies from the canonically C-A-B order that is found in methane and ammonium oxidizing bacteria (Arp *et al.*, 2007, Op den Camp *et al*., 2009). Predictions about the evolutionary origins of the non-canonically pXMO are difficult to make due to a lack of

sufficient operon datasets. Magnetic hybridization capture hybridization (MHC, Chapter 3) may provide operon datasets of pXMOs for evolutionary analysis. Additionally, operon structures of other iCuMMO groups could be achieved by this method. The non-canonically operon structure of the pXMO seem to be unusual. However, it has been shown earlier that operons of the ammonium monooxygenase (AMO) of Crenarcheota are arranged in alternative orders, too (Nicol & Schleper, 2006). The AMO of Crenarcheota and the iCuMMO are only very distantly related. Nevertheless, it could indicate that alternative operon structures are common within other iCuMMO groups, too. For further evolutionary predictions of the origins and the relationships of iCuMMOs additional operon structures are required.

MHC could also provide sequence information about the different subunits of the iCuMMOs. The PmoA subunit is mostly in the focus of the analysis of CuMMOs. This may be due to the fact that this subunit was believed to contain the active centre of the enzyme for a long time (DiSpirito *et al*., 1992). However, analyses of crystal structures of the pMMO revealed two possible active centers of the pMMO: A dicopper centre coordinated by histidine residues located within the PmoB and a metal centre formed by carboxylate and histidine residues within the PmoC (Hakemian *et al.*, 2008, Lieberman & Rosenzweig, 2005, Smith *et al.*, 2011). Liew *et al.* (2014) used site-directed mutations on a hydrocarbon monooxygenase (HMO) model system to predict the active site of the CuMMOs. They showed that a mutation in the metal binding residue in PmoB leaded to a reduced activity of the enzyme. A mutation in metal centre ligands in PmoC leaded to an inoperable enzyme. The active centre seems to be coordinated by PmoC**.** Additionally, a mutation in a strongly conserved area within PmoC

shifted the substrate preference of the enzyme towards smaller alkanes (Liew *et al.*, 2014). These findings lead to the conclusion that studies on the PmoA or PxmA might not be optimal for function related interpretations. Studies of the PmoC might be more appropriate. Magnetic hybridization capture (MHC) that could be able to capture long target sequences may provide sequences containing complete operons of the iCuMMOs that also contain *pmoC* sequences. This sequence information might give deeper insights in the phylogenetic relationship and the function of the iCuMMOs.

5.1 Outlook and concluding

This PhD work focused on the enzyme superfamily of CuMMOs that was believed to be restricted to methane and ammonium oxidizing bacteria for a long time. Several CuMMOs with alternative substrates or yet unknown functions have been found within the last years (Nakamura *et al*. BAH22833, BAH22839; Redmond *et al*., 2010, Suzuki *et al*., 2012, Tavormina *et al*., 2011, Vorobev *et al*., 2014, Hamamura *et al.*, 1999, Hamamura *et al.*, 2001, Sayavedra-Soto *et al.*, 2011, Swan *et al.*, 2011).

The methanotrophs and the CuMMOs have been already studied for a long time. However, the great potential of CuMMO in methanotrophs and iCuMMOs in nature arise during the last years. A main point that contributes to this fact may be the limitation of molecular techniques. Primers that are often used in environmental studies are only able to detect the

diversity that matches them. A process in detection of a new diversity of iCuMMOs might bring the use of capture probes (Chapter 3) that are not as sensitive to target sequences containing mismatches. However, they only detect matching sequences, too. To be independent on available sequence information for primer and probe design, metagenomic analysis is the method of choice to detect a new diversity of "hidden" CuMMOs (Fierer *et al.*, 2007). Another method that disclaims the use of primers or probes is single cell genomics. It is a powerful tool in environmental ecology that provides large-scale genomic information about individual microorganisms (Rinke *et al.*, 2014). An advantage of this method could be the possibility to link CuMMO sequences to organisms for the whole genome is available for analysis.

The primarily assumption that methanotrophic bacteria are obligate and can only grow on methane, methanol and in some cases on C_1 compounds could be disproven already several years ago. Facultative methanotrophs are known that are able to utilize multicarbon substrates like acetate, pyruvate, succinate, malate and ethanol (Belova *et al.*, 2011, Dedysh *et al.*, 2005, Dunfield *et al.*, 2010). In Chapter 4 we could show that type II methanotrophs could grow under short alkane incubation. If they use these alkanes directly as substrates or if they are part a consortium that degenerate the alkanes could not be discovered, terminatory. Stable isotope probing using $_{13}C$ labeled alkanes as substrates may provide information about the organisms involved in alkane degradation. This could also show if the methanotrophs assimilate the alkanes or use them for energy yield.

Summarized, the potential of the CuMMOs is enormous. We are just at the beginning of getting insights into the diversity and the importance of the CuMMOs that are aside of the

relatively well characterized ammonium and methane monooxygenases. Further studies need to be performed to get new insights in the substrate ranges of the CuMMOs and the organisms possessing these interesting enzymes.

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Erklärung

Ich versichere, dass ich meine Dissertation

“Novel deep branching Cu-containing membrane-bound monooxygenases: distribution and function”

selbstständig 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 ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

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