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From Nature to Nurture:

Isolation, Physiology and Preservation of Methane-Oxidizing Bacteria

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Sven Hoefman – From Nature to Nurture: Isolation, Physiology and Preservation of Methane-Oxidizing Bacteria

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List of abbreviations

AMO	ammonia monooxygenase
ANOVA	analysis of variance
AOB	ammonia-oxidizing bacteria
AUC	area under the curve
BLAST	basic local alignment search tool
CBB	Calvin-Benson-Bassham cycle
CFU	colony forming units
CPA	cryoprotectant
(d)AMS	(diluted) ammonium mineral salts
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethyl sulfoxide
(d)NMS	(diluted) nitrate mineral salts
DO	dissolved oxygen
EM	expectation maximization
FCM	flow cytometry
GC	gas chromatography
GTR	general time reversible
HAO	hydroxylamine oxidoreductase
HRT	hydraulic retention time
ICM	intracytoplasmic membrane
LPA	lyoprotectant
MALDI TOF	matrix assisted laser desorption ionisation time of flight
MS	mass spectrometry
ML	maximum likelihood
MOB	methane-oxidizing bacteria
MOR	methane oxidation rate
MPN	most probable number
NOB	nitrite-oxidizing bacteria

OD	optical density
PAR	photosynthetic active range
PFP	primary facultative pond
PI	propidium iodide
PLFA	phospholipid-derived fatty acids
(P)PCA	(probabilistic) principle component analysis
(p/s)MMO	(particulate/soluble) methane monooxygenase
rep-PCR	repetitive element sequence based polymerase chain reaction
ROC	receiver operating characteristic
RuMP	ribulose monophosphate
(s)COD	(soluble) chemical oxygen demand
SRT	sludge retention time
T-RFLP	terminal-restriction fragment length polymorphism
TSA	trypticase soy agar
TSB	trypticase soy broth
UPGMA	unweighted pair group method with arithmetic averages
VBNC	viable but non-culturable
VSS	volatile suspended solids
WSP	waste stabilization pond
WWTP	waste water treatment plant

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Part A:

General Introduction

Background & Scope

The surface of our planet is warming up. Iconic to this fact is the melting of the polar ice caps, but other direct major implications are observed that affect us all, including extreme drought, loss of biodiversity and an increase in weather extremities, to name a few. The temperature on Earth is governed by a balance of greenhouse gases, such as water vapor, carbon dioxide, methane and nitrous oxide, which absorb solar energy and reradiate it in the form of heat into the atmosphere. For ages, these gases are continuously being produced and broken down by a variety of chemical and biological processes. However, since the industrial revolution the atmospheric greenhouse gas concentration has gone up due to an increase in human-induced emissions. For example, extensive methane emissions are detected from rice paddies, animal husbandry and landfills. All around the world, microbes, termed methanotrophs, are found that can consume this greenhouse gas, and are thus viewed as gatekeepers preventing excessive methane emissions from escaping to the atmosphere. It is of vital importance to understand what factors may positively or negatively impact the methane consuming activity of these organisms. Therefore, tools are needed to efficiently bring and keep these organisms into culture and to catalog and safeguard the methane-oxidizing biodiversity. In the future, this might allow us to manage these human-related methane emissions, by stimulating these organisms to consume methane, which can potentially be coupled to the degradation of groundwater pollutants or the production of high-value biomass.

The work in this thesis describes (i) the successful long-term preservation of methane-utilizing bacteria and confirms the broad applicability of the applied methodology for fastidious bacteria in general, (ii) advocates the use of miniaturized tools for rapid isolation, screening and characterization of these methanotrophs, (iii) discusses the large variability and its implications of important physiological features between closely related strains and (iv) validates the research by depositing all characterized strains in public culture collections and describing several novel methanotrophic taxa.

The research was performed between May 2009 and March 2013 at the Laboratory of Microbiology (LM-UGent), Faculty of Science, Ghent University. The central research themes of this lab are diversity, classification and identification of a wide variety of bacteria, including food-associated, animal and human-associated and environmental strains. In 2009, a project of the Geconcerteerde Onderzoeksacties of Ghent University (BOF09/GOA/005) was initiated in collaboration with the Laboratory of Microbial Ecology and Technology (LabMET), Ghent University. The aim of this project, named "Sustainable methanotrophs: from ecology to microbial resource management", is to develop the concept of managing microbial methanotrophic communities by (i) the transposition and selection of natural methane-oxidizing inocula in diverse conditions, (ii) identifying, isolating and preserving the key microbial partners in these consortia and (iii) exploring methanotroph applications relevant to the society.

As LM-UGent had no prior experience or expertise in working with these organisms, several tools had to be implemented initially in order to perform the experiments described throughout the thesis and to initiate this exciting new research line at the lab.

An introduction to aerobic methane-oxidizing bacteria

METHANE-OXIDIZING BACTERIA

Definition

Methanotrophs are microorganisms defined by their ability to generate energy via the oxidation of the greenhouse gas methane (Semrau et al., 2010). In this way, all methanotrophs fully oxidize CH_4 to CO_2 (Chistoserdova, 2011; Joye, 2012). The organisms that fulfill this definition are remarkably diverse. In anaerobic environments, both archaea and bacteria can oxidize methane. Anaerobic methanotrophic archaea couple methane oxidation by reverse methanogenesis to sulphate, manganese or iron reduction, performed either by themselves or in obligate association with other microbial partners, such as sulphate-reducing bacteria (Joye, 2012; Milucka et al., 2012). The bacterium *Methylomirabilis oxyfera* oxidizes methane in anaerobic environments by producing its own oxygen by nitrite dismutation, thus oxidizing methane aerobically in an anaerobic setting (Ettwig et al., 2010). In ecosystems where oxygen is readily available, obligate aerobic bacteria from the phyla Proteobacteria and Verrucomicrobia have been found to oxidize methane with oxygen as electron acceptor (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008). While for most methanotrophs methane can also be utilized as sole carbon source, the verrucomicrobial methanotrophs grow autotrophically by fixing CO_2, while methane oxidation provides these cells with energy (Khadem et al., 2011).

The work described in this thesis focuses specifically on the aerobic proteobacterial methanotrophs. Throughout the thesis, we use the term aerobic Methane-Oxidizing Bacteria, or MOB in short, to refer to this subset of methanotrophic organisms.

Methane

Methane is the major component of biogas, an important fossil fuel for the industry and households. It is mostly produced through the anaerobic decomposition of organic matter

by methanogenic archaea, a natural process occurring mainly in wetlands (e.g. swamps and bogs) (Thauer and Shima, 2006). Methane can also be produced by plants or geologically within the Earth's crust (Keppler et al., 2006; Etiope, 2009). Methane is also an important greenhouse gas, molecules defined by their ability to capture part of the incoming solar energy by infrared absorption and to re-radiate this energy in the form of heat in the atmosphere (Forster et al., 2007). It has been observed that the concentration of several of these gases, mainly carbon dioxide, methane and nitrous oxide, has increased dramatically since the industrial revolution due to anthropogenic greenhouse gas emissions. As a result, the temperature of the Earth's surface increases, a process commonly termed global warming leading to climate change (Denman et al., 2007; Forster et al., 2007; Isaksen et al., 2011). The combustion of methane as a fuel results in major carbon dioxide emissions. More directly, common anthropogenic activities such as animal husbandry and rice cultivation favor an increase in methanogenic activity, resulting in overall higher atmospheric methane levels (Lelieveld et al., 1998; Forster et al., 2007). Alongside methane sources, several methane sinks exist, both chemically and biologically, that balance atmospheric methane levels (Lelieveld et al., 1998; Conrad, 2009). Methane is oxidized chemically in the troposphere by hydroxyl radicals, while biological methane oxidation is performed by methanotrophs (Hanson and Hanson, 1996; Lelieveld et al., 1998). While wetland soils (including rice paddies) are regarded as the most important source of methane, non-flooded upland soils (e.g. forests and grasslands) form the only net biological methane sink (Le Mer and Roger, 2001; Bodelier and Laanbroek, 2004). Although both methane and carbon dioxide are greenhouse gases, methane oxidation converting methane to carbon dioxide is regarded as a net greenhouse gas sink, as it has been estimated that one methane molecule is 20 to 25 times more efficient as a greenhouse gas than carbon dioxide (Lelieveld et al., 1998; Forster et al., 2007). Therefore, by studying the microbes responsible for biological methane oxidation, we can increase our knowledge of the global carbon cycle in order to better understand the challenges we face by continuously destabilizing the balance of greenhouse gases in the atmosphere.

MOB DIVERSITY & ECOLOGY

Phylogeny & Taxonomy

The first isolation of a methanotroph, *Bacillus methanicus* later renamed to *Methylomonas methanica*, was reported by Söhngen in 1906 and only three more methanotrophs were described in the following period until 1970 (Anthony, 1982). In that year, Whittenbury and colleagues reported the isolation and characterization of over 100 methanotrophic cultures, establishing the basic phylogenetic framework of the proteobacterial methane-oxidizing bacteria (Whittenbury et al., 1970b). Traditionally, the proteobacterial MOB were divided into three groups, type I, II and X MOB, mainly based on (i) the arrangement of their intracytoplasmic membranes, (ii) their pathway for carbon assimilation and (iii) their major cellular fatty acids (Hanson and Hanson, 1996). Currently, with 16S rRNA and functional gene phylogeny, these divisions still hold. The type II MOB are positioned within the *Alphaproteobacteria* and the type I and type X MOB form two distinct clades within the *Gammaproteobacteria*. Because of this, the type I and type X MOB are now sometimes referred to as type Ia and type Ib MOB, respectively. A 16S rRNA gene phylogenetic maximum likelihood tree of the 17 described type strains of the proteobacterial methanotrophic genera is displayed in Figure A-1. Currently, twelve methane-oxidizing genera have been formally described within the family *Methylococcaceae* of the *Gammaproteobacteria*: the genera *Methylomicrobium* (Ia), *Methylosarcina* (Ia), *Methylobacter* (Ia), *Methylomonas* (Ia), *Methylovulum* (Ia), *Methylosoma* (Ia), *Methylosphaera* (Ia), *Methylococcus* (Ib), *Methylogaea* (Ib), *Methylocaldum* (Ib), *Methylohalobius* and *Methylothermus* (Geymonat et al., 2011). Although the latter two genera have been classified as *Methylococcaceae*, these may not be monophyletic with this family (Op den Camp et al., 2009). In addition, filamentous methanotrophs have been discovered within the genera *Crenothrix* and *Clonothrix* of the *Methylococcaceae* family (Stoecker et al., 2006; Vigliotta et al., 2007), although so far no such pure cultures have yet been described. Within the *Alphaproteobacteria* (Type II), the genera *Methylosinus* and *Methylocystis* in the family *Methylocystaceae* (Bowman et al., 1993) and the genera *Methylocella, Methyloferula* and *Methylocapsa* in the family *Beijerinckiaceae* have been described (Dedysh, 2009; Vorobev et al., 2010). The three verrucomicrobial MOB strains that have been found are positioned within the genus *Methylacidiphilum* (Op den Camp et al., 2009).

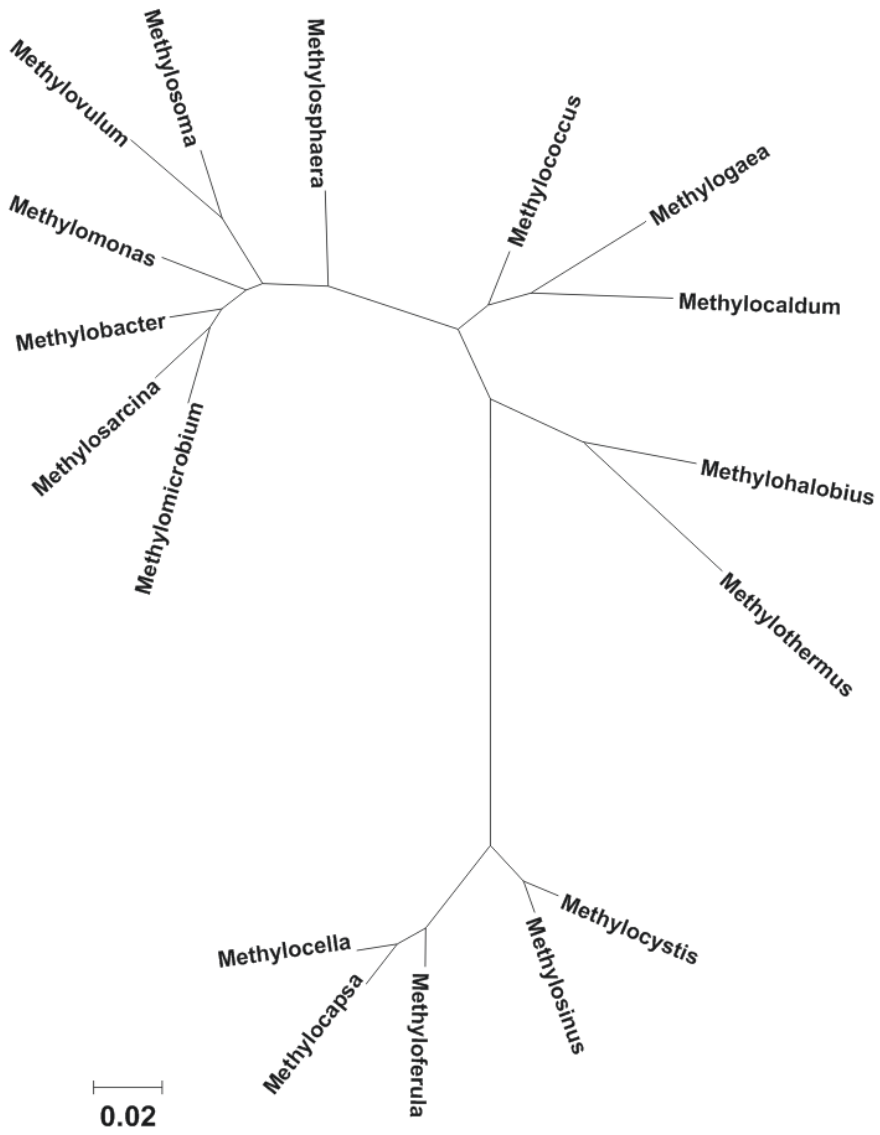

Figure A-1: 16S rRNA gene phylogenetic maximum likelihood tree [1403 nucleotide alignment; GTR substitution model; 1000-replicate bootstrapping; MEGA 5 software (Tamura et al., 2011)] of the 17 formally described type strains of the proteobacterial methanotrophic genera. The top 12 genera represent the gammaproteobacterial MOB, the bottom 5 genera represent the alphaproteobacterial MOB. Bar: 0.02 substitutions per nucleotide position.

Habitats of methane-oxidizing bacteria

Methane-oxidizing bacteria are ubiquitous in nature. In practically all places where methane is produced by methanogens in anaerobic environments such as different soils, sediments or eutrophic lakes, obligate aerobic methanotrophic bacteria are found in the oxic surface layer above the anoxic zone. As such, MOB have been isolated from rivers, lakes, ponds, marine environments, wetlands, rice paddies, ground water, waste water, coal mine drainage water and sludge (Hanson and Hanson, 1996; Semrau et al., 2010). When nitrogen is limited, such as in some natural soils and sediments and freshwater lakes, some

MOB can fix nitrogen by an oxygen-sensitive nitrogenase and are thus found in low dissolved oxygen zones (Hanson and Hanson, 1996). Most of the MOB thus obtained were mesophilic (growth optimum around 25-30°C) and neutrophilic (pH optimum around 7). However, to highlight the physiological diversity of these bacteria, MOB with the following characteristics have also been found:

(i) thermotolerant/philic strains isolated from thermal mud, soil and springs with growth optimum between 42°C to 60°C (Bodrossy et al., 1997; Tsubota et al., 2005; Op den Camp et al., 2009),

(ii) psychrotolerant/philic strains isolated from tundra soil and wetland, groundwater, acidic wetlands and marine low temperature lakes with growth optimum between 6°C to 20°C (Bowman et al., 1997; Kalyuzhnaya et al., 1999; Trotsenko and Khmelenina, 2005; Wartiainen et al., 2006; Dedysh, 2009),

(iii) halotolerant/philic strains isolated from Antarctic marine, hypersaline and soda lakes with NaCl optimum between 2.5 to 9%, requiring sea water or NaCl additions for growth and tolerating up to 15% NaCl amendments (Bowman et al., 1997; Heyer et al., 2005; Kalyuzhnaya et al., 2008),

(iv) alkalitolerant/philic strains isolated from soda lakes with pH optimum between 9 to 10.5 (Kalyuzhnaya et al., 2008),

(v) acidotolerant/philic methanotrophic strains isolated from thermal mud, soil and springs and acidic wetlands with growth optimum at pH 2 to 5.5 (Dedysh, 2009; Op den Camp et al., 2009; Danilova et al., 2012; Kip et al., 2012).

Most notably, the verrucomicrobial MOB are thermophilic growing as high as 65°C and acidophilic growing as low as at pH 0.8 (Op den Camp et al., 2009).

Non-flooded oxic soils (e.g. forest soils) that only receive methane from the atmosphere (1.8 ppmv) also display methane oxidation activity (Denman et al., 2007). Functional gene sequence analysis (*pmoA*-based, see further) detected groups of these so-called high-affinity methanotrophs most closely related to, but clearly distinct from, both alpha- and gammaproteobacterial methanotrophs (upland soil clusters α and γ, mainly present in acidic and neutral forest soils, respectively). These organisms have so far not been brought into pure culture (Kolb, 2009). However, growth at 10 ppmv and activity at atmospheric methane levels has been observed for a cultured *Methylocystis* strain [see further, (Baani and Liesack, 2008)].

Interaction with other organisms

Methanotrophic bacteria have been found to form close, often obligate, interactions with other organisms. Near hydrothermal vents and cold seeps in the deep sea, endosymbiotic gammaproteobacterial MOB have been detected in the tissues of mussels, sponges, snails and tubeworms (Childress et al., 1986; Cavanaugh et al., 1987; Petersen and Dubilier, 2009). These marine invertebrates rely on the methanotrophs to provide them with methane-derived carbon, and in return, the methanotrophs are provided a stable environment with sufficient oxygen and methane to support their growth (Petersen and Dubilier, 2009). These MOB have so far resisted isolation attempts, but would represent a novel genus within the *Methylococcaceae* family based on 16S rRNA and functional gene sequence analysis (Hanson and Hanson, 1996).

In wetlands, mainly alphaproteobacterial MOB were found to oxidize methane in the roots of many aquatic macrophytes. The roots are expected to act as channels from which methane produced in anaerobic zones can escape to the atmosphere. Therefore, MOB can oxidize the methane with oxygen provided by the plants (King, 1994; Hanson and Hanson, 1996; Raghoebarsing et al., 2005). Moreover, it was shown that up to 15% of the carbon uptake of submerged *Sphagnum* mosses was methane-derived as provided by methane oxidation of partly endophytic MOB (Raghoebarsing et al., 2005), a process which occurs all around the world (Kip et al., 2010). Furthermore, MOB were shown to form stable consortia with algae, whereby the algae provide oxygen for methane oxidation and the carbon dioxide thus formed can be fixed by the algae (van der Ha et al., 2011).

MOB can also form the basis of a methane-driven food web, providing carbon to heterotrophs, including heterotrophic bacteria (Hutchens et al., 2004; Murase and Frenzel, 2007; Qiu et al., 2009; Dumont et al., 2011). An unfortunate side effect of such interactions is the problematic purification of some MOB during isolation procedures, as heterotrophic bacteria, often non-methanotrophic methylotrophs such as methanol-utilizing hyphomicrobia, often copurify and can be notoriously hard to get rid of (Bowman, 2006). It is expected that such interactions can be mutually beneficial, as the MOB provides carbon to the heterotroph, while the heterotroph removes toxic methane oxidation intermediates (e.g. methanol) and/or produces stimulating complex compounds (e.g. vitamins) for the MOB (Hanson and Hanson, 1996; Iguchi et al., 2011).

PHYSIOLOGY & BIOCHEMISTRY

Carbon metabolism

A schematic overview of methane oxidation by MOB is presented in Figure A-2. In a first oxidation step methane is converted to methanol catalyzed by a methane monooxygenase (MMO). Two forms of this enzyme have been described, a membrane-associated particulate MMO (pMMO) and a soluble cytoplasmic MMO (sMMO). MMO splits the O-O bond of molecular oxygen using two reducing equivalents, delivered by NADH + H^+ (sMMO) or reduced cytochrome c (pMMO). One oxygen atom is reduced to H_2O while the other is incorporated into methane (CH_4) forming methanol (CH_3OH). Methanol is then further oxidized to formaldehyde (HCHO) by methanol dehydrogenase (Hanson and Hanson, 1996; Dalton, 2005; Semrau et al., 2010; Chistoserdova, 2011).

Formaldehyde can be further oxidized for energy generation or act as the starting point of carbon assimilation. For energy generation, formaldehyde is oxidized to formate (HCOOH) via a tetrahydromethanopterin pathway and formate is oxidized to carbon dioxide by formate dehydrogenase (Chistoserdova, 2011). Gammaproteobacterial methanotrophs assimilate carbon at the level of formaldehyde by the ribulose monophosphate pathway (RuMP), whereby formaldehyde is added onto ribulose monophosphate to generate glucose-6-phosphate. C6 sugars formed in this way are then converted into glyceraldehyde-3-phosphate (C3 molecule) for assimilation, oxidized for NAD(P)H generation and regenerated into RuMP. Alphaproteobacterial methanotrophs assimilate 2 moles of formaldehyde and 1 mole of CO_2 into 1 mole of 2-phosphoglycerate (C3 molecule) by the serine pathway (Hanson and Hanson, 1996; Chistoserdova, 2011). The formation of one C3 molecule requires less energy via the RuMP pathway than via the serine pathway (Anthony, 1982). Therefore, carbon assimilation is more efficient in gammaproteobacterial MOB than in alphaproteobacterial MOB.

Verrucomicrobial methanotrophs cannot assimilate carbon via the RuMP or serine pathway. Instead, these organisms assimilate carbon at the level of CO_2 by the Calvin-Benson-Bassham cycle (Khadem et al., 2011). Although, genes of this cycle have been detected in some proteobacterial MOB, these organisms were negative for growth on CO_2 as carbon source in physiological tests (Hanson and Hanson, 1996; Khadem et al., 2011).

Most MOB possess pMMO, except for members of the genera *Methylocella* and *Methyloferula* which only possess sMMO (Dedysh, 2009; Vorobev et al., 2010). Several MOB possess both pMMO and sMMO, and for these organisms, MMO expression is

regulated by the copper-to-biomass ratio, commonly referred to as the copper switch (Semrau et al., 2010). At higher copper levels (>4 µM), pMMO, a copper-containing enzyme, will be exclusively expressed. At lower copper levels (<0.8 µM), sMMO expression, an enzyme with a diiron center, will mainly occur (Hakemian and Rosenzweig, 2007). Due to the difference in redox potential of the electron donor, cells expressing pMMO (cytochrome c dependent) have a 35% higher growth yield than cells expressing sMMO ($NADH+H^+$ dependent) (Dalton, 2005). Therefore, sMMO expression in these organisms is considered as a survival strategy in environments deprived of copper. Several MOB produce small peptides which function as copper-siderophores or chalkophores (Kim et al., 2004; Semrau et al., 2010). At low copper-to-biomass ratios, these so-called methanobactins are excreted extracellularly to capture copper ions in order to enhance growth of the MOB (Semrau et al., 2010).

In the genomes of several MOB, sequence divergent pMMO encoding genes have been observed alongside the typical pMMO encoding genes. Several gammaproteobacterial MOB possess such a pMMO homolog of yet unknown function, named pXMO (Tavormina et al., 2008; Tavormina et al., 2011). However, in some alphaproteobacterial MOB, another type of divergent pMMO has been shown to be responsible for high affinity methane oxidation in a *Methylocystis* strain. While typical methane monooxygenases oxidize methane with lower apparent half saturation constants ($K_{m(app)}$ > 1 µM CH_4), the 'high affinity' pMMO of the *Methylocystis* strain had an apparent K_m of 0.11 µM, which corresponds with values observed for high affinity methane oxidation in upland soils (Baani and Liesack, 2008).

Besides methane, the proteobacterial MOB can also grow with methanol as sole carbon source (Hanson and Hanson, 1996). However, in a strain-dependent manner, methanol concentrations are toxic at certain levels, due to formaldehyde accumulation. Some strains only tolerate methanol fumes without any methanol actually added to the medium, while other strains can be trained to tolerate up to 5% (v/v) methanol (Bowman, 2006). Some strains can also utilize other C1 compounds, such as methylamine (Bowman, 2006). Only very few MOB utilize carbon sources with C-C bounds, i.e. display facultative methanotrophy (Dedysh et al., 2005). Members of the genus *Methylocella* can utilize acetate, pyruvate, succinate, malate and ethanol, while some strains within *Methylocapsa* and *Methylocystis* also utilize acetate (Dedysh et al., 2005; Dunfield et al., 2010; Belova et al., 2011; Belova et al., 2013). Some *Methylocella* strains show a preference of growth with multicarbon compounds over methane, while the acetate-utilizing *Methylocystis*

strains clearly prefer methane over acetate. Therefore, it has been proposed that acetate utilization could be a survival strategy when methane availability is limited or variable (Belova et al., 2011). As some *Methylocystis* strains also show a high affinity for methane, this could mean that in some non-flooded soils these strains can rely on acetate under normal atmospheric methane levels, while they could switch to methane utilization when methane levels are elevated, e.g. during heavy rainfall (Mohanty et al., 2006).

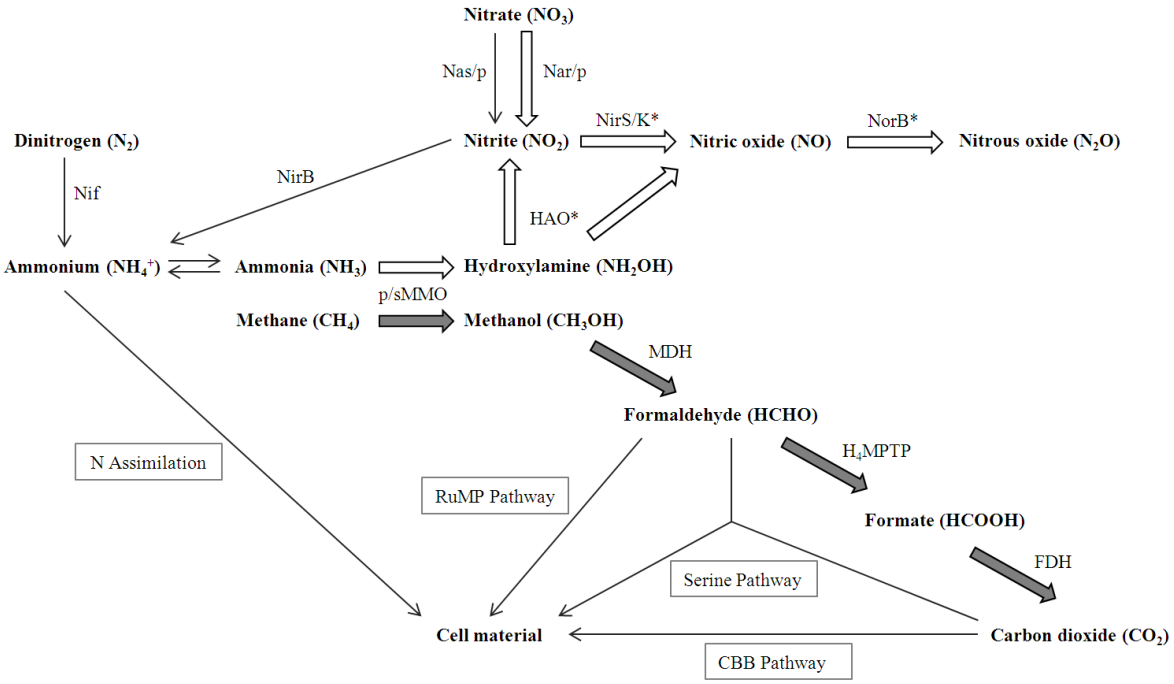

Figure A-2: Schematic overview of the carbon and nitrogen metabolism of MOB with methane monooxygenase as central enzyme. Assimilation (thin arrows), energy generation (grey arrows) and dissimilatory nitrogen conversions (white arrows) are indicated. Arrows between ammonia and ammonium indicate the pH dependent NH_3/NH_4^+ balance (pKa = 9.23). p/sMMO, particulate and soluble methane monooxygenase; MDH, methanol dehydrogenase, H_4MPTP, H_4MPT-linked pathway for formaldehyde oxidation; FDH, formate dehydrogenase; Nif, nitrogenase; NirB/D, assimilatory nitrite reductase; HAO, hydroxylamine oxidoreductase; Nas/p, assimilatory nitrate reductase; Nar/p, dissimilatory nitrate reductase; NirS/K, nitrite reductase; NorB, nitric oxide reductase, *and several other known and unknown enzymes, listed in Stein and Klotz (2011). Note that not all MOB can perform each reaction, most notably, the RuMP (Ribulose monophosphate) pathway is performed by Type I MOB, the serine pathway by Type II MOB, the CBB (Calvin-Benson-Bassham) pathway by verrucomicrobial MOB and the nitrogen metabolism is variable between different MOB. Designed based on Hanson and Hanson (1996), Stein and Klotz (2011) and Chistoserdova (2011).

Genes encoding for subunits of several of the above mentioned enzymes are often used to detect MOB in the environment and to study their functional gene phylogeny: the *pmoA*, *mmoX*, *pxmA* and *mxaF* genes. The *pmoA* gene encodes the 27 kDa peptide of pMMO, the *mmoX* gene encodes the α-subunit of the hydroxylase component of sMMO, the *pxmA*

encodes for the sequence-divergent particulate monooxygenase (pXMO) found in some gammaproteobacterial methanotrophs and the *mxaF* gene encodes the large subunit of methanol dehydrogenase (McDonald et al., 1995; Costello and Lidstrom, 1999; Hutchens et al., 2004; Tavormina et al., 2008). Also non-methanotrophic methylotrophs possess the latter gene, and therefore it is not useful as a detection tool for MOB specifically (McDonald et al., 2008).

Nitrogen metabolism

A schematic overview of the nitrogen and carbon metabolism of MOB is presented in Figure A-2. All MOB can grow with ammonium as sole nitrogen source via ammonium assimilation (Whittenbury et al., 1970b). Most, but not all MOB, can grow with nitrate as sole nitrogen via an assimilatory nitrate and nitrite reductase (Anthony, 1982; Stein and Klotz, 2011). In this way, also nitrite can function as nitrogen source, although nitrite is toxic at elevated levels (mM range) in a strain-dependent manner (Bowman et al., 1993; Hanson and Hanson, 1996).

Several methanotrophs can fix atmospheric nitrogen via an oxygen-sensitive nitrogenase. Although initially mostly Type II and Type Ib were thought to possess this trait (Murrell and Dalton, 1983), some Type I and verrucomicrobial MOB can also fix nitrogen (Auman et al., 2001; Boulygina et al., 2002; Khadem et al., 2010). As nitrogen fixation is an energetically costly process, it is thought to function as a survival strategy when nitrogen is limited, such as in some natural soils, sediments and freshwater lakes (Hanson and Hanson, 1996; Bodelier and Laanbroek, 2004). The *nifH* gene encoding the Fe protein of nitrogenase and the *nifD* gene encoding the α-subunit of dinitrogenase can be used to detect potential for nitrogen fixation in MOB and to study the gene phylogeny (Dedysh et al., 2004c).

The methane-oxidizing bacteria are closely evolutionary related with the ammonia-oxidizing bacteria (AOB) (Hanson and Hanson, 1996; Stein and Klotz, 2011). These lithoautotrophic AOB utilize ammonia and carbon dioxide as sole energy and carbon source, respectively (Koops et al., 2006). AOB catalyze the first step in the nitrification process, converting ammonia to nitrite, and are thus considered key organisms in the global nitrogen cycle, along with the ammonia-oxidizing archaea (Könneke et al., 2005). Their key enzyme, ammonia monooxygenase (AMO), can also oxidize methane although they cannot utilize methane as carbon and energy source (Hanson and Hanson, 1996), therefore, they are not considered to be MOB *sensu stricto*. Likewise, MMO of

methanotrophs can oxidize ammonia, although the process is not coupled to a proton motive force (Stein and Klotz, 2011). Therefore, ammonia is considered to be a competitive inhibitor of MMO for methane oxidation. Furthermore, the intermediate and end product of ammonia oxidation, hydroxylamine and nitrite, respectively, are toxic for MOB when accumulated (Hanson and Hanson, 1996). Therefore, although ammonium can be used to grow all cultured MOB, high ammonia levels inhibit the growth of these organisms. However, some MOB appear unable to oxidize ammonia, while others possess tools to detoxify nitrite and hydroxylamine (Figure A-2), traits which appear to differ largely between methanotrophic genera (Nyerges and Stein, 2009; Nyerges et al., 2010; Campbell et al., 2011). Some MOB detoxify hydroxylamine by hydroxylamine oxidoreductase (HAO), yielding nitrite or nitric oxide. Alternatively, the compound can be re-converted by a hydroxylamine reductase into ammonia (not shown on Figure A-2 for clarity). Nitrite reductase can convert nitrite to nitric oxide, while this product can be converted into nitrous oxide by various nitric oxide reductases (Stein and Klotz, 2011). Nitrous oxide is, along with methane and carbon dioxide, a potent greenhouse gas (Forster et al., 2007). The impact of MOB on the global nitrous oxide production remains unclear as (i) the conditions triggering these reactions are poorly understood, (ii) many of the involved enzymes are still unknown, (iii) the ability to detoxify is unpredictable by phylotype or taxon (Stein and Klotz, 2011). Moreover, MOB form associations with denitrifying bacteria, and can therefore also contribute indirectly to nitrous oxide production (Hanson and Hanson, 1996; Modin et al., 2007). Nonetheless, it is clear that MOB affect both the global carbon as well as the global nitrogen cycle and are therefore key organisms to study these biogeochemical cycles.

In the environment, the effects of nitrogen additions are still poorly understood (Bodelier and Laanbroek, 2004). Sometimes, ammonium addition inhibits methane oxidation activity. As explained above, this can be explained by MMO binding site competition of ammonia or product toxicity of ammonia oxidation (Schnell and King, 1994; Nyerges and Stein, 2009). This effect is likely to be elevated at a higher *in situ* pH, since such conditions shift the NH_3/NH_4^+ balance towards ammonia. Alternatively, non specific ionic effects have been suggested as a cause of activity inhibition, since NH_4Cl showed higher activity inhibition than equinormal $(NH_4)_2SO_4$ levels in forest soils (King and Schnell, 1998). In this way, high nitrate levels can also inhibit methane oxidation (Park et al., 1992; Reay and Nedwell, 2004; Lee et al., 2009; Fender et al., 2012). However, in some cases, nitrogenous fertilization led to enhanced activity, possibly due to MOB community shifts,

a relief of nitrogen limitation and a regulatory role of nitrogen on methane oxidation (Bodelier et al., 2000; Bodelier and Laanbroek, 2004; Mohanty et al., 2006).

Cell structure

Methanotrophic cells appear as rods, curved rods, cocci, coccibacilli, ellipsoidal or are pleomorphic, are typically 1 to 2 µm in cell size and some cells are motile (Bowman et al., 1993). Transmission electron micrographs reveal the presence of intracytoplasmic membranes (ICM, Figure A-3). In gammaproteobacterial MOB, these membranes appear as bundles of vesicular disks (Type I). In *Methylocystis* and *Methylosinus* strains the membranes are aligned parallel to the cell wall (Type II). In *Methylocapsa* strains, a single membrane stack parallel to the long axis of the cell is observed (Dedysh et al., 2002; Dunfield et al., 2010). In *Methyloferula* and *Methylocella* strains, cytoplasmic membrane vesicles are apparent (Dedysh et al., 2000; Dunfield et al., 2003; Dedysh et al., 2004a; Vorobev et al., 2010).

Particulate methane monooxygenase is bound on the intracytoplasmic membranes of pMMO-expressing methanotrophs, thus these membranes have an important function in methane oxidation. In methanotrophs expressing both sMMO and pMMO, these internal membranes develop more intensively when more copper, required for pMMO expression but not for sMMO expression, is available (Hanson and Hanson, 1996).

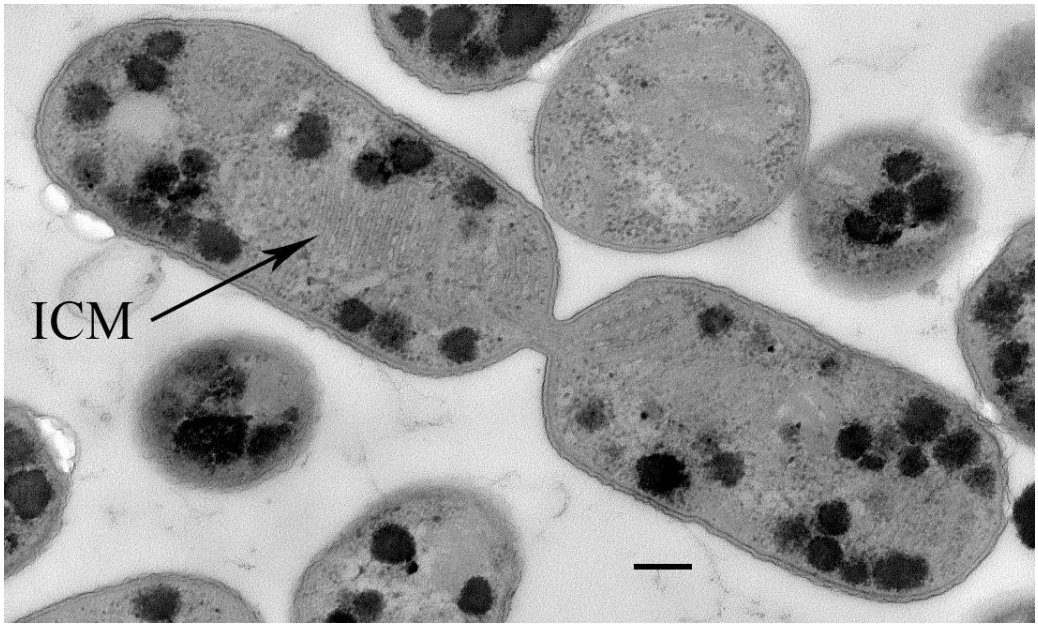

Figure A-3: Transmission electron micrograph of *Methylomonas* strain R-45377 (= LMG 26260) isolated in this study, showing rods that multiply by binary fission and possess intracytoplasmic membranes (ICM) that appear as stacks of discs, a typical feature of gammaproteobacterial MOB. Bar: 0.2 µM.

The initial division of the proteobacterial MOB into two types was also supported by cellular phospholipid-derived fatty acids (PLFA) profiles (Hanson and Hanson, 1996). Type I MOB (Gammaproteobacteria) mainly contain C-16 PLFA's while Type II MOB (Alphaproteobacteria) mainly contain C-18 PLFA's. Furthermore, both groups have signature PLFA biomarkers: $C_{16:1}$w8 and $C_{16:1}$w5 for Type I MOB and $C_{18:1}$w8 for Type II MOB. The specific PLFA profiles are used to differentiate methanotrophic species and assign pure cultures to a type. Additionally, methane labeling studies can be used to detect which type of MOB grows preferentially in different situations (McDonald et al., 2008). However, caution is advised when using such methods as exceptions to the above mentioned rules occur. *Methylothermus* and *Methylohalobius* strains (Gammaproteobacteria) possess significant C-18 fractions (Heyer et al., 2005; Tsubota et al., 2005), while *Methylocystis heyeri* (Alphaproteobacteria) strains contain significant $C_{16:1}$w8 fractions, a fatty acid long thought to be unique to Type I MOB (Dedysh et al., 2007).

Many MOB, but not all, form different types of resting stages (Bowman et al., 1993). Whereas vegetative cells die off within days when they are deprived of methane (Bowman, 2006), all forms of resting stages survive for weeks without methane (Whittenbury et al., 1970a), providing a useful survival strategy in environments with variable methane levels. *Methylosinus* cells elongate and form 'pear' and 'comma' shapes when entering the stationary phase, after which spores are budded off. These exospores are heat-resistant (surviving 15 minutes at 85°C) and desiccation-resistant (surviving at least 18 months in a dried state deprived of methane). Under optimal conditions, spores germinate within days up to two weeks. Besides spores, some MOB also form cysts, of which three types have been observed: lipid cysts (*Methylocystis* strains), *Azotobacter*-type cysts (*Methylobacter* strains) and immature cysts (*Methylomonas* and *Methylococcus* strains). While the lipid and *Azotobacter*-type cysts provide resistance to desiccation but not to heat, the immature cysts are neither desiccation nor heat resistant and seem to only provide survival in situations of famine (Whittenbury et al., 1970a).

CULTIVATION

Headspace

As mentioned above, MOB require two gases, methane and molecular oxygen, in the first step of methane oxidation. Therefore, liquid cultivation is performed in gastight bottles sealed with a rubber stopper, while cultivation on plates is performed in gastight jars and/or desiccators. Usually, the headspace is composed of 20 to 50 % methane in air. Similar methane oxidation rates are observed in this range (Whittenbury et al., 1970b; Bowman, 2006). From a practical point of view, 5 to 15 % methane concentrations in air are usually avoided, since such gas mixtures are explosive. Growth of the MOB is supported by normal oxygen levels in air (21%). However, these oxygen levels, combined with excessive shaking, can result in severe oxidative stress for some methanotrophs (Vorob'ev and Dedysh, 2008; Medvedkova et al., 2009). Therefore, it has been suggested that incubation at different oxygen levels can enrich different MOB populations (Bussmann et al., 2006). Both methane and oxygen are poorly soluble in aqueous solutions. Indeed, Henry's law constants at 25°C of 0.0014 M/atm for CH_4 and 0.0013 M/atm for O_2, compared to e.g. 0.032 M/atm for CO_2 were reported (Sander, 1999). Growth of MOB is thus expected to be limited by the low solubility of CH_4 and O_2 and the slow mass transfer of these gases to the liquid phase, resulting in generation times of 3 hours or more in a strain-dependent manner (Whittenbury et al., 1970b). Some MOB are also cultivated with CO_2 amended to the headspace in various concentrations (% level), since verrucomicrobial MOB rely on CO_2 as a carbon source (Khadem et al., 2011) and type II MOB also incorporate CO_2 via the serine pathway (Figure A-2).

Media

Since methane is provided to the headspace, MOB are usually cultivated in carbon-free mineral media. As an alternative to methane, methanol can be added to the medium (Bowman, 2006), or in the case of facultative MOB, the addition of multicarbon compounds is possible (Dedysh et al., 2005). Nitrate and/or ammonium is usually amended as nitrogen source, giving rise to widely used media such as nitrate mineral salts (NMS) medium and ammonium mineral salts (AMS) medium (Whittenbury et al., 1970b). Depending on the desired expression of sMMO or pMMO, low (<1 µM) or higher (>1 µM) copper levels are amended to the media, respectively. A strong phosphate buffer (>2 mM) is added, since CO_2 production and ammonia oxidation lead to acidification (Whittenbury

et al., 1970b; Koops et al., 2006). Additional compounds such as iron, $MgSO_4$ and $CaCl_2$ and a trace elements solution are added (Bowman, 2006). Growth via nitrogen fixation is assessed in nitrogen-free mineral media under low oxygen tension with methane added to the headspace (Auman et al., 2001). To enhance isolation success, the composition of the mineral media can be adapted depending on the natural environment (e.g. pH and salt composition), since MOB are physiologically diverse as described above and some strains for example require sea water or the addition of vitamins for growth (Bowman et al., 1993; Bowman et al., 1997; Dedysh et al., 1998).

Isolation

MOB are typically not the most dominant population in natural environments, as for example heterotrophic bacteria can rapidly outgrow the methanotrophs (Hanson and Hanson, 1996; Bowman, 2006). Therefore, samples are usually enriched in carbon-free media with methane in the headspace as sole carbon source. After one or several enrichment steps, cultures are serially diluted onto plates (e.g. NMS agar plates). Following incubation, separate colonies are picked up and spread onto new plates and subcultured to purity. In practice, purification is laborious and sometimes problematic since (i) non-methanotrophic heterotrophs can grow on traces of carbon and thus can be falsely mistaken for MOB and (ii) some heterotrophs, typically methylotrophs, tend to co-purify with the methanotrophs. The ability to oxidize methane of the cultures is assessed by gas chromatography monitoring the drop in methane and oxygen levels and the subsequent rise in carbon dioxide levels. Cultures positive for methane oxidation and appearing to be pure as determined by standard tests, should be further confirmed for absence of growth on different carbon-rich media to ensure the absence of a co-purifying heterotroph. Although, facultative MOB have been described, so far no MOB has been found that grows on carbon-rich media such as trypticase soy agar or nutrient agar in the absence of methane (Dedysh et al., 2005). When an MOB appears to grow facultatively, this finding needs to be confirmed with additional tests (Dedysh et al., 2004b).

Identification & Taxonomy

The goal of taxonomy (from *taxis*, meaning order in Greek) is to create order to deal with the astonishing complexity of microbial diversity. Order is created by a classification system and by giving a unique name to a group of isolates with similar characteristics. This provides context when communicating with other scientists as well as allows to extrapolate

information gathered for a certain strain to similar strains with a reasonable degree of certainty (Moore et al., 2010). Ideally, the order system is based on a combination of evolutionary relationship between organisms (phylogeny) supported by phenotypic traits. In practice, this is often difficult to achieve, for example due to horizontal gene transfer or when very closely related strains reveal a distinct phenotype (e.g. pathogenic versus non-pathogenic strains).

Currently, a bacterial species is defined as a group of strains that (i) share similar phenotypic and chemotaxonomic characteristics, (ii) share a whole genome DNA relatedness above 70% as measured by DNA-DNA hybridizations, and (iii) have a 16S rRNA gene sequence similarity above 97% to 98% (Coenye et al., 2005; Konstantinidis and Tiedje, 2005; Stackebrandt and Ebers, 2006). Conversely, a researcher can motivate the description of a novel species when a group of strains is isolated that meets the above-mentioned criteria amongst each other, but are distinct from other known species. This means that based on a comparison with the type strains of the most closely related species, these type strains show (i) a 16S rRNA gene relatedness below 97% to 98%, (i) a DNA-DNA based genome relatedness below 50% to 70% and (iii) distinct phenotypic characteristics compared with the novel strains. For higher taxonomic ranks, e.g. genus level, clear definitions are lacking, and are mostly based on an evaluation of 16S rRNA gene phylogeny of the most closely related genera and their species supported by phenotypic characteristics (Konstantinidis and Tiedje, 2007).

For identification of MOB or potential description of a novel methanotrophic species, the above mentioned polyphasic approach is applied (Vandamme et al., 1996). Morphological techniques are performed, such as determination of cell shape and size, motility and type of flagella, colonial characteristics, pigmentation, resting stage formation and their characteristics, determination of ICM type by transmission electron microscopy and gram staining. Furthermore, physiological and biochemical characteristics are assessed: pH range and optimum, temperature range and optimum, salt tolerance, carbon and nitrogen source utilization. PLFA analysis, a chemotaxonomic technique, is performed by FAME-GC. The presence of several genes is analyzed and their phylogeny is evaluated: 16S rRNA gene, *pmoA*, *mmoX*, *nifH*, *pxmA* and *mxaF*. The %G+C content is analyzed by HPLC and DNA-DNA hybridizations should be performed when comparing strains that share over 97% 16S rRNA gene relatedness.

Additional tests can be advised based on a comparison of the available data of the most closely related species. Chemotaxonomic techniques besides PLFA can be included but

mostly do not have sufficient resolution to distinguish species or even genera (e.g. quinone analysis). Additionally, enzyme tests can be performed determining the carbon assimilation pathway (RuMP, serine and CBB pathway), although this is usually also governed by a higher taxonomic level (Semrau et al., 2010).

Potential for biotechnology

Methane-oxidizing bacteria have many potential biotechnological applications, mainly in three fields: (i) biological mitigation of the greenhouse gas methane, (ii) production of high-value products from methane and (iii) bioremediation of pollutants (Jiang et al., 2010; Wendlandt et al., 2010).

Certain anthropogenic activities, such as rice cultivation, animal husbandry, landfill sites and waste water treatment, stimulate anaerobic methane production by methanogens (Lelieveld et al., 1998; Thauer and Shima, 2006; Forster et al., 2007). Therefore, atmosphere methane levels have been rising since the industrial age, contributing to global warming and climate change (Forster et al., 2007). Methane emissions in landfills can be effectively mitigated by the use of biocovers, permeable material, e.g. compost, amended over the surface of a landfill to stimulate methane oxidation activity by MOB (Gebert and Grongroft, 2006; Scheutz et al., 2009). Biofilters can also be applied, for example in animal breeding farms, whereby oxygen and the to-be-treated methane-rich gas mixture are pumped over the filter containing methanotrophs embedded on packing material (Petersen et al., 2009; Semrau et al., 2010). In wetland-type areas, e.g. rice paddies, such applications are more difficult to achieve given the vast area over which methane is produced. Here, it is vital to understand the factors that limit methane oxidation in order to stimulate activity efficiently: e.g. nitrogenous fertilization can enhance activity due to a relief of nitrogen limitation in some situations (Bodelier and Laanbroek, 2004; Mohanty et al., 2006).

MOB also show potential for the production of biomass or high-value products. Methanotrophs can be applied for the production of single-cell protein which can be used as a protein source in the animal feed market (Dalton, 2005). This approach is attractive as methane is relatively cheap as a substrate and methanotrophic biomass is rich in essential amino acids. Industrial efforts have been made by Norferm Denmark A/S producing single-cell protein, named BioProtein, by a mixed culture of the methanotroph *Methylococcus capsulatus* Bath with heterotrophs responsible for the removal of metabolites inhibiting the MOB (Bothe et al., 2002). However, currently the process is not cost-efficient, in part due to the slow mass transfer of methane to the liquid phase and the

low solubility of methane and oxygen. Perhaps, these challenges can be overcome in the future, as for example the addition of paraffin oil has been observed to promote mass transfer (Han et al., 2009). Alternatively, the low substrate-specificity of methane monooxygenases can be exploited for high-value product formation by co-metabolism, i.e. substrate conversion in the obligate presence of methane (Dalton, 2005; Trotsenko et al., 2005). For example, propene can be converted into the more valuable product epoxypropane. However, in many cases the substrate, intermediates and/or end-products can be toxic to the applied methanotrophic strains, resulting in the requirement of reactor modifications reducing cost-efficiency. However, if methanotrophic strains were to be found with a higher tolerance to such metabolites, or in the specific case of epoxypropane, if a strain would produce an enantiopure form of the epoxide, methanotrophic product production by co-metabolism might become economically viable (Dalton, 2005). Some methanotrophs have also been shown to produce other high-value products such as ectoine or the biopolymers polyhydroxybutyrate and polyhydroxybutyrate/valerate (Trotsenko et al., 2005; Helm et al., 2006; Zhang et al., 2008; Reshetnikov et al., 2011). The production of these and potentially new products by methanotrophs could be exploited in the future.

The low substrate specificity of sMMO and, to a lesser extent, pMMO can also be applied for bioremediation purposes (Jiang et al., 2010; Wendlandt et al., 2010; Semrau, 2011). For example, both MMO's have been shown to degrade trichloroethylene, a major groundwater pollutant. As explained above, such co-metabolism processes can be toxic to the MOB, due to enzyme competition with methane or toxic product formation. Although traditionally most biodegradation experiments focused on sMMO expressing cells, as these have a broader substrate specificity and degrade components like trichloroethylene much faster, pMMO expressing cells seem to be more resilient to these toxic effects and are potentially more useful for trichloroethylene degradation on a longer term (Semrau et al., 2010). Therefore in the future, the focus of bioremediation might switch towards specific MOB strains that are more stable under these harsh conditions.

MAINTENANCE & PRESERVATION

Cryopreservation

Two main approaches are used for successful long-term (years) preservation of bacteria: cryopreservation and lyophilization (Tindall, 2007). In cryopreservation, low temperatures are applied to induce inactivity by freezing while cells remain alive and can be resuscitated by thawing to regain activity (Gao and Critser, 2000). The fate of a cell by lowering the temperature goes as follows. Depending on the solutes present that can depress the freezing point, this point will be reached a few degrees below 0°C. Below the freezing point, supercooling will occur: while the solution is below its freezing point, no ice crystals are yet formed. When further lowering the temperature, extracellular ice will be formed spontaneously. This occurs before any intracellular ice can be formed. The extracellular formation of ice concentrates the solutes extracellularly, causing water to escape from the cells. This dehydration of the cells causes a concentration of solutes intracellularly, rendering the cells no longer supercooled, and thus preventing intracellular ice to be formed spontaneously upon further lowering the temperature (Mazur, 1984). This appears to be crucial for survival of cells: intracellular ice formation results in immediate cell death (Karlsson et al., 1993). Therefore, it is essential that cooling does not happen too fast, since this would not allow sufficient time to lose the supercooled state and cells would freeze internally (Mazur, 1984). Although rarely applied in bacteriology, equipment exists for controlled cooling of cells, e.g. at a rate of 1°C per minute (Shu et al., 2010). As the temperature is lowered further at a sufficiently slow rate, more extracellular ice is formed, leading to further dehydration of cells and concentration of solutes up to a solute saturation point. Below this eutectic point, solutes can also crystallize resulting in strong solution stress and injury to cells. Finally, below -135°C, the glass-transition temperature of water is reached: traces of liquid water within the dehydrated cells are converted into a ‘glassy’ state. In this state, cells that survived the freezing process have been calculated to remain intact for thousands of years. In fact, it is not the low temperatures at such that harm cells, but rather reaching these low temperatures (cooling) firstly, and then afterwards traversing the same temperature gradient during thawing (resuscitation). Resuscitating cultures should be performed rapidly, as small ice crystals tend to collide causing re-crystallization intracellularly, when given sufficient time to do so (Mazur, 1984).

Thus, four main causes of cell injury are detected upon freezing and resuscitating the cells: (i) extracellular ice formation which can crush cells, (ii) dehydration of cells which can

destabilize membranes and proteins, (iii) solution effects due to high salt concentrations, (iv) intracellular ice formation resulting in immediate cell death (Gao and Critser, 2000; Hubalek, 2003). To facilitate the cells on their journey towards the safe 'glassy' state, cryoprotectants (CPA) are added (Fuller, 2004). The most well-known and applied CPA in bacteriology is glycerol. However, DMSO appears superior over glycerol in protecting both bacterial and eukaryotic cells, but is toxic to cells (and humans) at room temperature (Hubalek, 2003). Both CPA's can penetrate the cells, although DMSO penetrates cells much faster, and their protective capabilities are diverse and attributed to different properties, including: (i) depression of the freezing point, (ii) protection against dehydration due to their hydrophilic nature, (iii) reduction in salt toxicity, (iv) prevention of intracellular ice formation and (v) formation of a gel-type state below the eutectic point preventing hyperosmotic injury (Hubalek, 2003; Fuller, 2004). Also non-permeable compounds such as several sugars, proteins and polymers, can function as efficient CPA's, by water retention and the formation of a viscous layer on the cell surface inhibiting growth of ice crystals. Often a combination of permeable and non-permeable CPA's proves to be successful (Hubalek, 2003; Morgan et al., 2006).

Following the above, it is clear that only temperatures below -135°C offer stable long-term cryopreservation. This can be achieved by freezing cells in liquid nitrogen (-196 °C) containers, above the liquid phase. However, often bacteria are also preserved at -20°C and at -80°C. While the latter can be recommended for preservation of cultures up to a maximum of a few years, the former is never recommended (Heylen et al., 2012).

Another type of cryopreservation is vitrification (Kasai et al., 1990; Kuwayama et al., 2005). This process is achieved by rapid cooling (10,000 °C min^{-1}) of cells to below the glass transition temperature using a highly concentrated vitrification solution, comparable with the above mentioned CPA. Due to rapid cooling, ice formation is completely avoided and the solution turns to its glassy state below -135°C ensuring successful long-term preservation. Needless to say, this process requires specialized equipment employing very small volumes to ensure successful vitrification. Although for bacteria, cells are just placed in -80°C freezers or in liquid nitrogen containers (Figure A-4; A, B, C), both freezing with controlled slow cooling rates, or extremely rapid cooling rates (vitrification) has resulted in successful preservation of many types of eukaryotic cells, such as oocytes, spermatozoa, embryos, stem cells, blood cells, cell lines and plant seeds (Mazur et al., 2008; Engelmann, 2011; Gonzalez et al., 2012).

Lyophilization

An alternative to cryopreservation of bacterial cells is lyophilization or freeze-drying (Adams, 2007), which is typically performed in three steps (Morgan et al., 2006). Cells are initially frozen, subsequently, ice is removed by sublimation to water vapor by lowering the pressure and in a second drying step at ambient temperature residual moisture is removed. The following program is applied by the BCCM-LMG Bacteria collection: initial freezing at -50°C for 60 min, following by primary drying at -18°C, 0.5 mbar, 410 min and secondary drying at 20°C, 0.012 mbar, 746 min. The ampoules thus obtained can be kept in the dark at 4°C (Figure A-4; D). As such, lyophilization requires specialized equipment and expertise and is therefore mainly applied solely by culture collections. Although lyophilization offers the advantage of easy distribution and low maintenance costs, cryopreservation is more widely applicable as it is easy to perform and requires limited expertise and equipment (Heylen et al., 2012).

Figure A-4: Liquid nitrogen containers, cryotubes, and racks for cryopreservation at temperatures below -135°C (A, C). Cryopreservation at -80°C in electrical freezers (B). Lyophilization equipment and freeze-dried ampoules (D). Pictures obtained via the BCCM LMG Bacteria Collection.

During the lyophilization process, cells are exposed to similar harsh conditions, both freezing and dehydration, as explained for cryopreservation. Therefore, similar protectants are applied, termed lyoprotectants (LPA), although usually these protectants focus on desiccation resistance (Morgan et al., 2006). A well-known sugar that offers protection against drying is trehalose, which stabilizes proteins and membranes, has a high glass-transition temperature and promotes the formation of an amorphous glassy state (Leslie et al., 1995; Arguelles, 2000; Streeter, 2003; Morgan et al., 2006; Hengherr et al., 2008). The desiccation properties of trehalose are also applied in nature. For example, tardigrades (i.e. small invertebrate animals commonly called water bears) can enter a complete state of suspended animation by desiccation, i.e. anhydrobiosis (Hengherr et al., 2008). In this state, water bears shrink and curl up to a small ball and can withstand complete dehydration by trehalose accumulation. This gives the water bears a remarkable resistance against extremely harsh conditions of varying temperature, pressure and radiation, and as such these organisms even survive space vacuum (Jonsson et al., 2008).

Public Culture Collections

Public culture collections are mostly public funded institutions that safeguard thousands of strains available for the scientific community upon request (Emerson and Wilson, 2009). A researcher can deposit novel isolates with interesting features in a collection, to ensure his strains do not get lost and to allow other researchers to further characterize the strains (Smith, 2003; Janssens et al., 2010). Additionally, most collections offer a wide variety of services, such as strain identification and phenotypic characterization. Moreover, since collections usually specialize in specific bacterial groups (e.g. pathogenic strains), they possess invaluable in house expertise on these organisms. Furthermore, when describing a novel taxon, researchers are obliged to deposit a type strain in at least two public collections in different countries in order for the taxon to become widely accepted. Strains used for a patent application also need to be deposited, although these are obviously not made publically available. Sadly, many strains were never deposited in a collection, some of which are no longer extant, making it impossible for other researchers to verify previously obtained results or difficult to compare closely related strains in taxonomic studies. Even if strains remain extant, continuous culturing and/or distribution between different research labs holds great risk for (i) change of culture over time due to gene mutations or loss of plasmids, (ii) contamination of strains and (iii) mixing up strains. In contrast, public collections function with a certified quality management system to ensure

that such human errors are limited. Finally, also mixed cultures can be deposited, although currently a collection cannot guarantee identical culture composition or function upon resuscitation (Heylen et al., 2012).

Preservation of Methane-Oxidizing Bacteria

Prior to the experiments performed in this study, only a very limited amount of data was available regarding the long-term preservation of MOB, or fastidious organisms in general (Bowman, 2006; Koops et al., 2006). Reports on freeze-drying of MOB were mostly unsuccessful, and cryopreservation was usually only successful for short periods, e.g. several months (Green and Woodford, 1992; Bowman, 2006). This latter finding is strange, considering the above explained mechanism behind cryopreservation: freezing and thawing as such determine survival mostly, while freezing cells a few months longer should matter little when preserving cells in liquid nitrogen containers or in stable -80°C freezers. Large strain variations are observed, which can perhaps be attributed to the formation of different resting stages by different MOB. In general, preservation conditions appear randomly chosen, and methods applied are not standardized, rendering it very difficult to compare or apply data from literature. Therefore, many of these cultures could only be maintained effectively by periodic sub-cultivation or were kept at low metabolic rates (4°C, under a methane:air or N_2 atmosphere) (Bowman, 2006). As a result, reference works such as The Prokaryotes and Bergey's Manual of Systematic Bacteriology list numerous methanotrophic taxa that no longer have extant representatives. Literature search of the preservation of other fastidious functional bacterial groups, such as ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), yields a similar amount of limited data. For both AOB and NOB, a lack of a suitable preservation protocol hampers the recognition of novel taxa since type strains first need to be successfully incorporated by culture collections before novel species can be described (A. Pommerening-Röser and E. Spieck, personal communication). Unfortunately, despite its importance for both applied and environmental microbiology, assessment of long-term preservation success of these and other microorganisms is often neglected.

Aims & Outline

Part of the PhD title “From Nature to Nurture” summarizes best the central theme of the work presented, in the sense that the focus lay on nurturing or taking care of MOB both in culture and during storage as well as on isolating methanotrophic strains from nature.

The research aimed at (i) improving the efficiency of the isolation of methanotrophic strains dominant in methanotrophic enrichments, (ii) formally describing isolates that belong to novel methanotrophic species and/or genera, (iii) revealing a large strain dependency in regard to the nitrogen metabolism and growth preference of closely related MOB within a genus, and (iv) establishing the use miniaturized cultivation as a simple tool to rapidly gather valuable information for a set of MOB regarding their growth in pure culture or when interacting with heterotrophs. A second part of the thesis aimed at establishing a standardized cryopreservation and freeze-drying protocol that enables the successful long-term preservation of the cultured methanotrophic diversity. In addition, the general applicability of the best preservation conditions thus obtained, was evaluated on a diverse set of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria.

The presented data is divided over three main research parts: Isolation of methane-oxidizing bacteria (Part B), Physiology of methane-oxidizing bacteria (Part C) and Preservation of methane-, ammonia- and nitrite-oxidizing bacteria (Part D). Each part is represented by several chapters, and at the end of each part a reflection and discussion section is included:

Part B - Isolation of methane-oxidizing bacteria

In **Chapter I**, the isolation procedure by conventional dilution plating was compared with miniaturized extinction culturing. Via culturing in 96-well microtiter plates several MOB were rapidly isolated from four different environments. Many isolates were immediately pure, making laborious purification redundant.

In **Chapter II**, the efficiency of miniaturized extinction isolation was confirmed by rapidly isolating MOB from the three major types of proteobacterial methanotrophs (Type Ia, Ib and II) from a facultative pond in South Africa.

Part C - Physiology of methane-oxidizing bacteria

In **Chapter III**, it was demonstrated that a miniaturized approach for medium optimization can be a simple tool to quickly generate strain-specific growth preference data. Batch cultivation in optimized media confirmed an increase in growth activity or cell yield for several strains.

In **Chapter IV**, a high physiological diversity regarding the nitrogen metabolism of 14 closely related strains within *Methylomonas* was observed. Several strains showed a high tolerance to salt, ammonium, nitrate, nitrite and hydroxylamine amendments, fixed atmospheric nitrogen and produced nitrous oxide and nitrite from nitrate and ammonium media.

In **Chapter V**, co-cultivation experiments of methanotrophs and heterotrophs revealed a strain-dependent preference of methanotrophs to show enhanced growth parameters with certain heterotrophs. Data mining tools were applied to group the organisms according to their interaction patterns, and to explore the potential to predict the outcome of unknown co-cultivation combinations.

In **Chapter VI**, a proposal for the description of a novel methanotrophic genus is presented: *Methyloparacoccus murrellii*, isolated from pond water in South Africa and Japan.

In **Chapter VII**, a proposal for the description of a novel species within the genus *Methylomonas* is presented: *Methylomonas lenta*, isolated from manure and a denitrification tank in Belgium.

Part D – Preservation of methane-, ammonia- and nitrite-oxidizing bacteria

In **Chapter VIII**, it was shown that long-term preservation induced a viable but non-culturable state in a diverse set of MOB type strains. The MOB could be significantly resuscitated from the VBNC state using the TT preservation medium. All MOB were successfully preserved by cryopreservation and by lyophilization.

In **Chapter IX**, the most successful cryopreservation conditions obtained in Chapter 8 for MOB, were confirmed for their broad applicability by successfully preserving a diverse set of ammonia-oxidizing bacteria.

In **Chapter X**, the first report on the successful preservation of a diverse set of nitrite-oxidizing bacteria is presented, based on results obtained in Chapter 8. Additionally, the influence of carbon and DMSO concentration for preservation of these organisms was assessed.

Following Part D, general conclusions are formulated, and a summary of the work is provided.

References

Adams, G. 2007. The Principles of Freeze-Drying. In *Cryopreservation and Freeze-Drying Protocols*. Day, J., and Stacey, G. (eds). Totowa, New Jersey: Humana Press, pp. 15-38.

Anthony, C. 1982. Biochemistry of methylotrophs. London: The Academic Press.

Arguelles, J.C., 2000. Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. Arch Microbiol 174, 217-224.

Auman, A.J., Speake, C.C.,Lidstrom, M.E., 2001. nifH sequences and nitrogen fixation in type I and type II methanotrophs. Appl Environ Microbiol 67, 4009-4016.

Baani, M.,Liesack, W., 2008. Two isozymes of particulate methane monooxygenase with different methane oxidation kinetics are found in Methylocystis sp strain SCZ. Proc Natl Acad Sci U S A 105, 10203-10208.

Belova, S.E., Baani, M., Suzina, N.E., Bodelier, P.L.E., Liesack, W.,Dedysh, S.N., 2011. Acetate utilization as a survival strategy of peat-inhabiting Methylocystis spp. Environmental Microbiology Reports 3, 36-46.

Belova, S.E., Kulichevskaya, I.S., Bodelier, P.L., Dedysh, S.N., 2013. *Methylocystis bryophila* sp. nov., a facultatively methanotrophic bacterium from acidic Sphagnum peat, and emended description of the genus Methylocystis (ex Whittenbury et al. 1970) Bowman et al. 1993. Int J Syst Evol Microbiol 63, 1096-1104.

Bodelier, P.L., Roslev, P., Henckel, T.,Frenzel, P., 2000. Stimulation by ammonium-based fertilizers of methane oxidation in soil around rice roots. Nature 403, 421-424.

Bodelier, P.L.E.,Laanbroek, H.J., 2004. Nitrogen as a regulatory factor of methane oxidation in soils and sediments. FEMS Microbiol Ecol 47, 265-277.

Bodrossy, L., Holmes, E.M., Holmes, A.J., Kovacs, K.L.,Murrell, J.C., 1997. Analysis of 16S rRNA and methane monooxygenase gene sequences reveals a novel group of thermotolerant and thermophilic methanotrophs, Methylocaldum gen. nov. Arch Microbiol 168, 493-503.

Bothe, H., Jensen, K.M., Mergel, A., Larsen, J., Jorgensen, C.,Jorgensen, L., 2002. Heterotrophic bacteria growing in association with Methylococcus capsulatus (Bath) in a single cell protein production process. Appl Microbiol Biotechnol 59, 33-39.

Boulygina, E.S., Kuznetsov, B.B., Marusina, A.I., Turova, T.P., Kravchenko, I.K., Bykova, S.A. et al., 2002. [Study of nucleotide sequences of nifH genes in methanotrophic bacteria]. Mikrobiologiia 71, 500-508.

Bowman, J. 2006. The Methanotrophs - The Families *Methylococcaceae* and *Methylocystaceae*. Chapter 3.1.14. In *The Prokaryotes, third ed.* Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E. (eds), pp. 266-289.

Bowman, J.P., Sly, L.I., Nichols, P.D.,Hayward, A.C., 1993. Revised taxonomy of the methanotrophs - Description of *Methylobacter* Gen-Nov, emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group-I methanotrophs. International Journal of Systematic Bacteriology 43, 735-753.

Bowman, J.P., McCammon, S.A.,Skerratt, J.H., 1997. *Methylosphaera hansonii* gen nov, sp nov, a

psychrophilic, group I methanotroph from Antarctic marine-salinity, meromictic lakes. Microbiology 143, 1451-1459.

Bussmann, I., Rahalkar, M.,Schink, B., 2006. Cultivation of methanotrophic bacteria in opposing gradients of methane and oxygen. FEMS Microbiol Ecol 56, 331-344.

Campbell, M.A., Nyerges, G., Kozlowski, J.A., Poret-Peterson, A.T., Stein, L.Y.,Klotz, M.G., 2011. Model of the molecular basis for hydroxylamine oxidation and nitrous oxide production in methanotrophic bacteria. FEMS Microbiol Lett 322, 82-89.

Cavanaugh, C.M., Levering, P.R., Maki, J.S., Mitchell, R.,Lidstrom, M.E., 1987. Symbiosis of methylotrophic bacteria and deep-sea mussels. Nature 325, 346-348.

Childress, J.J., Fisher, C.R., Brooks, J.M., Kennicutt, M.C., Bidigare, R.,Anderson, A.E., 1986. A methanotrophic marine molluscan (*Bivalvia, Mytilidae*) symbiosis - Mussels fueled by gas. Science 233, 1306-1308.

Chistoserdova, L., 2011. Modularity of methylotrophy, revisited. Environ Microbiol 13, 2603-2622.

Coenye, T., Gevers, D., Van de Peer, Y., Vandamme, P.,Swings, J., 2005. Towards a prokaryotic genomic taxonomy. FEMS Microbiol Rev 29, 147-167.

Conrad, R., 2009. The global methane cycle: recent advances in understanding the microbial processes involved. Environmental Microbiology Reports 1, 285-292.

Costello, A.M.,Lidstrom, M.E., 1999. Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. Appl Environ Microbiol 65, 5066-5074.

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

Danilova, O.V., Kulichevskaya, I.S., Rozova, O.N., Detkova, E.N., Bodelier, P.L., Trotsenko, Y.A.,Dedysh, S.N., 2012. Methylomonas paludis sp. nov., the first acid-tolerant member of the genus Methylomonas, from an acidic wetland. Int J Syst Evol Microbiol.

Dedysh, S.N., Panikov, N.S.,Tiedje, J.M., 1998. Acidophilic methanotrophic communities from Sphagnum peat bogs. Appl Environ Microbiol 64, 922-929.

Dedysh, S.N., Liesack, W., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Semrau, J.D. et al., 2000. Methylocella palustris gen. nov., sp nov., a new methane-oxidizing acidophilic bacterium from peat bags, representing a novel subtype of serine-pathway methanotrophs. Int J Syst Evol Microbiol 50, 955-969.

Dedysh, S.N., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Semrau, J.D., Liesack, W.,Tiedje, J.M., 2002. Methylocapsa acidiphila gen. nov., sp nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from Sphagnum bog. Int J Syst Evol Microbiol 52, 251-261.

Dedysh, S.N., Berestovskaya, Y.Y., Vasylieva, L.V., Belova, S.E., Khmelenina, V.N., Suzina, N.E. et al., 2004a. *Methylocella tundrae* sp nov., a novel methanotrophic bacterium from acidic tundra peatlands. Int J Syst Evol Microbiol 54, 151-156.

Dedysh, S.N., Dunfield, P.F.,Trotsenko, Y.A., 2004b. Methane utilization by Methylobacterium species: new evidence but still no proof for an old controversy. Int J Syst Evol Microbiol 54, 1919-1920.

Dedysh, S.N., Ricke, P.,Liesack, W., 2004c. NifH and NifD phylogenies: an evolutionary basis for understanding nitrogen fixation capabilities of methanotrophic bacteria. Microbiology-Sgm 150, 1301-1313.

Dedysh, S.N., Knief, C.,Dunfield, P.F., 2005. *Methylocella* species are facultatively methanotrophic. J Bacteriol 187, 4665-4670.

Dedysh, S.N., Belova, S.E., Bodelier, P.L.E., Smirnova, K.V., Khmelenina, V.N., Chidthaisong, A. et al., 2007. *Methylocystis heyeri* sp nov., a novel type II methanotrophic bacterium possessing 'signature' fatty acids of type I methanotrophs. Int J Syst Evol Microbiol 57, 472-479.

Dedysh, S.N., 2009. Exploring methanotroph diversity in acidic northern wetlands: Molecular and cultivation-based studies. Microbiology 78, 655-669.

Denman, K.L., Brasseur, G., Chidthaisong, A., Ciais, P., Cox, P.M., Dickinson, R.E. et al. (2007) Couplings Between Changes in the Climate System and Biogeochemistry. In *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment. Report of the Intergovernmental Panel on Climate Change*. Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B. et al. (eds): Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Dumont, M.G., Pommerenke, B., Casper, P.,Conrad, R., 2011. DNA-, rRNA- and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment. Environ Microbiol 13, 1153-1167.

Dunfield, P.F., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A.,Dedysh, S.N., 2003. *Methylocella silvestris* sp nov., a novel methanotroph isolated from an acidic forest cambisol. Int J Syst Evol Microbiol 53, 1231-1239.

Dunfield, P.F., Yuryev, A., Senin, P., Smirnova, A.V., Stott, M.B., Hou, S.B. et al., 2007. Methane oxidation by an extremely acidophilic bacterium of the phylum Verrucomicrobia. Nature 450, 879-U818.

Dunfield, P.F., Belova, S.E., Vorob'ev, A.V., Cornish, S.L.,Dedysh, S.N., 2010. Methylocapsa aurea sp. nov., a facultative methanotroph possessing a particulate methane monooxygenase, and emended description of the genus Methylocapsa. Int J Syst Evol Microbiol 60, 2659-2664.

Emerson, D.,Wilson, W., 2009. Giving microbial diversity a home. Nat Rev Microbiol 7, 758-U758.

Engelmann, F., 2011. Cryopreservation of embryos: an overview. Methods Mol Biol 710, 155-184.

Etiope, G., 2009. Natural emissions of methane from geological seepage in Europe. Atmos Environ 43, 1430-1443.

Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M.M. et al., 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature 464, 543-+.

Fender, A.C., Pfeiffer, B., Gansert, D., Leuschner, C., Daniel, R.,Jungkunst, H.F., 2012. The inhibiting effect of nitrate fertilisation on methane uptake of a temperate forest soil is influenced by labile carbon. Biol Fertil Soils 48, 621-631.

Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., Fahey, D.W. et al. 2007 Changes in atmospheric constituents and in radiative forcing. Cambridge, United Kingdom: Cambridge University Press.

Fuller, B.J., 2004. Cryoprotectants: The essential antifreezes to protect life in the frozen state. Cryoletters 25, 375-388.

Gao, D.,Critser, J.K., 2000. Mechanisms of cryoinjury in living cells. ILAR J 41, 187-196.

Gebert, J.,Grongroft, A., 2006. Performance of a passively vented field-scale biofilter for the microbial oxidation of landfill methane. Waste Manag 26, 399-407.

Geymonat, E., Ferrando, L.,Tarlera, S.E., 2011. Methylogaea oryzae gen. nov., sp nov., a mesophilic methanotroph isolated from a rice paddy field. Int J Syst Evol Microbiol 61, 2568-2572.

Gonzalez, C., Boada, M., Devesa, M.,Veiga, A., 2012. Concise review: fertility preservation: an update. Stem Cells Transl Med 1, 668-672.

Green, P.N.,Woodford, S.K., 1992. Preservation studies on some obligately methanotrophic

bacteria. Lett Appl Microbiol 14, 158-162.

Hakemian, A.S.,Rosenzweig, A.C. (2007) The biochemistry of methane oxidation. In *Annu Rev Biochem*, pp. 223-241.

Han, B., Su, T., Wu, H., Gou, Z.X., Xing, X.H., Jiang, H. et al., 2009. Paraffin oil as a "methane vector" for rapid and high cell density cultivation of Methylosinus trichosporium OB3b. Appl Microbiol Biotechnol 83, 669-677.

Hanson, R.S.,Hanson, T.E., 1996. Methanotrophic bacteria. Microbiol. Rev 60, 439-471.

Helm, J., Wendlandt, K.D., Rogge, G.,Kappelmeyer, U., 2006. Characterizing a stable methane-utilizing mixed culture used in the synthesis of a high-quality biopolymer in an open system. J Appl Microbiol 101, 387-395.

Hengherr, S., Heyer, A.G., Kohler, H.R.,Schill, R.O., 2008. Trehalose and anhydrobiosis in tardigrades - evidence for divergence in responses to dehydration. FEBS J 275, 281-288.

Heyer, J., Berger, U., Hardt, M.,Dunfield, P.F., 2005. *Methylohalobius crimeensis* gen. nov., sp. nov., a moderately halophilic, methanotrophic bacterium isolated from hypersaline lakes of Crimea. Int J Syst Evol Microbiol 55, 1817-1826.

Heylen, K., Hoefman, S., Vekeman, B., Peiren, J.,De Vos, P., 2012. Safeguarding bacterial resources promotes biotechnological innovation. Appl. Microbiol. Biotechnol 94, 565-574.

Hubalek, Z., 2003. Protectants used in the cryopreservation of microorganisms. Cryobiology 46, 205-229.

Hutchens, E., Radajewski, S., Dumont, M.G., McDonald, I.R.,Murrell, J.C., 2004. Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. Environ Microbiol 6, 111-120.

Iguchi, H., Yurimoto, H.,Sakai, Y., 2011. Stimulation of Methanotrophic Growth in Cocultures by Cobalamin Excreted by Rhizobia. Appl Environ Microbiol 77, 8509-8515.

Isaksen, I.S.A., Gauss, M., Myhre, G., Anthony, K.M.W.,Ruppel, C., 2011. Strong atmospheric chemistry feedback to climate warming from Arctic methane emissions. Global Biogeochemical Cycles 25.

Islam, T., Jensen, S., Reigstad, L.J., Larsen, O.,Birkeland, N.K., 2008. Methane oxidation at 55 degrees C and pH 2 by a thermoacidophilic bacterium belonging to the Verrucomicrobia phylum. Proc Natl Acad Sci U S A 105, 300-304.

Janssens, D., Arahal, D.R., Bizet, C.,Garay, E., 2010. The role of public biological resource centers in providing a basic infrastructure for microbial research. Res Microbiol 161, 422-429.

Jiang, H., Chen, Y., Jiang, P., Zhang, C., Smith, T.J., Murrell, J.C.,Xing, X.-H., 2010. Methanotrophs: Multifunctional bacteria with promising applications in environmental bioengineering. Biochem Eng J 49, 277-288.

Jonsson, K.I., Rabbow, E., Schill, R.O., Harms-Ringdahl, M.,Rettberg, P., 2008. Tardigrades survive exposure to space in low Earth orbit. Curr Biol 18, R729-R731.

Joye, S.B., 2012. Microbiology: A piece of the methane puzzle. Nature 491, 538-539.

Kalyuzhnaya, M.G., Khmelenina, V.N., Kotelnikova, S., Holmquist, L., Pedersen, K.,Trotsenko, Y.A., 1999. Methylomonas scandinavica sp. nov., a new methanotrophic psychrotrophic bacterium isolated from deep igneous rock ground water of Sweden. Syst Appl Microbiol 22, 565-572.

Kalyuzhnaya, M.G., Khmelenina, V., Eshinimaev, B., Sorokin, D., Fuse, H., Lidstrom, M.,Trotsenko, Y., 2008. Classification of halo(alkali)philic and halo(alkali)tolerant methanotrophs provisionally assigned to the genera Methylomicrobium and Methylobacter and emended description of the genus Methylomicrobium. Int J Syst Evol Microbiol 58, 591-596.

Karlsson, J.O.M., Cravalho, E.G., Rinkes, I., Tompkins, R.G., Yarmush, M.L.,Toner, M., 1993. Nucleation and growth of ice crystals inside cultured-hepatocytes during freezing in the presences

of dimethy-sulfoxide. Biophys J 65, 2524-2536.

Kasai, M., Komi, J.H., Takakamo, A., Tsudera, H., Sakurai, T.,Machida, T., 1990. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. Journal of Reproduction and Fertility 89, 91-97.

Keppler, F., Hamilton, J.T.G., Brass, M.,Rockmann, T., 2006. Methane emissions from terrestrial plants under aerobic conditions. Nature 439, 187-191.

Khadem, A.F., Pol, A., Jetten, M.S.,Op den Camp, H.J., 2010. Nitrogen fixation by the verrucomicrobial methanotroph 'Methylacidiphilum fumariolicum' SolV. Microbiology 156, 1052-1059.

Khadem, A.F., Pol, A., Wieczorek, A., Mohammadi, S.S., Francoijs, K.J., Stunnenberg, H.G. et al., 2011. Autotrophic methanotrophy in verrucomicrobia: Methylacidiphilum fumariolicum SolV uses the Calvin-Benson-Bassham cycle for carbon dioxide fixation. J Bacteriol 193, 4438-4446.

Kim, H.J., Graham, D.W., DiSpirito, A.A., Alterman, M.A., Galeva, N., Larive, C.K. et al., 2004. Methanobactin, a copper-acquisition compound from methane-oxidizing bacteria. Science 305, 1612-1615.

King, G.M., 1994. Associations of methanotrophs with the roots and rhizomes of aquatic vegetation. Appl Environ Microbiol 60, 3220-3227.

King, G.M.,Schnell, S., 1998. Effects of ammonium and non-ammonium salt additions on methane oxidation by Methylosinus trichosporium OB3b and Maine forest soils. Appl Environ Microbiol 64, 253-257.

Kip, N., van Winden, J.F., Pan, Y., Bodrossy, L., Reichart, G.J., Smolders, A.J.P. et al., 2010. Global prevalence of methane oxidation by symbiotic bacteria in peat-moss ecosystems. Nature Geoscience 3, 617-621.

Kip, N., Ouyang, W., van Winden, J., Raghoebarsing, A., van Niftrik, L., Pol, A. et al., 2012. Detection, isolation, and characterization of acidophilic methanotrophs from Sphagnum mosses. Appl Environ Microbiol 77, 5643-5654.

Kolb, S., 2009. The quest for atmospheric methane oxidizers in forest soils. Environmental Microbiology Reports 1, 336-346.

Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B.,Stahl, D.A., 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437, 543-546.

Konstantinidis, K.T.,Tiedje, J.M., 2005. Genomic insights that advance the species definition for prokaryotes. Proc Natl Acad Sci U S A 102, 2567-2572.

Konstantinidis, K.T.,Tiedje, J.M., 2007. Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. Curr Opin Microbiol 10, 504-509.

Koops, H.-P., Purkhold, U., Pommerening-Röser, A., Timmermann, G.,Wagner, M. (2006) The Lithoautotrophic Ammonia-Oxidizing Bacteria. Chapter 3.2.12. In *The Prokaryotes, third ed*, pp. 778-811.

Kuwayama, M., Vajta, G., Kato, O.,Leibo, S.P., 2005. Highly efficient vitrification method for cryopreservation of human oocytes. Reproductive Biomedicine Online 11, 300-308.

Le Mer, J.,Roger, P., 2001. Production, oxidation, emission and consumption of methane by soils: A review. Eur J Soil Biol 37, 25-50.

Lee, S.W., Im, J., DiSpirito, A.A., Bodrossy, L., Barcelona, M.J.,Semrau, J.D., 2009. Effect of nutrient and selective inhibitor amendments on methane oxidation, nitrous oxide production, and key gene presence and expression in landfill cover soils: characterization of the role of methanotrophs, nitrifiers, and denitrifiers. Appl Microbiol Biotechnol 85, 389-403.

Lelieveld, J., Crutzen, P.J.,Dentener, F.J., 1998. Changing concentration, lifetime and climate

forcing of atmospheric methane. Tellus B 50, 128-150.

Leslie, S.B., Israeli, E., Lighthart, B., Crowe, J.H.,Crowe, L.M., 1995. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl Environ Microbiol 61, 3592-3597.

Mazur, P., 1984. Freezing of living cells - mechanisms and implications. Am. J. Physiol 247, C125-C142.

Mazur, P., Leibo, S.P.,Seidel, G.E., Jr., 2008. Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. Biol Reprod 78, 2-12.

McDonald, I.R., Kenna, E.M.,Murrell, J.C., 1995. Detection of methanotrophic bacteria in environmental-samples with the PCR. Appl Environ Microbiol 61, 116-121.

McDonald, I.R., Bodrossy, L., Chen, Y.,Murrell, J.C., 2008. Molecular ecology techniques for the study of aerobic methanotrophs. Appl Environ Microbiol 74, 1305-1315.

Medvedkova, K.A., Khmelenina, V.N., Suzina, N.E.,Trotsenko, Y.A., 2009. Antioxidant systems of moderately thermophilic methanotrophs Methylocaldum szegediense and Methylococcus capsulatus. Microbiology 78, 670-677.

Milucka, J., Ferdelman, T.G., Polerecky, L., Franzke, D., Wegener, G., Schmid, M. et al., 2012. Zero-valent sulphur is a key intermediate in marine methane oxidation. Nature 491, 541-546.

Modin, O., Fukushi, K.,Yamamoto, K., 2007. Denitrification with methane as external carbon source. Water Res 41, 2726-2738.

Mohanty, S.R., Bodelier, P.L.E., Floris, V.,Conrad, R., 2006. Differential effects of nitrogenous fertilizers on methane-consuming microbes in rice field and forest soils. Appl Environ Microbiol 72, 1346-1354.

Moore, E.R.B., Mihaylova, S.A., Vandamme, P., Krichevsky, M.I.,Dijkshoorn, L., 2010. Microbial systematics and taxonomy: relevance for a microbial commons. Res Microbiol 161, 430-438.

Morgan, C.A., Herman, N., White, P.A.,Vesey, G., 2006. Preservation of micro-organisms by drying; A review. J Microbiol Methods 66, 183-193.

Murase, J.,Frenzel, P., 2007. A methane-driven microbial food web in a wetland rice soil. Environmental Microbiology 9, 3025-3034.

Murrell, J.C.,Dalton, H., 1983. Nitrogen-fixation in obligate methanotrophs. Journal of General Microbiology 129, 3481-3486.

Nyerges, G.,Stein, L.Y., 2009. Ammonia cometabolism and product inhibition vary considerably among species of methanotrophic bacteria. FEMS Microbiol Lett 297, 131-136.

Nyerges, G., Han, S.K.,Stein, L.Y., 2010. Effects of Ammonium and Nitrite on Growth and Competitive Fitness of Cultivated Methanotrophic Bacteria. Appl Environ Microbiol 76, 5648-5651.

Op den Camp, H.J.M., Islam, T., Stott, M.B., Harhangi, H.R., Hynes, A., Schouten, S. et al., 2009. Environmental, genomic and taxonomic perspectives on methanotrophic Verrucomicrobia. Environmental Microbiology Reports 1, 293-306.

Park, S.H., Shah, N.N., Taylor, R.T.,Droege, M.W., 1992. Batch cultivation of *Methylosinus trichosporium* OB3b. 2. Production of particulate methane monooxygenase. Biotechnol Bioeng 40, 151-157.

Petersen, J.M.,Dubilier, N., 2009. Methanotrophic symbioses in marine invertebrates. Environmental Microbiology Reports 1, 319-335.

Petersen, S.O., Skov, M., Droscher, P.,Adamsen, A.P.S., 2009. Pilot Scale Facility to Determine Gaseous Emissions from Livestock Slurry during Storage. J Environ Qual 38, 1560-1568.

Pol, A., Heijmans, K., Harhangi, H.R., Tedesco, D., Jetten, M.S.M.,den Camp, H., 2007. Methanotrophy below pH1 by a new Verrucomicrobia species. Nature 450, 874-U817.

Qiu, Q., Conrad, R.,Lu, Y., 2009. Cross-feeding of methane carbon among bacteria on rice roots revealed by DNA-stable isotope probing. Environmental Microbiology Reports 1, 355–361.

Raghoebarsing, A.A., Smolders, A.J.P., Schmid, M.C., Rijpstra, W.I.C., Wolters-Arts, M., Derksen, J. et al., 2005. Methanotrophic symbionts provide carbon for photosynthesis in peat bogs. Nature 436, 1153-1156.

Reay, D.S.,Nedwell, D.B., 2004. Methane oxidation in temperate soils: effects of inorganic N. Soil Biol Biochem 36, 2059-2065.

Reshetnikov, A.S., Khmelenina, V.N., Mustakhimov, II, Kalyuzhnaya, M., Lidstrom, M.,Trotsenko, Y.A., 2011. Diversity and phylogeny of the ectoine biosynthesis genes in aerobic, moderately halophilic methylotrophic bacteria. Extremophiles 15, 653-663.

Sander, R., 1999. Compilation of Henry's Law Constants for Inorganic and Organic Species of Potential Importance in Environmental Chemistry. Version 3. Max-Planck Institute of Chemistry, Mainz, Germany: http://www.mpch-mainz.mpg.de/~sander/res/henry.html Version 3.

Scheutz, C., Kjeldsen, P., Bogner, J.E., De Visscher, A., Gebert, J., Hilger, H.A. et al., 2009. Microbial methane oxidation processes and technologies for mitigation of landfill gas emissions. Waste Manag Res 27, 409-455.

Schnell, S.,King, G.M., 1994. Mechanistic analysis of ammonium inhibition of atmospheric methane consumption in forest soils. Appl Environ Microbiol 60, 3514-3521.

Semrau, J.D., DiSpirito, A.A.,Yoon, S., 2010. Methanotrophs and copper. FEMS Microbiol Rev 34, 496-531.

Semrau, J.D., 2011. Bioremediation via Methanotrophy: Overview of Recent Findings and Suggestions for Future Research. Front Microbiol 2, 209.

Shu, Z., Kang, X., Chen, H., Zhou, X., Purtteman, J., Yadock, D. et al., 2010. Development of a reliable low-cost controlled cooling rate instrument for the cryopreservation of hematopoietic stem cells. Cytotherapy 12, 161-169.

Smith, D., 2003. Culture collections over the world. Int Microbiol 6, 95-100.

Stackebrandt, E.,Ebers, J., 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 33, 152-155.

Stein, L.Y.,Klotz, M.G., 2011. Nitrifying and denitrifying pathways of methanotrophic bacteria. Biochem Soc Trans 39, 1826-1831.

Stoecker, K., Bendinger, B., Schoning, B., Nielsen, P.H., Nielsen, J.L., Baranyi, C. et al., 2006. Cohn's Crenothrix is a filamentous methane oxidizer with an unusual methane monooxygenase. Proc Natl Acad Sci U S A 103, 2363-2367.

Streeter, J.G., 2003. Effect of trehalose on survival of *Bradyrhizobium japonicum* during desiccation. J. Appl. Microbiol 95, 484-491.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M.,Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28, 2731-2739.

Tavormina, P.L., Ussler, W.,Orphan, V.J., 2008. Planktonic and sediment-associated aerobic methanotrophs in two seep systems along the North American margin. Appl Environ Microbiol 74, 3985-3995.

Tavormina, P.L., Orphan, V.J., Kalyuzhnaya, M.G., Jetten, M.S.M.,Klotz, M.G., 2011. A novel family of functional operons encoding methane/ammonia monooxygenase-related proteins in gammaproteobacterial methanotrophs. Environmental Microbiology Reports 3, 91-100.

Thauer, R.K.,Shima, S., 2006. Biogeochemistry - Methane and microbes. Nature 440, 878-879.

Tindall, B. (2007) Vacuum-Drying and Cryopreservation of Prokaryotes. In *Cryopreservation and Freeze-Drying Protocols*. Day, J., and Stacey, G. (eds). Totowa, New Jersey: Humana Press, pp. 73-98.

Trotsenko, Y.A., Doronina, N.V.,Khmelenina, V.N., 2005. Biotechnological potential of aerobic methylotrophic bacteria: A review of current state and future prospects. Applied Biochemistry and Microbiology 41, 433-441.

Trotsenko, Y.A.,Khmelenina, V.N., 2005. Aerobic methanotrophic bacteria of cold ecosystems. FEMS Microbiol Ecol 53, 15-26.

Tsubota, J., Eshinimaev, B.T., Khmelenina, V.N.,Trotsenko, Y.A., 2005. *Methylothermus thermalis* gen. nov., sp nov., a novel moderately thermophilic obligate methanotroph from a hot spring in Japan. Int J Syst Evol Microbiol 55, 1877-1884.

van der Ha, D., Bundervoet, B., Verstraete, W.,Boon, N., 2011. A sustainable, carbon neutral methane oxidation by a partnership of methane oxidizing communities and microalgae. Water Res 45, 2845-2854.

Vandamme, P., Pot, B., Gillis, M., DeVos, P., Kersters, K.,Swings, J., 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiological Reviews 60, 407-+.

Vigliotta, G., Nutricati, E., Carata, E., Tredici, S.M., De Stefano, M., Pontieri, P. et al., 2007. Clonothrix fusca Roze 1896, a filamentous, sheathed, methanotrophic gamma-proteobacterium. Appl Environ Microbiol 73, 3556-3565.

Vorob'ev, A.V.,Dedysh, S.N., 2008. Inadequacy of enrichment culture technique for assessing the structure of methanotrophic communities in peat soil. Microbiology 77, 504-507.

Vorobev, A.V., Baani, M., Doronina, N.V., Brady, A.L., Liesack, W., Dunfield, P.F.,Dedysh, S.N., 2010. Methyloferula stellata gen. nov., sp. nov., an acidophilic, obligately methanotrophic bacterium possessing only a soluble methane monooxygenase. Int J Syst Evol Microbiol.

Wartiainen, I., Hestnes, A.G., McDonald, I.R.,Svenning, M.M., 2006. *Methylobacter tundripaludum* sp nov., a methane-oxidizing bacterium from Arctic wetland soil on the Svalbard islands, Norway (78 degrees N). Int J Syst Evol Microbiol 56, 109-113.

Wendlandt, K.D., Stottmeister, U., Helm, J., Soltmann, B., Jechorek, M.,Beck, M., 2010. The potential of methane-oxidizing bacteria for applications in environmental biotechnology. Eng Life Sci 10, 87-102.

Whittenbury, R., Davies, S.L.,Davey, J.F., 1970a. Exospores and cysts formed by methane-utilizing bacteria. J Gen Microbiol 61, 219-226.

Whittenbury, R., Phillips, K.C.,Wilkinson, J.F., 1970b. Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61, 205-217.

Zhang, Y.X., Xin, J.Y., Chen, L.L., Song, H.,Xia, C.U., 2008. Biosynthesis of poly-3-hydroxybutyrate with a high molecular weight by methanotroph from methane and methanol. Journal of Natural Gas Chemistry 17, 103-109.

Part B

Isolation of methane-oxidizing bacteria

Chapter I

Miniaturized extinction culturing for rapid isolation of methane-oxidizing bacteria

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Author Contributions:

SH and DvdH contributed equally. SH, DvdH, KH, NB designed the experiments. SH and DvdH performed the experiments, analyzed the data and wrote the paper. SH performed the isolation, purification, 16S rRNA and functional gene sequence analysis, rep-PCR fingerprinting, Type strain PCR analysis and sMMO activity tests. DvdH performed the DGGE and activity tests. KH, NB and PDV commented on the manuscript.

SUMMARY

Methane-oxidizing bacteria (MOB) have a large potential as a microbial sink for the greenhouse gas methane as well as for biotechnological purposes. However, their application in biotechnology has so far been hampered, in part due to the relative slow growth rate of the available strains. To enable the availability of novel strains, this study compares the isolation of MOB by conventional dilution plating with miniaturized extinction culturing, both performed after an initial enrichment step. The extinction approach rendered 22 MOB isolates from four environmental samples, while no MOB could be isolated by plating. In most cases, extinction culturing immediately yielded MOB monocultures making laborious purification redundant. Both type I (*Methylomonas* spp.) and type II (*Methylosinus* sp.) MOB were isolated. The isolated methanotrophic diversity represented at least 11 different strains and several novel species based on 16S rRNA gene sequence dissimilarity. These strains possessed the particulate (100%) and soluble (64%) methane monooxygenase gene. Also, 73% of the strains could be linked to a highly active fast-growing mixed MOB community. In conclusion, miniaturized extinction culturing was more efficient in rapidly isolating numerous MOB requiring little effort and fewer materials, compared with the more widely applied plating procedure. This miniaturized approach allowed straightforward isolation and could be very useful for subsequent screening of desired characteristics, in view of their future biotechnological potential.

INTRODUCTION

Next to carbon dioxide, methane is the second most important greenhouse gas contributing to one fifth of global warming (Dalal and Allen, 2008). Yearly, 30 Tg of atmospheric methane is removed through oxidation by aerobic methanotrophic bacteria (MOB), a unique group of bacteria capable of utilizing methane as their sole carbon and energy source (Trotsenko and Murrell, 2008). The key enzyme of these microorganisms, particulate or soluble methane monooxygenase (pMMO and sMMO), is remarkable in its broad substrate specificity (Semrau et al., 2010). To date, MOB have shown potential in three fields of industrial biotechnology, namely (i) geoengineering the climate through assimilation of methane and thus mitigating greenhouse effects (Scheutz et al., 2009), (ii) bioremediation of pollutants via co-metabolism by MMOs (Wendlandt et al., 2010) and (iii) production of commercially relevant metabolites (Zhang et al., 2008; Jiang et al., 2010). Despite the multifunctional potential of these bacteria, there are still several factors limiting large-scale applicability in industrial processes, which are mostly related to the MOB themselves, such as slow growth rates and low substrate affinity (Jiang et al., 2010). In order to achieve the full potential of MOB and communities for such biotechnological applications, a straightforward isolation technique is of high value, as it allows the optimization of the growth conditions of the MOB of interest. However, their cultivation is still laborious (Bowman, 2006). To date, only few MOB have been studied and examined thoroughly for further biotechnological applications (Jiang et al., 2010). The examined cultures were often not selected for their optimal use in these bioprocesses but were just the only available methanotrophic cultures at the time.

The first MOB were isolated by Söhngen (1906), but it was not until 1970 that Whittenbury and co-workers established a successful procedure to isolate and characterize methane-oxidizing bacteria by plating on Nitrate- or Ammonium Mineral Salts (NMS/AMS) medium (Whittenbury et al., 1970). Since then, novel research on MOB mainly encompassed exploring new environments and using cultivation conditions adapted to the corresponding environment (Dedysh et al., 1998b; Wise et al., 1999; Svenning et al., 2003; Bussmann et al., 2004). Most of the methanotrophic diversity thus obtained could be phylogenetically positioned within the *Alphaproteobacteria* (such as *Methylosinus* (Type II)) and the *Gammaproteobacteria* (such as *Methylomonas* (Type Ia) and *Methylococcus* (Type Ib)) (Dedysh, 2009). Since the recognition of "The Great Plate Count Anomaly" (Staley and Konopka, 1985), alternative ways to increase the general cultivability of the

microbial diversity were explored, for example by application of growth conditions that closely mimic the natural environment (Dedysh et al., 1998b), prolonged incubation at low temperatures (Song et al., 2009) or the use of alternative gelling agents replacing agar (Janssen et al., 2002; Dedysh et al., 2007; Stott et al., 2008; Tamaki et al., 2009). Another approach was extinction culturing by diluting a sample to the point of extinction and thereby purifying to a less complex sample containing only one or a few organisms (Button et al., 1993; Schut et al., 1993), which was further optimized by the development of high-throughput culturing methods (Connon and Giovannoni, 2002; Rappe et al., 2002; Stingl et al., 2007). To date, MOB are still mostly isolated via plate methods, whereby one or several liquid enrichment steps are followed by a serial dilution onto plates (Dunfield et al., 2003; Dedysh et al., 2004; Heyer et al., 2005; Tsubota et al., 2005; Wartiainen et al., 2006). However, these procedures are very laborious and almost always require elaborate purification.

The main objective of this study was to combine and optimize several of the above mentioned cultivation approaches for the specific isolation of methane-oxidizing bacteria, and to compare the isolation efficiency of the resulting two-step liquid isolation procedure, consisting of an initial prolonged enrichment and subsequent miniaturized extinction culturing, with conventional dilution plating. Initial enrichment followed by extinction culturing greatly simplified purification procedures and easily rendered novel methanotrophic strains, which were further characterized by sequence analysis of the 16S rRNA gene, repetitive element sequence based PCR fingerprinting (rep-PCR), *pmoA* and *mmoX* gene amplification (encoding for pMMO and sMMO, respectively) as well as sMMO activity assays. Most of the strains were traced back to highly active and fast-growing methanotrophic communities through denaturing gradient gel electrophoresis (DGGE) targeting the *pmoA* gene.

EXPERIMENTAL PROCEDURES

Isolation of methane-oxidizing bacteria

Samples were taken from (i) the top layer of a denitrification tank of a wastewater treatment plant (WWTP, Ossemeersen, Gent, Belgium), (ii) a covered but aerobic slurry pit of a cow stable (Melle, Belgium), (iii) the top litter layer of a wetland (Bourgoyen, Gent, Belgium) and (iv) the biofilter of an anaerobic digester (DRANCO-process, Brecht, Belgium).

On the day of sampling, 3 g of each sample was homogenized in 27 mL of a 5 times diluted Nitrate Mineral Salts (dNMS) medium (Dunfield et al., 2003), with a modified copper concentration (0.8 µM Cu^{2+}) and a 2 mM Na_2HPO_4/KH_2PO_4 buffer. The pH of the medium was adjusted to the pH of each sample (biofilter material: pH 7.8; other samples: pH 6.8). Dilution series of samples (10^{-2} up to 10^{-11}) were prepared in dNMS medium in triplicate. The resulting 120 vials were sealed and 20% (v/v) CH_4 was added to the headspace. The cultures were incubated for five weeks at 20°C while shaken (90 rpm). Weekly, concentrations of CH_4, O_2, CO_2 and N_2O in the headspace were analyzed with a Compact GC (Global Analyser Solutions, Belgium). Dilutions were considered positive for methanotrophic growth when CH_4 and O_2 were consumed (drop below initial average subtracted by five times the standard deviation), with subsequent rise in CO_2 levels, and observation of turbidity. Available in triplicate for each dilution, this information was used to estimate the abundance of cultivable MOB using Most Probable Number (MPN) tables (USDA-FSIS, 2008).

To retrieve MOB from the enrichment cultures showing methanotrophic activity, dilution plating and extinction culturing were carried out in parallel. A schematic overview of the followed methodology is shown in Figure I-1.

Dilution plating was performed by inoculation of the enrichment cultures (10^{-2}, 10^{-4} & 10^{-6}; 100 µL $plate^{-1}$) on dNMS medium with 0.9% gellan gum and 1% $MgSO_4.7H_2O$. After incubation for 2 weeks at 20°C in gas-tight jars under a CH_4:air (1:1) atmosphere, 200 colonies were randomly selected and subcultured to purity. The isolates were checked for methanotrophy by (i) growth on solid dNMS with methane in the headspace, (ii) absence of growth on solid dNMS under air and (iii) absence of growth on 1/10 Trypticase Soy Agar (TSA) under air.

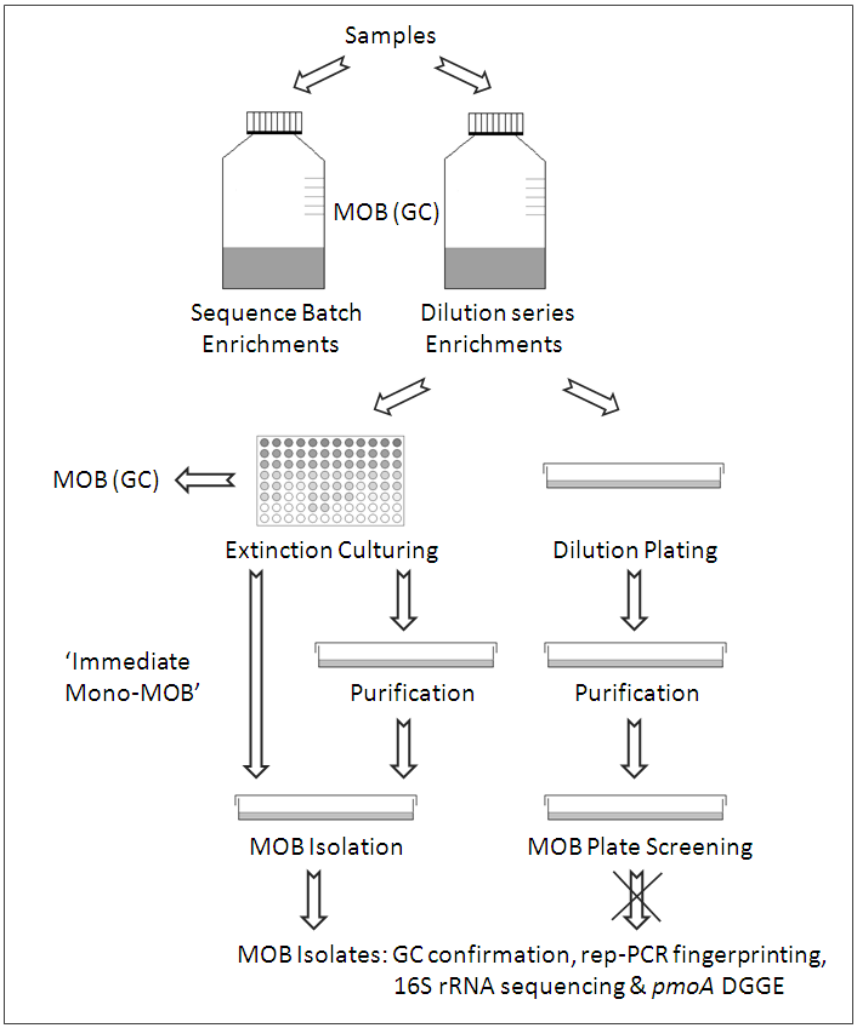

Figure I-1: Flow chart of the followed methodology.

Extinction culturing was performed in duplicate by serially diluting the enrichment cultures (10^{-2} to 10^{-9}) with liquid dNMS medium (dilution-to-extinction) in sterile 96-well microtiter plates. After incubation for two weeks at 20°C under a CH_4:air (1:1) atmosphere, turbidity indicative of growth was checked visually and by measuring the optical density at 600 nm. For each dilution series, the highest dilution showing growth was (i) confirmed for methane oxidation by GC analysis (see above), (ii) plated on solid dNMS medium (with gellan gum) and subcultured to purity if necessary. MOB purity was evaluated by (i) colony morphology, (ii) phase contrast microscopy and (iii) absence of growth on 1/10 TSA and dNMS plates supplemented with 0.1% glucose, 0.1% fructose and 0.1% yeast extract under air. The isolates were confirmed for methane oxidation by GC analysis.

Methane oxidation rate of sequence batch enrichments

In parallel with the dilution series enrichments used for MOB isolation, sequence batch enrichments using the same cultivation conditions were set-up in triplicate on the day of sampling for each of the four original samples, allowing the estimation of methane oxidation rates (MOR) of the methanotrophic communities cultivable under set conditions. Gastight Schott-bottles with a total volume of 1150 mL were filled with 200 mL dNMS medium. Inoculation was performed with 2 mL of the original sample. For the sample of biofilter material, 0.5 g of inoculum was added. After sealing the reactors, 20% (v/v) CH_4 was added to the headspace (950 mL). The cultures were placed on a shaker (100 rpm) at 20 °C and GC analysis of the headspace was performed daily. When the activity dropped to almost zero, reactors were opened under non-sterile conditions after which 160 mL of liquid phase was removed for physico-chemical analysis or stored at -20°C for *pmoA* gene DGGE analysis (see further)**.** The remainder of the liquid phase (40 mL) of the triplicate sequence batch enrichments was merged together and subsequently distributed equally over the three reactors. Freshly made dNMS medium was added to a total volume of 200 mL after which 20% (v/v) CH_4 was again added to the headspace. In total, this cycle was repeated two times. The hydraulic retention time (HRT) and sludge retention time (SRT) for each cycle were 90 h.

Identification and characterization of MOB isolates

Dereplication of all isolates was performed to assess genetic heterogeneity and group isolates with identical genomic fingerprints, further referred to as strains. Rep-PCR was performed as described by Ghyselinck et al. (2011) with a $(GTG)_5$-primer (Versalovic et al., 1994). The clustering method was supported by visual inspection: isolates were considered as genomically identical when they demonstrated identical fingerprints, which led to a cut-off value of 93%. For each group, a representative strain was selected randomly and deposited in the BCCM/LMG Culture Collection (LMG 26258-26263 & LMG 26612-26616). Furthermore, the representative strains were granted an R-collection number, which represents the research collection of LM-UGent (Table I-2).

Each strain was identified to the genus level through 16S rRNA gene sequence analysis. PCR amplification and sequencing of the 16S rRNA gene was performed as described by Heyrman and Swings (2001). The sequences were analyzed using a 3130 XL Genetic Analyzer (Applied Biosystems, USA) and assembled with BioNumerics 5.1 software (Applied Maths, Belgium). A reliable genus identification was obtained in two steps: (i)

query in the "Classifier" program of the Ribosomal Database Project II (Cole et al., 2005) of the 16S rRNA gene sequence of each new strain, (ii) all type strains of all species of all genera mentioned in the Classifier report were compared in an exhaustive pair wise manner with the query sequence of each new strain in BioNumerics 5.1. Strains were provisionally assigned to the genus of their closest type strain based on the obtained 16S rRNA gene sequence.

A slightly modified version of the naphthalene oxidation assay of Brusseau et al. (1990) was used to measure sMMO activity of the MOB strains. A crystal of naphthalene was added to 5 mL freshly grown culture, in dNMS without copper addition, and incubated at 28°C on a shaker (150 rpm) for 2 h. After incubation, 20 µL of freshly prepared tetrazotized-o-dianisidine solution (2.68 g L^{-1}) was added to 180 µL of each cell suspension in duplicate in microtiter plates, and the formation of a colored diazo-dye was immediately monitored by recording the absorbance at a wavelength of 525 nm via spectrophotometry. The assay was validated using four MOB reference type strains (Table I-1) that possess sMMO (DSM 17706^T, DSM 15673^T, DSM 18500^T, NCIMB 11131^T) and four that only possess pMMO (DSM 13736^T, DSM 17261^T, NCIMB 11914^T, NCIMB 11130^T). Primers described in literature for amplification of the *mmoX* gene were also tested using these type strains (Table I-1). Primers described by Hutchens et al. (2004) were selected for *mmoX* gene amplification of the isolates positive for the sMMO activity assay. Amplification was confirmed by subsequent sequencing of the *mmoX* gene.

Table I-1: 16S rRNA, *pmoA* and *mmoX* gene primer set evaluation using 13 MOB reference type strains.

		MOB characteristics[a]			16S rRNA gene[b]					*pmoA* gene[b]			*mmoX* gene[b]		
					Type I		Type II								
Type strain	Species	MOB type[c]	Presence *pmoA* gene[c]	Presence *mmoX* gene[d]	MethT1dF/MethT1bR[e]	Type IF/Type IR[f]	27F/MethT2R[e]	533f/Am976[g]	Type IIF/Type IIR[f]	A189f/A682r[h]	A189f/mb661r[i]	A189f/mb661r_nd[j]	534f /1393r[k]	mmoXA/mmoXB[l]	mmoX206f/mmoX886r[m]
DSM 13736^T	*Methylosarcina fibrata*	I	+	-	+	+	-	-	-	x	+	FN	-	-	-
NCIMB 11914^T	*Methylobacter luteus*	I	+	-	+	+	x	FP	-	+	+	+	-	-	-
NCIMB 11912^T	*Methylocaldum gracile*	I	+	-	x	+	-	/	-	+	+	/	-	/	-
DSM 19304^T	*Methylomicrobium alcaliphilum*	I	+	-[n]	+	+	-	-	-	+	+	+	-	-	-
NCIMB 11130^T	*Methylomonas methanica*	I	+	-[o]	+	+	x	FP	-	x	x	FN	-	-	-
NCIMB 11853^T	*Methylococcus capsulatus*	I	+	+	/	/	/	FP	/	/	+	+	+	+	/
NCIMB 11129^T	*Methylocystis parvus*	II	+	-[p]	-	-	+	+	+	x	+	+	-	-	-
DSM 17261^T	*Methylocystis rosea*	II	+	-[q]	x	-	+	+	+	+	+	+	-	-	-
DSM 17706^T	*Methylosinus sporium*	II	+	+	x	-	+	+	+	x	+	+	+	FN	+
NCIMB 11131^T	*Methylosinus trichosporium*	II	+	+	x	-	+	+	+	+	+	+	+	FN	+
DSM 18500^T	*Methylocystis hirsuta*	II	+	+[r]	/	/	/	+	/	/	+	+	+	+	/
DSM 15510^T	*Methylocella silvestris*	II	-	+[b]	-	-	+	+	+	-	-	-	+	+	FN
DSM 15673^T	*Methylocella tundrae*	II	-	+[b]	-	-	FN	+	+	x	-	-	FN	+	FN

a. Literature based characteristics: **c.** Dedysh (2009), **d.** Murrell et al. (2000), **n.** Kalyuzhnaya et al. (2008), **o.** Koh et al. (1993); **p.** McDonald et al. (1997); **q.** Wartiainen et al. (2006); **r.** Lindner et al. (2007)

b. Results: **+** Positive result; **-** Negative result; FP False positive; FN False negative; x Aspecific amplification (Multiple bands, including at correct length); **/** Not tested
Primer sets: **e.** Wise et al. (1999); **f.** Chen et al. (2007); **g.** Bodelier et al. (2005); **h.** Holmes et al. (1995); **i.** Costello & Lidstrom (1999); **j.** Lin et al. (2005); **k.** Horz et al. (2001); **l.** Auman et al. (2000); **m.** Hutchens et al. (2004)

pmoA gene DGGE analysis

Primers described in literature were tested for suitability for DGGE analysis targeting either 16S rRNA or *pmoA*, since most known MOB, except for members of *Methylocella* and *Methyloferula* (Dedysh et al., 2005; Vorobev et al., 2010), as well as all novel strains from this study contained *pmoA*. Thirteen type strains (six type I and seven type II MOB) were used as positive controls for the evaluation. These strains, their main properties, the primers tested and results obtained are listed in Table I-1. The PCR mix and temperature-time profiles from the original description were tested, as well as the PCR mix used for 16S rRNA gene amplification (Heyrman and Swings, 2001). Only one *pmoA* primer set (A189f/mb661r) could correctly detect the pMMO gene in all tested strains. The 16S rRNA gene could be amplified in all strains with both the Type IF/Type IR and Type IIF/Type IIR sets of Chen et al. (2007), however, amplicons were too long for DGGE analysis. The *pmoA* set A189f/mb661r (Costello and Lidstrom, 1999) with GC clamp 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3' was selected for further DGGE analysis.

The DNA extraction procedure was adapted from Gevers et al. (2001) and El Fantroussi et al. (1999). DGGE analysis of the PCR amplicons was performed with an INGENY phorU2X2 DGGE-system (Goes, The Netherlands). A 6.5% (w/v) polyacrylamide gel with a 30 to 80% denaturing gradient (a 100% denaturant solution contains 7 M urea and 40% (w/v) formamide) was applied. Gels were run in 1x TAE buffer for 16 h at 150 V and stained afterwards with SYBR Green I nucleic acid gel stain. The resulting DGGE patterns were processed using BioNumerics 5.1. Band position analysis was used to track the obtained isolates back to the sequence batch cultures and the dilution series enrichments used for MOB isolation and was performed by visual comparison of band location in the gel.

Nucleotide sequence accession numbers

The 16S rRNA gene sequence data generated in this study has been deposited in GenBank/EMBL/DDBJ with accession numbers FR798952 to FR798973.

RESULTS

Isolation of fast-growing MOB

Four environmental samples (wastewater treatment plant, wetland, biofilter and slurry pit) were serially diluted and enriched, while monitoring methane and oxygen consumption and carbon dioxide production. During the first four weeks, higher dilutions were gradually found positive with each subsequent measurement, stabilizing by the fifth week (Figure I-2). These data were used to calculate the abundance of cultivable MOB under given growth conditions (MPN g^{-1} expressed as MPN index [95% confidence]): cultivable MOB were most abundant (although not supported statistically) in the wastewater treatment plant (4.3×10^4 MOB g^{-1} [9.0×10^3 MOB g^{-1} - 1.8×10^5 MOB g^{-1}]), followed by wetland (4.3×10^3 MOB g^{-1} [9.0×10^2 MOB g^{-1} - 1.8×10^4 MOB g^{-1}]) and then the slurry pit and biofilter samples (both 2.3×10^2 MOB g^{-1} [4.6×10^1 MOB g^{-1} - 9.4×10^2 MOB g^{-1}]). After five weeks of incubation at 20°C, 23 dilutions were found positive for methane oxidation and were used further for MOB isolation.

The extinction culturing procedure was performed in duplicate to isolate MOB from these enrichment cultures, resulting in 46 extinction series, of which only four did not show growth after two weeks of incubation. The highest dilutions of the remaining 42 series were transferred to gas-tight vials and dNMS medium solidified with gellan gum for confirmation of methane consumption and purity check, respectively. Methane oxidation was observed in 27 series, of which 14 were immediately monocultures. Seven methane-oxidizing cultures consisted of an MOB in co-culture with a non-methanotrophic bacterium (identified as a member of *Nocardioides* with 16S rRNA gene sequence analysis) forming a distinct colony morphology surrounding the methanotrophic colonies. From the remaining six methane-oxidizing cultures, several different colony morphologies were found upon plating. Subsequent purification of these MOB was achieved after a maximum of 3 sub-cultivation steps. One dilution series resulted in a monoculture (identified as *Ancylobacter* with 16S rRNA gene sequence analysis), but did not oxidize CH_4. Fourteen dilutions were discarded since these were not able to oxidize CH_4 and did not result in a pure culture upon plating.

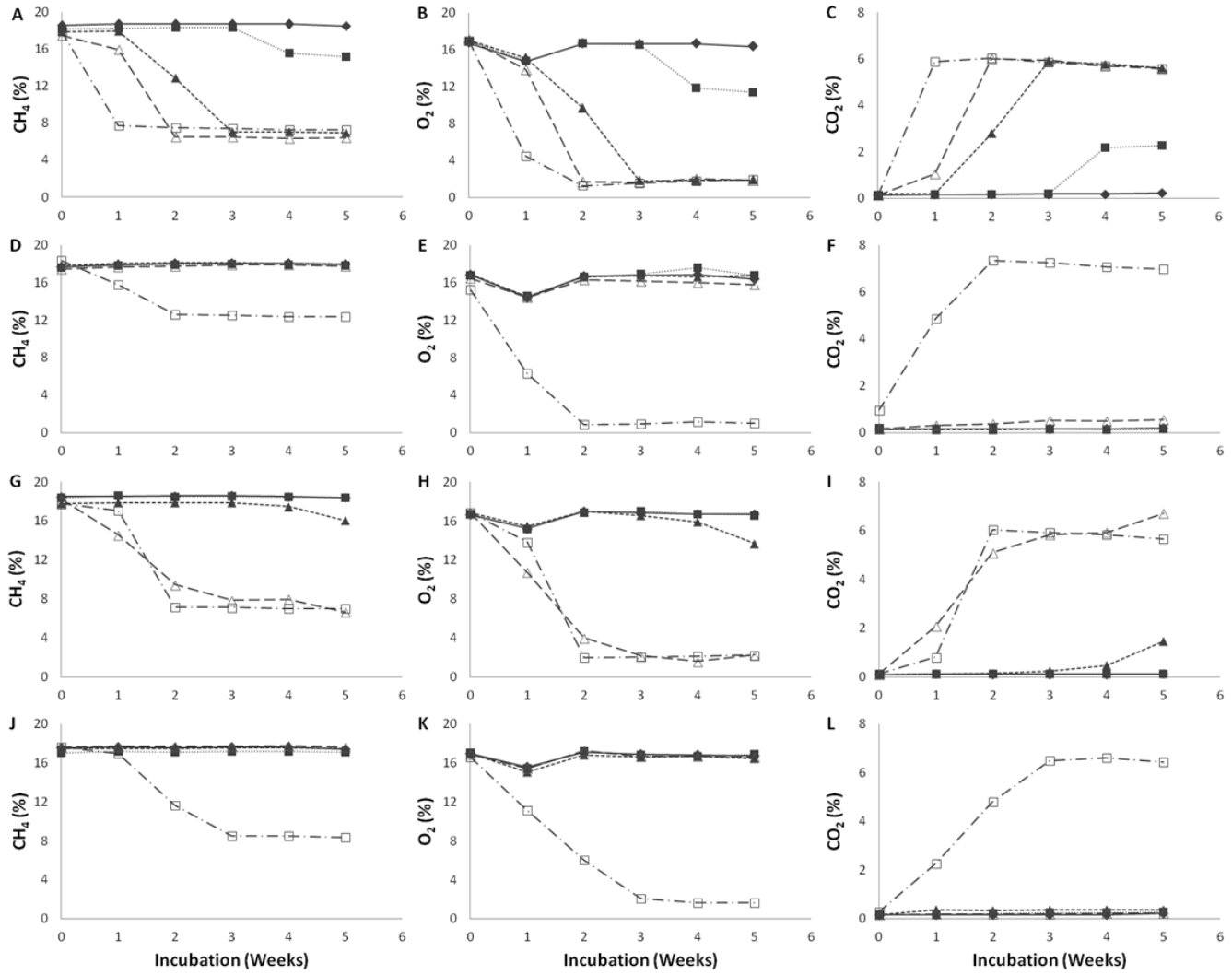

Figure I-2: Average methane (A, D, G, J), oxygen (B, E, H, K) and carbon dioxide (C, F, I, L) levels (%) during 5 weeks incubation in dNMS at 20°C (90 rpm) under a CH_4:air (1:4) atmosphere for the WWTP (A, B, C) slurry pit (D, E, F), wetland (G, H, I) and biofilter sample (J, K, L) for the dilution series 10^{-2} (white square), 10^{-3} (white triangle), 10^{-4} (black triangle) 10^{-5} (black square) and 10^{-6} (black diamond) in triplicate (error bars not shown for clarity).

Dilution plating was performed in parallel. The 23 initial methane-oxidizing enrichments were diluted, plated on dNMS (solidified with gellan gum) and incubated under atmospheric conditions supplemented with CH_4. Randomly, 200 colonies were picked up and purified. Almost all purified isolates (197 out of 200) showed heterotrophic growth on diluted TSA without CH_4 and were therefore not considered as potential MOB. The three remaining isolates, not able to grow on diluted TSA, also failed to grow on solid dNMS with or without CH_4 added to the headspace. To confirm these results, eight randomly selected isolates obtained via dilution plating, were identified to the genus level through 16S rRNA gene sequence analysis. These sequences were affiliated with *Nocardioides*, *Zoogloea*, *Rhizobium*, *Pseudomonas*, *Polaromonas*, *Rhodobacter* and *Enterobacter*, genera not harboring known MOB.

Identification and characterization of MOB isolates

In total, 22 purified MOB isolates were retrieved from four different samples (Table I-2). Dereplication with rep-PCR fingerprint analysis grouped the isolates into 11 distinct clusters, representing 11 unique strains (Figure I-3). Randomly chosen representatives of each strain were further identified to the genus level with 16S rRNA gene sequence analysis. Ten out of eleven methanotrophic strains were assigned to the Gammaproteobacterial genus *Methylomonas*, while the remaining strain was assigned to the Alphaproteobacterial genus *Methylosinus* (Table I-2). Pairwise comparisons of the 16S rRNA gene sequences of the 10 newly isolated strains with the type strains of all species of *Methylomonas* suggested that representatives of potentially novel *Methylomonas* species were isolated from all four environments, with 16S rRNA gene sequence similarities below 98% (Stackebrandt and Ebers, 2006). The eleven reference strains contained the *pmoA* gene, seven of which also harbored the *mmoX* gene and showed sMMO activity (Table I-2): six *Methylomonas* strains isolated from the WWTP sample as well as the *Methylosinus* sp. strain.

Table I-2: Genus assignment of 11 representative MOB strains (rep-PCR; Figure I-3) based on 16S rRNA gene sequence analysis (> 1,400 bp). Similarity values of 16S rRNA gene sequence to closest type strain, origin of the strains and results of naphthalene oxidation assay and *mmoX* gene amplification are given.

Sample	Representative Strain	sMMO & *mmoX*	Genus identification	Type strain with highest 16S rRNA gene sequence similarity to query sequence			
				Species name	Strain number	Sequence similarity	Accession number
WWTP	D1 (R-45371)[d]	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.5%	AF304196
	C1 (R-45372)	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.4%	AF304196
	G1 (R-45474)	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.6%	AF304196
	E1 (R-45363)	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.6%	AF304196
	H1 (R-45362)	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.6%	AF304196
	K1 (R-45364)	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.3%	AF304196
	B1 (R-45370)	No	*Methylomonas*	*Methylomonas scandinavica*	SR5^T	97.5%	AJ131369
Slurry Pit	I1 (R-45377)	No	*Methylomonas*	*Methylomonas scandinavica*	SR5^T	97.5%	AJ131369
Wetland	A6[a] (R-45378)	No	*Methylomonas*	*Methylomonas fodinarum*[b]	ACM 3268^T	96.2%	X72778
	J1 (R-45383)	No	*Methylomonas*	*Methylomonas fodinarum*[c]	ACM 3268^T	95.6%	X72778
	F1 (R-45379)	Yes	*Methylosinus*	*Methylosinus sporium*	NCIMB 11126^T	98.9%	Y18946
Biofilter	A6[a]	No	*Methylomonas*	*Methylomonas fodinarum*[b]	ACM 3268^T	96.2%	X72778

[a]Five wetland and two biofilter isolates clustered together according to rep-PCR, wetland isolate A6 was randomly selected as representative strain.

[b]Update March 2013: the newly described *Methylomonas koyamae* NCIMB 14606^T (AB538964) is currently the most closely related type strain with 99.5% 16S rRNA gene sequence similarity with A6 (R-45378).

[c] Update March 2013: the newly described *Methylomonas koyamae* NCIMB 14606^T (AB538964) is currently the most closely related type strain with 97.9% 16S rRNA gene sequence similarity with J1 (R-45383).

[d] The original numbers A6, B1, C1, D1, E1, F1, G1, H1, I1, J1, K1 are exclusively used in Chapter I, while the corresponding R-numbers (Research collection, LM-UGent; between brackets) are used throughout the thesis.

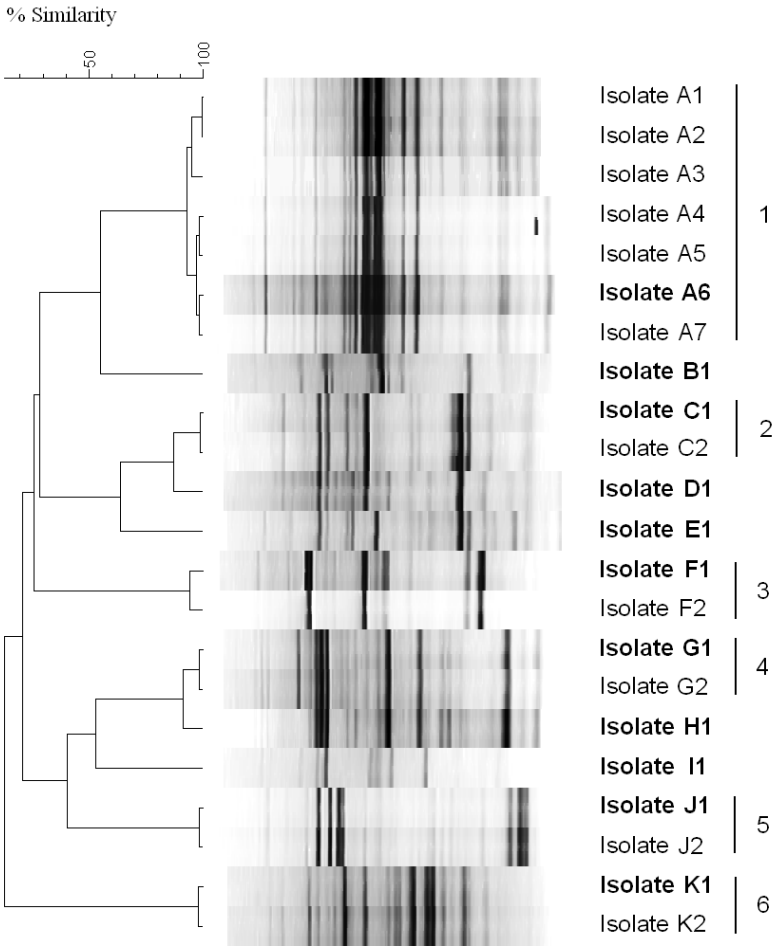

Figure I-3: GTG_5 rep-PCR fingerprinting using Pearson product moment correlation coefficient and UPGMA. MOB isolates were divided into 11 groups: 5 isolates showing unique profiles (B1, D1, E1, H1 and I2) and 6 groups of isolates (1-6) showing identical profiles. F1 and F2 isolates belonged to the genus *Methylosinus*, all the other isolates were identified as members of *Methylomonas* (Table I-2). A representative of each cluster was selected randomly (indicated in bold) for further characterization.

Detection of the isolated MOB in fast-growing methanotrophic communities

Sequence batch enrichments from the original samples were set up in parallel with the dilution series enrichments used for MOB isolation, with the same cultivation conditions.

To evaluate the presence of the isolates, under conditions selecting for highly active fast-growing MOB in a mixed counity, a sequence batch set-up with a relatively low sludge retention time of 90 h was chosen. All sequence batch enrichments showed methane oxidation activity, although the moment that a significant methane oxidation (a drop below the initial average methane concentration subtracted by five times the standard deviation) was observed differed between samples: 72 h for the WWTP cultures, 96 h for the wetland cultures and 144 h for the cultures inoculated with samples from the slurry pit or the biofilter material (Figure I-4).

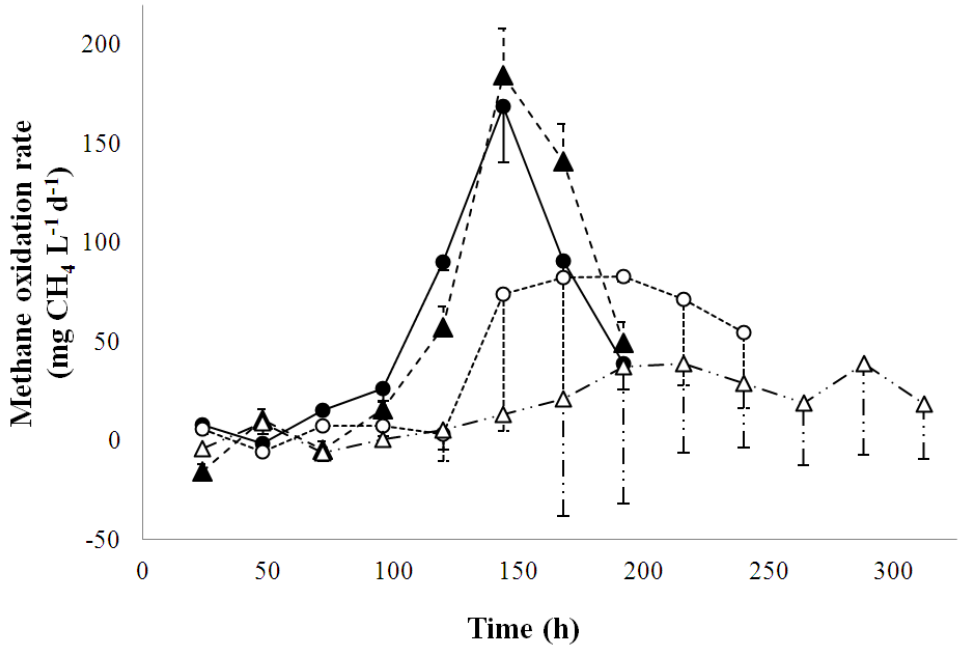

Figure I-4: The average methane oxidation rate (n=3, mg CH_4 $L^{-1}_{liquid\ phase}$ day^{-1}) of sequence batch enrichments of the original samples during the first cycle, for the sample of the wastewater treatment plant (black circle), slurry pit (white circle), wetland (black triangle) and biofilter material (white triangle). One-sided error bars are shown for clarity, with the same line type.

A steep rise in the methane oxidation rate over time was observed, with a maximum after 144 h of 169±28 mg CH_4 L^{-1} day^{-1} and 184±24 mg CH_4 L^{-1} day^{-1} for the WWTP and wetland cultures, respectively. The maximal methane oxidation rate (MOR) was lower for the two other cultures, with 83±3 mg CH_4 L^{-1} day^{-1} after 192 h and 39±45 mg CH_4 L^{-1} day^{-1} after 216 h for the slurry pit cultures and the biofilter cultures, respectively. When the oxygen concentration in the reactors became limiting, a decrease in the methane oxidation rate was observed and a second cycle was started. The observed daily MOR for the second cycle was of the same order for all four inocula with a minimum of 108±16 (biofilter) and a maximum of 266±7 (wetland) mg CH_4 L^{-1} day^{-1} (Table I-3).

PmoA DGGE analyses (Figure I-5) were performed on the eleven representative strains selected following rep-PCR fingerprinting (Figure I-3) and SBR enrichments after the first cycle of each environmental sample.

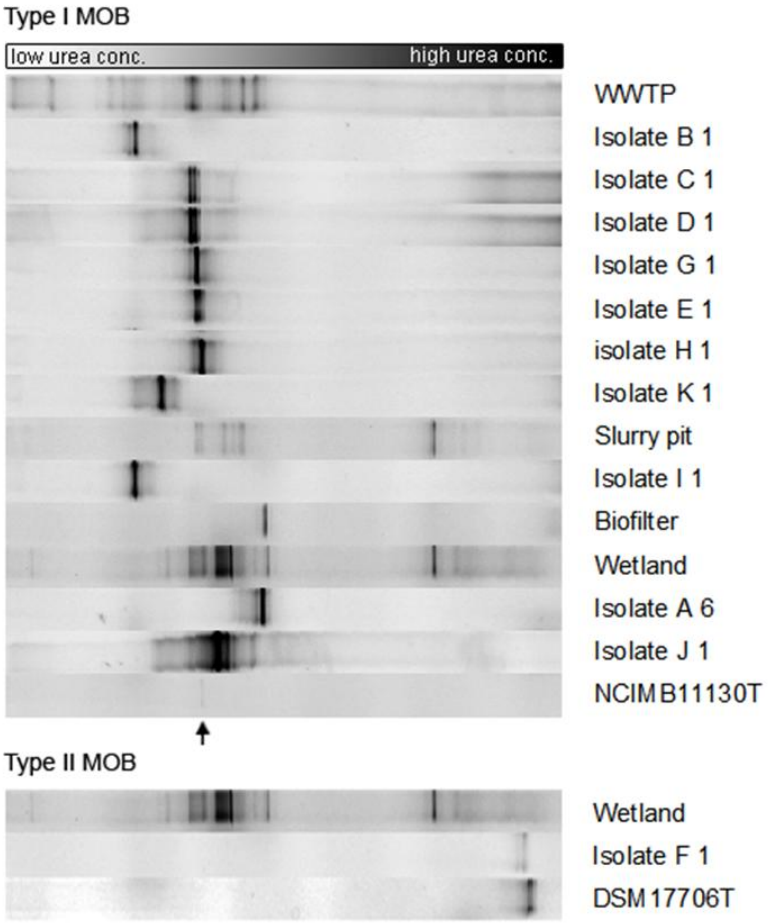

Figure I-5: DGGE analysis, based on the *pmoA* primer set A189fGC/mb661r for type I and type II MOB, respectively. The community in the active enrichment from one sequence batch reactor is shown after the first cycle for each of the four samples, i.e. a sample from the wastewater treatment plant (WWTP), a slurry pit, biofilter material and a wetland. The 11 representative strains selected based on rep-PCR fingerprinting (Figure I-3) are shown in relation to their corresponding active enrichment. Strains NCIMB11130^{T} (*Methylomonas methanica*, band indicated by arrow) and DSM17706^{T} (*Methylosinus sporium*) are shown as reference. Based on the band position of the strains, eight out of eleven representative strains could be linked to their active community, demonstrating the abundance of these strains in a mixed community selecting for fast-growing methane-oxidizing bacteria.

Table I-3: Overview of activity parameters of enriched cultures during the second cycle in sequence batch reactors: the methane oxidation rate (mg CH_4 L^{-1} liquid day^{-1}) during the first and second day of the second cycle for the four samples, the ratio of produced CO_2 over consumed CH_4 (mg CO_2-C mg^{-1} CH_4-C), the ratio of produced volatile suspended solids over consumed CH_4 (mg VSS mg^{-1} CH_4-C) and the ratio of consumed CH_4 over consumed NO_3^- (mg CH_4-C mg^{-1} NO_3^--N).

	WWTP	Slurry pit	Wetland	Biofilter
MOR_{day1}	182±29	203±20	266±7	108±16
MOR_{day2}	165±25	91±18	110±10	194±10
CO_2-C:CH_4-C ratio	0.56±0.02	0.53±0.04	0.55±0.02	0.51±0.03
VSS:CH_4-C ratio	0.60±0.09	0.77±0.19	0.68±0.12	0.79±0.14
CH_4-C:NO_3^--N ratio	15.7±1.3	13.0±3.3	15.6±2.4	16.5±3.5

DGGE profiles of WWTP and wetland were more diverse than those of the biofilter and slurry pit. From the seven *Methylomonas* strains isolated from the WTTP only one (represented by isolate K1) could not be traced back to the complete profile of the SBR enrichment, while this was the case for the other six strains, suggesting that these were dominantly present. It is however clear that different strains (isolates C1, D1, G1, E1 and H1) as proven by rep analysis, did show a *pmoA* band at a similar height, indicating that one band of the complete profile from the SBR enrichment covered a diversity of different methanotrophic strains. From the wetland, the two retrieved *Methylomonas* strains (represented by isolate A6 and J1) could be traced back to the SBR enrichment, while this was not the case for the single *Methylosinus* strain (isolate F1). The two isolates retrieved from the biofilter formed a single stable rep cluster with isolates from wetland, represented by isolate A6. The *pmoA* band of this strain was also observed in the DGGE profile of the biofilter, again suggesting the dominant presence in the SBR enrichment of this sample. From the slurry pit, only one strain was isolated for which the matching *pmoA* band could not be observed in the DGGE profile of the SBR enrichment. However, the *pmoA* band of this strain had a similar GC content as isolate B1 from WWTP, of which rep analysis already showed that they represented different strains.

DISCUSSION

Methane-oxidizing bacteria can serve as important sinks for the greenhouse gas methane or as key players in different biotechnological industries (Semrau et al., 2010). Currently, their applicability is limited by the number of suitable strains readily available, which were not specifically isolated for this purpose, and the lack of the necessary properties for efficient use in large-scale industrial applications (Jiang et al., 2010). Therefore, novel methanotrophic strains need to be easily obtained in culture and characterized. However, the isolation of MOB with the conventional plating approach is laborious and time consuming, requiring one or more liquid enrichment steps followed by serial dilution plating and extensive purification (Whittenbury et al., 1970; Bowman et al., 1993; Bodrossy et al., 1995; Dedysh et al., 1998a; Iguchi, 2010). Therefore, in this study, a simple and miniaturized isolation protocol was applied to efficiently isolate MOB by a combination of several recently developed cultivation procedures: a prolonged initial enrichment at low temperatures (Song et al., 2009) and adaptation of incubation conditions for desired MOB (Dedysh et al., 1998b), followed by high-throughput extinction culturing (Button et al., 1993; Wise et al., 1999; Connon and Giovannoni, 2002; Bodelier et al., 2005) and subsequent purification using gellan gum plates (Janssen et al., 2002; Dedysh et al., 2007). Other high-throughput culturing approaches were designed to favor the isolation of abundant bacteria *in situ* (Connon and Giovannoni, 2002; Rappe et al., 2002; Stingl et al., 2007; Song et al., 2009), while our protocol specifically targeted a certain sub-population of a specific functional group with a custom-made prolonged enrichment. This made the organisms of interest, in this case fast-growing MOB, abundant *ex situ* before extinction culturing. This approach resulted in immediate pure cultures, avoiding elaborate purification, which is known to be problematic for the isolation of MOB (Bowman, 2006). Without this approach, no immediate MOB pure culture would be obtained since heterotrophic bacteria are more abundantly present in environmental samples (Wise et al., 1999). In perspective of biotechnological applications, varying the cultivation parameters of the initial enrichment in combination with miniaturization in 96-well plates has the potential to select for MOB with specific desired characteristics. As such, these wanted MOB can become abundant, even if non-abundant *in situ*, and can then be rapidly isolated via miniaturized extinction culturing. For example, the cultivation conditions chosen in this study, a diluted NMS medium under a high concentration of CH_4 and a relatively low hydraulic retention time, are known to select for highly active fast-growing MOB, a

characteristic which is important for industrial use of bacteria (Begonja and Hrsak, 2001; Schrader et al., 2009; Wendlandt et al., 2010). Indeed, the mixed SBR communities showed a high methane removal rate from 108±16 to 266±7 mg CH_4 L^{-1} day^{-1} respectively, which is in the range of reported highly active methane-oxidizing communities (Melse and Van der Werf, 2005; Nikiema et al., 2005; Gebert and Grongroft, 2006; Scheutz et al., 2009). By band position analysis of *pmoA*-targeted DGGE, most isolated MOB could be traced back to this active community, demonstrating that the isolated strains were able to rapidly oxidize methane and grow to higher densities in a competitive setting. Most isolated MOB were indeed closely related to *Methylomonas* (Type Ia), known to harbor fast-growing MOB with a short generation time of 3.5 h (Whittenbury et al., 1970). Fast-growing Type Ib MOB (f.e. *Methylococcus*) with similar doubling times (Whittenbury et al., 1970) were not expected to be retrieved because they require higher isolation temperatures, while type II MOB are generally known to grow slower, with generation times ranging from 5 h up to several days (Whittenbury et al., 1970; Dedysh et al., 2000; Dedysh et al., 2002; Vorobev et al., 2010). Since DGGE patterns mainly represent the major constituents of a community (Muyzer and Smalla, 1998), the few strains that could not be traced back by band position analysis probably were expected to have longer generation times and as such were not abundantly present in the highly-active methane-oxidizing communities. Not all *pmoA* bands in the mixed communities had isolated representatives, therefore an up-scaling of the isolation campaign, with more extinction cultures per enrichment, would detect more novel MOB cultivable under the set conditions. This was confirmed as extinction series from initial enrichments did lead to the isolation of two different MOB strains (strains E1 & H1 and strains B1 & D1).

In total, 22 MOB isolates belonging to eleven distinct strains were obtained, which is a relatively high number compared to other studies (Dianou and Adachi, 1999; Auman et al., 2000; Bussmann et al., 2004; Miller et al., 2004). Members of *Methylomonas* (type I MOB) were isolated from all four samples, while *Methylosinus* representatives (type II MOB) were only obtained from the wetland sample. Despite that isolates were assigned to known genera, they represented at least 10 different *Methylomonas* strains, several of which belonging to novel species within the genus based on 16S rRNA gene sequence dissimilarity with *Methylomonas* type strains. Currently, four species have been validly described, although only the type strain of *Methylomonas methanica* (NCIMB 11130^T) is still accessible for the scientific community. This issue on availability of fastidious microorganisms such as MOB is a widely recognized problem and greatly hampers the in-

depth investigation of their biotechnological potential, especially because properties such as substrate affinity, growth rate, substrate range or degradation of xenobiotics are strain-dependent features. For example, six different *Methylomonas* strains isolated in this study possessed sMMO, in addition to pMMO, while the type strain of the genus does not possess sMMO (Koh et al., 1993). Both sMMO and pMMO are known to degrade pollutants, such as chlorinated hydrocarbons (Bowman et al., 1993; Jiang et al., 2010): sMMO has a broader substrate range and is known to rapidly degrade pollutants, pMMO degrades compounds at slower rates but over an extended time frame (Semrau et al., 2010). While common in type II & Ib MOB (Hanson and Hanson, 1996), few type Ia MOB such as *Methylomonas* contain sMMO although it has been reported (Koh et al., 1993; Shen et al., 1997; Auman et al., 2000; Bussmann et al., 2006). Since type I MOB have a higher efficiency in carbon conversion (Scheutz et al., 2009), these *Methylomonas* isolates, which possess both sMMO and pMMO, could particularly be of interest to screen for degradation of recalcitrant compounds.

In this study, the isolation efficiency for the retrieval of fast-growing MOB between miniaturized extinction culturing and conventional dilution plating was compared. To our knowledge, such a comparison has not been previously reported. The plating approach, which is the most applied method for isolation of MOB (Dunfield et al., 2003; Dedysh et al., 2004; Heyer et al., 2005; Tsubota et al., 2005; Wartiainen et al., 2006), did not render any MOB in this study when performed in parallel with extinction culturing from the same initial enrichments. However, the applied methodology should allow their isolation since MOB isolates obtained from extinction culturing and reference strains from public bacteria collections were cultivated successfully on solid medium in the same manner. Therefore, it is likely that additional plating trials investigating more colonies would allow the isolation of MOB. However, even then miniaturized extinction culturing from initial enrichments will be more time and labour efficient than conventional plating in retrieving numerous methane-oxidizing bacteria with specific desired characteristics.

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REFERENCES

Auman, A.J., Stolyar, S., Costello, A.M.,Lidstrom, M.E., 2000. Molecular characterization of methanotrophic isolates from freshwater lake sediment. Appl Environ Microbiol 66, 5259-5266.

Begonja, A.,Hrsak, D., 2001. Effect of growth conditions on the expression of soluble methane monooxygenase. Food Technol Biotechnol 39, 29-35.

Bodelier, P.L.E., Meima-Franke, M., Zwart, G.,Laanbroek, H.J., 2005. New DGGE strategies for the analyses of methanotrophic microbial communities using different combinations of existing 16S rRNA-based primers. FEMS Microbiol Ecol 52, 163-174.

Bodrossy, L., Murrell, J.C., Dalton, H., Kalman, M., Puskas, L.G.,Kovacs, K.L., 1995. Heat-tolerant methanotrophic bacteria from the hot-water effluent of a natural-gas field. Appl Environ Microbiol 61, 3549-3555.

Bowman, J. (2006) The Methanotrophs - The Families Methylococcaceae and Methylocystaceae. Chapter 3.1.14. *Prokaryotes* **5**: 266-289, DOI: 10.1007/0-387-30745-1_15.

Bowman, J.P., Jimenez, L., Rosario, I., Hazen, T.C.,Sayler, G.S., 1993. Characterization of the methanotrophic bacterial community present in a trichloroethylene-contaminated subsurface groundwater site. Appl Environ Microbiol 59, 2380-2387.

Brusseau, G.A., Tsien, H.C., Hanson, R.S.,Wackett, L.P., 1990. Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. Biodegradation 1, 19-29.

Bussmann, I., Pester, M., Brune, A.,Schink, B., 2004. Preferential cultivation of type II methanotrophic bacteria from littoral sediments (Lake Constance). FEMS Microbiol Ecol 47, 179-189.

Bussmann, I., Rahalkar, M.,Schink, B., 2006. Cultivation of methanotrophic bacteria in opposing gradients of methane and oxygen. FEMS Microbiol Ecol 56, 331-344.

Button, D.K., Schut, F., Quang, P., Martin, R.,Robertson, B.R., 1993. Viability and isolation of marine-bacteria by dilution culture - Theory, procedures, and initial results. Appl Environ Microbiol 59, 881-891.

Chen, Y., Dumont, M.G., Cebron, A.,Murrell, J.C., 2007. Identification of active methanotrophs in a landfill cover soil through detection of expression of 16S rRNA and functional genes. Environ Microbiol 9, 2855-2869.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M. et al., 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res 33, D294-D296.

Connon, S.A.,Giovannoni, S.J., 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl Environ Microbiol 68, 3878-3885.

Costello, A.M.,Lidstrom, M.E., 1999. Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. Appl Environ Microbiol 65, 5066-5074.

Dalal, R.C.,Allen, D.E., 2008. Greenhouse gas fluxes from natural ecosystems. Aust J Bot 56, 369-407.

Dedysh, S.N., Panikov, N.S., Liesack, W., Grosskopf, R., Zhou, J.Z.,Tiedje, J.M., 1998a. Isolation of acidophilic methane-oxidizing bacteria from northern peat wetlands. Science 282, 281-284.

Dedysh, S.N., Panikov, N.S.,Tiedje, J.M., 1998b. Acidophilic methanotrophic communities from Sphagnum peat bogs. Appl Environ Microbiol 64, 922-929.

Dedysh, S.N., Liesack, W., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Semrau, J.D. et al., 2000. Methylocella palustris gen. nov., sp nov., a new methane-oxidizing acidophilic bacterium from peat bags, representing a novel subtype of serine-pathway methanotrophs. Int J Syst Evol Microbiol 50, 955-969.

Dedysh, S.N., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Semrau, J.D., Liesack, W.,Tiedje, J.M., 2002. Methylocapsa acidiphila gen. nov., sp nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from Sphagnum bog. Int J Syst Evol Microbiol 52, 251-261.

Dedysh, S.N., Berestovskaya, Y.Y., Vasylieva, L.V., Belova, S.E., Khmelenina, V.N., Suzina, N.E. et al., 2004. *Methylocella tundrae* sp nov., a novel methanotrophic bacterium from acidic tundra peatlands. Int J Syst Evol Microbiol 54, 151-156.

Dedysh, S.N., Knief, C.,Dunfield, P.F., 2005. *Methylocella* species are facultatively methanotrophic. J Bacteriol 187, 4665-4670.

Dedysh, S.N., Belova, S.E., Bodelier, P.L.E., Smirnova, K.V., Khmelenina, V.N., Chidthaisong, A. et al., 2007. *Methylocystis heyeri* sp nov., a novel type II methanotrophic bacterium possessing 'signature' fatty acids of type I methanotrophs. Int J Syst Evol Microbiol 57, 472-479.

Dedysh, S.N., 2009. Exploring methanotroph diversity in acidic northern wetlands: Molecular and cultivation-based studies. Microbiology 78, 655-669.

Dianou, D.,Adachi, K., 1999. Characterization of methanotrophic bacteria isolated from a subtropical paddy field. FEMS Microbiol Lett 173, 163-173.

Dunfield, P.F., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A.,Dedysh, S.N., 2003. *Methylocella silvestris* sp nov., a novel methanotroph isolated from an acidic forest cambisol. Int J Syst Evol Microbiol 53, 1231-1239.

El Fantroussi, S., Verschuere, L., Verstraete, W.,Top, E.M., 1999. Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. Appl Environ Microbiol 65, 982-988.

Gebert, J.,Grongroft, A., 2006. Performance of a passively vented field-scale biofilter for the microbial oxidation of landfill methane. Waste Manage 26, 399-407.

Gevers, D., Huys, G.,Swings, J., 2001. Applicability of rep-PCR fingerprinting for identification of Lactobacillus species. FEMS Microbiol Lett 205, 31-36.

Ghyselinck, J., Van Hoorde, K., Hoste, B., Heylen, K.,De Vos, P., 2011. Evaluation of MALDI-TOF MS as a tool for high-throughput dereplication. J Microbiol Methods.

Hanson, R.S.,Hanson, T.E., 1996. Methanotrophic bacteria. Microbiol. Rev 60, 439-471.

Heyer, J., Berger, U., Hardt, M.,Dunfield, P.F., 2005. *Methylohalobius crimeensis* gen. nov., sp. nov., a moderately halophilic, methanotrophic bacterium isolated from hypersaline lakes of Crimea. Int J Syst Evol Microbiol 55, 1817-1826.

Heyrman, J.,Swings, J., 2001. 16S rDNA sequence analysis of bacterial isolates from biodeteriorated mural paintings in the Servilia tomb (necropolis of Carmona, Seville, Spain). Syst Appl Microbiol 24, 417-422.

Holmes, A.J., Owens, N.J.P.,Murrell, J.C., 1995. Detection of novel marine methanotrophs using phylogenetic and functional gene probes after methane enrichment. Microbiology 141, 1947-1955.

Horz, H.P., Yimga, M.T.,Liesack, W., 2001. Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of pmoA, mmoX, mxaF, and 16S rRNA and ribosomal DNA, including pmoA-based terminal restriction fragment length polymorphism profiling. Appl Environ Microbiol 67, 4177-4185.

Hutchens, E., Radajewski, S., Dumont, M.G., McDonald, I.R.,Murrell, J.C., 2004. Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. Environ Microbiol 6, 111-120.

Iguchi, H., Yurimoto H., Sakai Y. (2010) *Methylovulum miyakonense* gen. nov., sp. nov., a novel type I methanotroph from a forest soil in Japan. *Int J Syst Evol Microbiol*, DOI: 10.1099/ijs.0.019604-0.

Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M.,Sait, M., 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. Appl Environ Microbiol 68, 2391-2396.

Jiang, H., Chen, Y., Jiang, P., Zhang, C., Smith, T.J., Murrell, J.C.,Xing, X.-H., 2010. Methanotrophs: Multifunctional bacteria with promising applications in environmental bioengineering. Biochem Eng J 49, 277-288.

Kalyuzhnaya, M.G., Khmelenina, V., Eshinimaev, B., Sorokin, D., Fuse, H., Lidstrom, M.,Trotsenko, Y., 2008. Classification of halo(alkali)philic and halo(alkali)tolerant methanotrophs provisionally assigned to the genera *Methylomicrobium* and *Methylobacter* and emended description of the genus *Methylomicrobium*. Int J Syst Evol Microbiol 58, 591-596.

Koh, S.C., Bowman, J.P.,Sayler, G.S., 1993. Soluble methane monooxygenase production and trichloroethylene degradation by a Type-I methanotroph, *Methylomons methanica*-68-1. Appl Environ Microbiol 59, 960-967.

Lin, J.L., Joye, S.B., Scholten, J.C.M., Schafer, H., McDonald, I.R.,Murrell, J.C., 2005. Analysis of methane monooxygenase genes in mono lake suggests that increased methane oxidation activity may correlate with a change in methanotroph community structure. Appl Environ Microbiol 71, 6458-6462.

Lindner, A.S., Pacheco, A., Aldrich, H.C., Staniec, A.C., Uz, I.,Hodson, D.J., 2007. Methylocystis hirsuta sp nov., a novel methanotroph isolated from a groundwater aquifer. Int J Syst Evol Microbiol 57, 1891-1900.

McDonald, I.R., Uchiyama, H., Kambe, S., Yagi, O.,Murrell, J.C., 1997. The soluble methane monooxygenase gene cluster of the trichloroethylene-degrading methanotroph Methylocystis sp. strain M. Appl Environ Microbiol 63, 1898-1904.

Melse, R.W.,Van der Werf, A.W., 2005. Biofiltration for mitigation of methane emission from animal husbandry. Environ Sci Technol 39, 5460-5468.

Miller, D.N., Yavitt, J.B., Madsen, E.L.,Ghiorse, W.C., 2004. Methanotrophic activity, abundance, and diversity in forested swamp pools: Spatiotemporal dynamics and influences on methane fluxes. Geomicrobiol J 21, 257-271.

Murrell, J.C., McDonald, I.R.,Gilbert, B., 2000. Regulation of expression of methane monooxygenases by copper ions. Trends Microbiol 8, 221-225.

Muyzer, G.,Smalla, K., 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Anton Leeuw Int J G 73, 127-141.

Nikiema, J., Bibeau, L., Lavoie, J., Brzezinski, R., Vigneux, J.,Heitz, M., 2005. Biofiltration of methane: An experimental study. Chem Eng J 113, 111-117.

Rappe, M.S., Connon, S.A., Vergin, K.L.,Giovannoni, S.J., 2002. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature 418, 630-633.

Scheutz, C., Kjeldsen, P., Bogner, J.E., De Visscher, A., Gebert, J., Hilger, H.A. et al., 2009. Microbial methane oxidation processes and technologies for mitigation of landfill gas emissions. Waste Manage Res 27, 409-455.

Schrader, J., Schilling, M., Holtmann, D., Sell, D., Villela, M., Marx, A.,Vorholt, J.A., 2009. Methanol-based industrial biotechnology: current status and future perspectives of methylotrophic bacteria. Trends Biotechnol 27, 107-115.

Schut, F., Devries, E.J., Gottschal, J.C., Robertson, B.R., Harder, W., Prins, R.A.,Button, D.K., 1993. Isolation of typical marine-bacteria by dilution culture - growth, maintenance, and characteristics of isolates under laboratory conditions. Appl Environ Microbiol 59, 2150-2160.

Semrau, J.D., DiSpirito, A.A.,Yoon, S., 2010. Methanotrophs and copper. FEMS Microbiol Rev 34, 496-531.

Shen, R.N., Yu, C.L., Ma, Q.Q.,Li, S.B., 1997. Direct evidence for a soluble methane monooxygenase from type I methanotrophic bacteria: Purification and properties of a soluble methane monooxygenase from *Methylomonas* sp. GYJ3. Arch Biochem Biophys 345, 223-229.

Söhngen, N.L., 1906. Uber Bakterien, welche methan ab kohlenstoffnahrung und energiequelle gerbrauchen. Parasitenkd. Infectionskr. Abt. 2. 15, 513-517.

Song, J., Oh, H.M.,Cho, J.C., 2009. Improved culturability of SAR11 strains in dilution-to-extinction culturing from the East Sea, West Pacific Ocean. FEMS Microbiol Lett 295, 141-147.

Stackebrandt, E.,Ebers, J., 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 8, 6-9.

Staley, J.T.,Konopka, A., 1985. Measurement of in sity activities of non photysynthetic microorganisms in aquatic and terrestrial habitats. Annu Rev Microbiol 39, 321-346.

Stingl, U., Tripp, H.J.,Giovannoni, S.J., 2007. Improvements of high-throughput culturing yielded novel SAR11 strains and other abundant marine bacteria from the Oregon coast and the Bermuda Atlantic Time Series study site. Isme Journal 1, 361-371.

Stott, M.B., Crowe, M.A., Mountain, B.W., Smirnova, A.V., Hou, S.B., Alam, M.,Dunfield, P.F., 2008. Isolation of novel bacteria, including a candidate division, from geothermal soils in New Zealand. Environ Microbiol 10, 2030-2041.

Svenning, M.M., Wartiainen, I., Hestnes, A.G.,Binnerup, S.J., 2003. Isolation of methane oxidising bacteria from soil by use of a soil substrate membrane system. FEMS Microbiol Ecol 44, 347-354.

Tamaki, H., Hanada, S., Sekiguchi, Y., Tanaka, Y.,Kamagata, Y., 2009. Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment. Environ Microbiol 11, 1827-1834.

Trotsenko, Y.A.,Murrell, J.C., 2008. Metabolic aspects of aerobic obligate methanotrophy. Adv Appl Microbiol 63, 183-229.

Tsubota, J., Eshinimaev, B.T., Khmelenina, V.N.,Trotsenko, Y.A., 2005. *Methylothermus thermalis* gen. nov., sp nov., a novel moderately thermophilic obligate methanotroph from a hot spring in Japan. Int J Syst Evol Microbiol 55, 1877-1884.

USDA-FSIS 2008 Most Probable Number Procedure and Tables. S.W. Washington, D.C.

Versalovic, J., Schneider, M., De Bruijn, F.J.,Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5, 25-40.

Vorobev, A.V., Baani, M., Doronina, N.V., Brady, A.L., Liesack, W., Dunfield, P.F.,Dedysh, S.N., 2010. Methyloferula stellata gen. nov., sp. nov., an acidophilic, obligately methanotrophic bacterium possessing only a soluble methane monooxygenase. Int J Syst Evol Microbiol.

Wartiainen, I., Hestnes, A.G., McDonald, I.R.,Svenning, M.M., 2006. *Methylobacter tundripaludum* sp nov., a methane-oxidizing bacterium from Arctic wetland soil on the Svalbard islands, Norway (78 degrees N). Int J Syst Evol Microbiol 56, 109-113.

Wendlandt, K.D., Stottmeister, U., Helm, J., Soltmann, B., Jechorek, M.,Beck, M., 2010. The potential of methane-oxidizing bacteria for applications in environmental biotechnology. Eng Life Sci 10, 87-102.

Whittenbury, R., Phillips, K.C.,Wilkinson, J.F., 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61, 205-217.

Wise, M.G., McArthur, J.V.,Shimkets, L.J., 1999. Methanotroph diversity in landfill soil: Isolation of novel type I and type II methanotrophs whose presence was suggested by culture-independent 16S ribosomal DNA analysis. Appl Environ Microbiol 65, 4887-4897.

Zhang, Y., Xin, Y., Chen, L., Song, H.,Xia, C., 2008. Biosynthesis of poly-3-hydroxybutyrate with a high molecular weight by methanotroph from methane and methanol. J Nat Gas Chem 17, 103-109.

Chapter II

Facultative ponds harbor methane oxidizing communities supported by algae

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Redrafted from:

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Author Contributions:

DvdH, AKC and NB designed the experiments. SH performed the miniaturized extinction culturing, dereplication and identification of MOB isolates, and wrote these sections. DvdH performed all other experiments, analyzed the data and wrote the manuscript. MLI helped with the experiments. SH, PDV, IML, AKC and NB commented on the manuscript.

SUMMARY

Waste stabilization ponds are key units of many low-cost wastewater treatment plants. Typically, anaerobic processes taking place in the sludge blanket result in emissions of the greenhouse gas CH_4. A South African pilot facultative waste stabilization pond was used to evaluate the fate of CH_4 in the oxygenated pond surface layer. On site emission analyses showed that such facultative ponds are substantial CH_4 sources. Yet, part of the produced CH_4 is consumed by aerobic methane oxidizing bacteria (MOB) before it can reach the atmosphere. Both type Ia, Ib and II MOB were isolated out of enriched samples originating from the surface layer of 25 cm, wherein at least 10^4 cultivable MOB mL^{-1} were observed. These enrichments also showed a high methane oxidizing activity of 190±5 mg CH_4 L^{-1}_{liquid} d^{-1}. The algal community had an important indirect effect on the methanotrophic activity as the algae provided the MOB with O_2. In laboratory simulations with a stagnant water column, methane oxidation rates were significantly higher when algae were able to produce O_2, which was subsequently consumed by MOB. As such, algae have an essential supportive role in the CH_4 oxidation of facultative ponds and could therefore influence their carbon footprint to a substantial extent.

INTRODUCTION

All over the world, treatment of wastewater is a necessity to obtain a sustainable way of living for an ever-increasing population (Verstraete et al., 2009). While industrialized countries have the possibility to invest in state-of-the art technologies like activated sludge systems or anaerobic digesters, sewage treatment with a low capital and operational cost is often the only possibility in many developing countries (Aiyuk et al., 2004; Mara, 2004; De Almeida et al., 2009). Therefore, waste stabilization ponds (WSP) have been successfully implemented for wastewater treatment, largely due to the pioneering work of Oswald and Marais (Caldwell, 1946; Marais, 1966; Oswald, 1976). WSP are characterized by long retention times to allow for the gradual breakdown of organic waste by microorganisms (Mara, 2005). In addition to anaerobic and aerobic ponds, facultative versions exist and these are the most common WSP types in the USA (Reed, 1995). They differ from anaerobic ponds as there is an aerobic surface layer present. Aside from wind effects, this layer is mainly oxygenated by algae and cyanobacteria, which produce oxygen as a product of photosynthesis (Mara, 2005). Often, these algae enter the system naturally and a clear selection frequently occurs for motile algae that can optimize their vertical position, therefore having an advantage over non-motile algae (Pearson et al., 1987; Mara, 2005). At the bottom of the pond however, an almost permanent anaerobic sludge blanket results in the release of large amounts of biogas comprising H_2S, NH_3, CO_2 and CH_4 (Craggs et al., 2008). Due to the nature and construction of these ponds, large emissions of CO_2 and CH_4 from the surface area can be expected and CH_4 production rates of 0.17 kg CH_4 kg^{-1} BOD_{waste} were found in models of anaerobic ponds fed with municipal waste (DeGarie et al., 2000; van der Steen et al., 2003). Picot et al. (2003) estimated that about 3.3 m^3 $capita^{-1}$ y^{-1} of CH_4 is produced in real scale ponds, when a daily sewage production of 100 g chemical oxygen demand (COD) $capita^{-1}$ d^{-1} is assumed. Taking into account that CH_4 is a 25 times stronger greenhouse gas than CO_2 (Forster et al., 2007), a production of 0.25 ton CO_2-equivalents of CH_4 y^{-1} per household of four persons can be estimated.

Due to these high emissions and the presence of O_2 in the surface layer of facultative ponds, aerobic methane oxidizing bacteria (MOB) might be expected to be part of the resident microbial community. Although van der Steen et al. (2003) did not find evidence of significant microbial CH_4 oxidation activity in simulated WSP bottles, MOB were identified in the root zone of *Lemna minor*, a common aquatic plant regularly floating on the surface of such ponds (Hanson et al., 1993). It has been observed at laboratory scale

that algae can provide the MOB with molecular O_2, but this interaction is still poorly described under natural conditions (Bahr et al., 2011; van der Ha et al., 2011).

The present study focuses on a pilot scale Primary Facultative Pond (PFP), receiving domestic wastewater. In an effort to evaluate the influence of the algal photosynthetic activity on the methanotrophic activity, pond samples were enriched with CH_4 under illuminated and dark conditions. Furthermore, the amount of cultivable MOB in the pond was estimated by means of a most probable number approach. Finally, an isolation campaign on the enriched pond samples allowed to positively identify cultivable MOB.

EXPERIMENTAL PROCEDURES

Study site

The facultative pond used in this study is a component of an Integrated Algae Pond System located at the Institute for Environmental Biotechnology Rhodes University (EBRU), adjacent to the Grahamstown Wastewater Treatment Plant (33° 19' 07" South, 26° 33' 25" East), which operates uninterrupted and treats 80 m^3 d^{-1} of domestic effluent. The complete system comprises a 840 m^2 PFP, a single fermentation pit, two 500 m^2 high rate algal ponds and two algal settling ponds. Upflow velocity in the fermentation pit is maintained at 1-1.5 m d^{-1} while the Hydraulic Retention Times (HRT) in the fermentation pit and PFP are 3 and 20 d, respectively. All experiments and sampling took place in the period from February 2nd until April 25th, 2012.

Estimation of methane emissions

Emission of CH_4 from the facultative pond was estimated by means of a free-floating gas capture device positioned in an area equidistant from the centre and the border of the pond. The device was constructed of polystyrene (2 dm^2) with an attached gastight polyethylene terephthalate bottle (420 mL) and an open cross section of 20 cm^2. Biogas produced from the PFP expelled the water from the bottle allowing quantification of produced CH_4 over time. Extrapolated quantification of CH_4 production was based on measurements during six different periods of 24 to 120h.

Influence of algae on the physico-chemical conditions in the pond

In order to estimate the physico-chemical conditions in the pond, depth profiles of temperature, dissolved oxygen (DO) and pH were constructed at different time intervals at a predefined spot approximately 6 m from the centre of the pond, thereby not disturbing the water column. The mentioned parameters were measured every 1 cm from the surface to a depth of 50-60 cm. DO and temperature were measured using a handheld dissolved oxygen meter (Model 85, YSI Inc., OH, USA), while pH was measured with a WTW330 (WTW, Weilheim, Germany) instrument.

On March 13th 2012, three samples (1 L) were harvested aseptically from the PFP using a pump. Two samples ($sample_{5cm,\ no\ visible\ algae}$ and $sample_{25cm,\ no\ algae}$), from a depth of 5 cm and 25 cm respectively, were taken 6 m from the centre of the pond where no algae were visibly present. A third sample ($sample_{5\ cm,\ visable\ algae)}$ was taken at 5 cm depth, 50 cm away

from the edge of the pond, where a large amount of motile algae were visibly present. At the time of sampling, the light intensity at the water surface was 1604 µmol m^{-2} s^{-1} (PAR photosynthetic active range), while ambient air temperature was 28.4°C. The pond was at that time largely anaerobic, with the exception of some zones where motile algae appeared to gather. The water temperature, pH and DO were measured *in situ.* Chlorophyll content, VSS and COD analyses were performed within 1h of sampling. Subsamples were immediately filtered using a 0.22 µm filter (Millipore, Belgium) and stored at 4°C until analyses of anion concentration, soluble COD (sCOD) and NH_4^+-N, respectively.

Amount and activity of MOB

Based on physico-chemical analyses of the pond water, diluted Ammonium Mineral Salts medium (dAMS) was chosen as medium for enrichment of the pond samples, as it had a similar NH_4^+-content and pH value as the pond water, without the presence of organic C sources, which would make the enrichment and isolation of MOB more difficult. Sterile dAMS medium (pH 6.9) was prepared by adding 0.1 g NH_4Cl; 0.2 g $MgSO_4.7H_2O$; 7.17 g $Na_2HPO_4.2H_2O$; 2.72 g KH_2PO_4; 30 mg $CaCl_2.6H_2O$; 4.5 mg Na_2-EDTA; 3.5 mg $FeCl_3.6H_2O$; 2.5 mg $CuSO_4.5H_2O$ (10 µM) and other trace elements to one liter of demineralized water up to a volume of 1 L, in accordance to Bowman (2006).

Quantification of living MOB in the three mentioned pond samples was done by dilution to extinction (*n*=3), up to a 10^7 fold dilution. Therefore, 900 µL of sterile dAMS medium and 100 µL sample from the lower dilution were pooled into 12 mL vacutainers (BD, South Africa) under an atmosphere of 20% (v/v) CH_4 in air. After 12 days, presence of MOB was evaluated based on measurements of the absorbance (A_{600nm}), supported by gas phase analyses. Therefore, control vials were used that were treated similarly, but without addition of CH_4 to the gas phase. Dilutions were considered positive for methanotrophic activity when the absorbance increased with a factor 10 and the CH_4 concentration dropped below the average initial value, substracted by five times the standard deviation. This information was used to estimate the abundance of cultivable MOB under the given growth conditions using most probable number tables and is expressed together with its 95% confidence interval (Anonymous, 2008).

The methanotrophic activity was evaluated by measuring the CH_4 oxidation rate of enrichments from the three mentioned pond samples. Therefore, six Schott bottles with a total volume of 590 mL were sterilized and sealed with butyl stoppers. To three bottles, 100 mL sterile dAMS medium was added while the three remaining bottles were filled

with 100 mL filtered (0.22 µm) supernatant (pH 7.1), obtained after centrifugation of pond water (15 min, 10,000 × g, Beckman Coulter Avanti J-E Centrifuge). All bottles were inoculated with 5% (v/v) of one of three fresh pond samples, filled with 20% (v/v) CH_4 in air and placed on a shaker (80 rpm) at 28°C in the dark. The CH_4 oxidation rate and apparent O_2 consumption were measured daily until the O_2 concentration was below 3% (v/v). To indicate the microbial nature of the CH_4 oxidation, the same approach was used after autoclaving the cultures (20 min, 121°C, 1 bar). No significant CH_4 oxidation was observed in this bottle during a period of 96h.

Isolation and identification of cultivable MOB

After four days of incubation, the enriched samples with dAMS were subsampled (25 mL) and MOB were isolated according to Hoefman et al. (2012a). Briefly, samples were serially diluted in 12-fold to the point of extinction (10^{-3} up to 10^{-10}) in 96-well microtiter plates with dAMS. After incubation for two weeks at 28°C under an atmosphere of 50 % (v/v) in air in gastight jars (Oxoid), the highest dilution of each series that was visually positive for growth, was plated onto dAMS agar and sub-cultured to purity. Pure cultures were confirmed for methane oxidation by CH_4 and O_2 consumption and CO_2 production through GC analysis (Compact GC; Global Analyzer Solutions, Belgium) of batch cultures. Pure cultures positive for CH_4 oxidation were identified via 16S rRNA gene sequence analysis and dereplicated via $(GTG)_5$ rep-PCR fingerprinting. Purity of the MOB isolates was confirmed by (i) colony morphology, (ii) phase-contrast microscopy, (iii) absence of heterotrophic growth on 1/10 trypticase soy agar under air and (iv) absence of growth on dAMS under air.

Algae assisted methane oxidation

To evaluate the influence of algal photosynthetic activity on the CH_4 oxidation rate, pond samples were taken 5 cm below the surface (DO = 0.4 mg O_2 L^{-1}). Into three darkened bottles and three illuminated bottles with a volume of 590 mL, 80 mL of filtered pond water was added. These bottles were incubated with 10 mL of an active MOB enrichment on filtered pond water and 10 mL of a native mixed algae culture, both originating from the pond. All bottles were sealed, filled with 20% (v/v) CH_4 in air and placed in an incubation room at 28±2°C without shaking. While the dark bottles were shielded from the light, the illuminated bottles were placed continuously under fluorescent lamps for 72h, (71 **µ**mol m^{-2} s^{-1}, PAR) and the CH_4 and O_2 concentrations in the gas phase measured daily.

Control bottles without algal inoculation indicated that the supplied light intensity had no inhibitory effect on the methanotrophic activity as such.

Physico-chemical analyses of the liquid and gas phase

The concentration of volatile suspended solids (VSS) was assessed according to Greenberg et al. (1992). For chlorophyll extraction, a volume of 20-200 mL was filtered using a Whatman GF/C filter and the filter cake extracted in darkness with 90% acetone for 24h according to Eaton et al. (1998). The absorbance was measured using an AquaMate Plus Spectrophotometer (Thermo Scientific, Waltham, MA). The concentrations of NH_4^+, NO_3^-, NO_2^- and PO_4^{3-} and the concentration of chemical oxygen demand (COD) were analyzed by means of the respectable Merck Spectroquant® test kit (Merck, Whitehouse Station, NJ) and analyzed with the same spectrophotometer. The soluble COD (sCOD) content was measured with a Nanocolor COD test kit 160 (Macherey-Nagel, Germany) with a measuring range between 0 and 160 mg COD L^{-1}. Absorbance was measured with a Nanocolor vario 4 (Macherey-Nagel, Germany). Identification of algae species was done by means of a Carl Zeiss Axiostar Plus light microscope (400× and 1000× magnification).
Analyses of the O_2 and CH_4 concentrations in the gas phase was by injection of 250 µL into an Agilent 6820 gas chromatograph (Agilent Technologies, Santa Clara CA) equipped with a flame ionization and thermal conductivity detector and fitted with either a Molsieve 5A packed column (6 ft × 2.1 mm i.d., Restek Corporation, Bellefonte, PA) or a GS-GasPRO capillary column (60 m × 0.32 mm i.d., Agilent Technologies, Santa Clara, CA). The thermal conductivity detector was set at 300°C with helium as the carrier gas at a flow rate of 0.9 mL min^{-1}. The gas pressure in the bottles was measured with an Infield 7 pressure meter (UMS, Germany) to compensate for over- or under pressure.

Statistical analysis

Results were statistically analyzed by performing a Welch modified two-sample t-test with equal variance and at a significance level of 95% (*n* replicates, $p<0.05$). Significant CH_4 removal was defined as a difference of 5% compared to the former sampling point. Analyses were performed with SPSS (Duncan, version 19.0).

Nucleotide sequence accession numbers

The 16S rRNA gene sequence data generated in this study has been deposited in GenBank/EMBL/DDBJ with accession numbers HF558987 to HF558991.

RESULTS

Influence of algae on the physico-chemical conditions in the pond

Depth profiles over time showed that the physico-chemical conditions in the pond fluctuated constantly: temperature, DO and pH shifted drastically over short periods, depending on the preceding weather conditions (Figure II-1 and II-2). The maximal depth where O_2 saturation occurred depended strongly on the algal activity and was positioned between 55 and less than 1 cm below the pond surface. On sunny days, O_2 levels in the surface layer regularly reached oversaturated values above 25 mg O_2 L^{-1}, though the pond became completely anaerobic during shaded days. Microscopic determination showed that most algae present belonged to the motile *Pyrobotris* species while small amounts of *Chlorella* sp., *Scenedesmus* sp., *Pediastrum* sp. and cyanobacterial species were also observed.

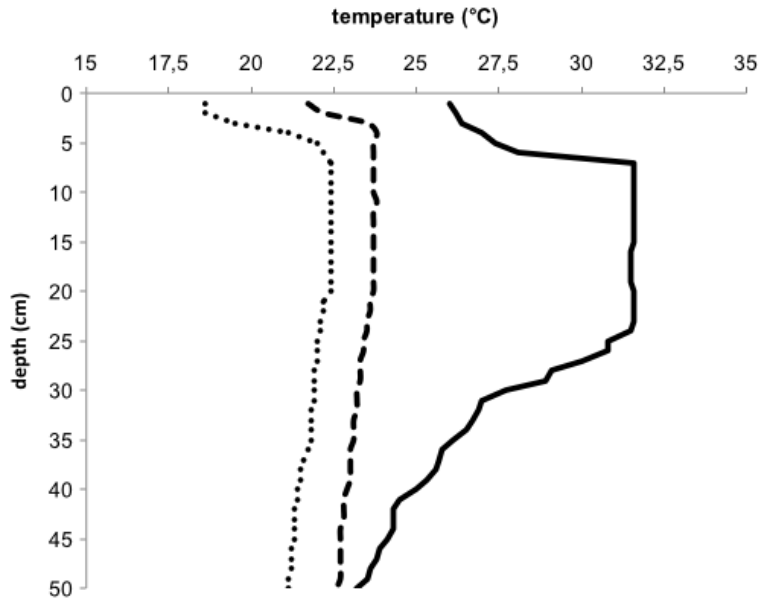

Figure II-1. Temperature profiles of the pond (6 m away from the centre of the pond) in steps of 10 mm, at three different times (February, 7th: solid line, February, 9th: dashed line, February, 10th: dotted line).

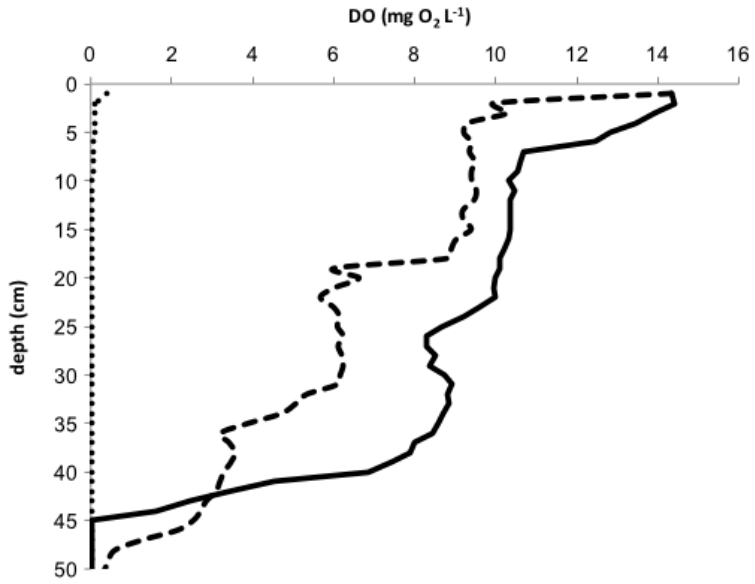

Figure II-2. Dissolved oxygen (DO, mg O_2 L^{-1}) profiles of the pond (6 m away from the centre of the pond) in steps of 10 mm at three different times (February, 7th: solid line, February, 9th: dashed line, February, 10th: dotted line).

To evaluate the influence of algal presence on the physico-chemical conditions in the pond, three samples were taken at a time when the pond was largely anaerobic: sample$_{5cm,visible\ algae}$ was taken at 5 cm depth and algae were visibly observed. Sample$_{5cm,\ no\ visible\ algae}$ was also taken at 5 cm depth but without visibly observable algae while sample$_{25cm,\ no\ algae}$ was taken at 25 cm depth and also without visibly observable algae. In the latter, no chlorophyll was detected and the DO concentration in that sample was below the detection limit of 0.05 mg O_2 L^{-1} (Table II-1). In the other two samples, taken at 5 cm depth, algae were present and concentrations of 0.1±0.1 (sample$_{5cm,\ no\ visible\ algae}$) and 4.4±0.7 (sample$_{5cm,visible\ algae}$) mg chlorophyll L^{-1} were measured. The DO concentrations of these samples were 3.5 and 13.6 mg O_2 L^{-1} respectively, demonstrating the correlation between algal presence and DO. Higher amounts of algae also resulted into increased COD and VSS concentrations (Table II-1).

Table II-1. Overview of physico-chemical parameters for the sample taken at 25 cm depth and samples taken at 5 cm depth with and without visible algae presence, respectively.

	$Sample_{5,\ no\ visible\ algae}$	$Sample_{5,\ visible\ algae}$	$Sample_{25,\ no\ algae}$	Units
Water temp.	27.1	26.4	23.7	°C
pH	7.4	8.4	7.1	-
DO	3.5	13.6	<0.05	mg O_2 L^{-1}
VSS	282±52	584±64	248±26	mg VSS L^{-1}
COD	186±13	430±17	180±6	mg COD L^{-1}
sCOD	99	122	84	mg sCOD L^{-1}
Chlorophyll	0.12±0.02	4.38±0.71	<0.02	mg Chl L^{-1}
NO_3^--N	<0.05	<0.05	<0.05	mg NO_3^--N L^{-1}
NO_2^--N	<0.05	<0.05	<0.05	mg NO_2^--N L^{-1}
NH_4^+-N	17.0	18.5	21.0	mg NH_4^+-N L^{-1}
PO_4^{3-}	4.8	5.2	4.8	mg PO_4^{3-} L^{-1}

Amount and activity of MOB

To assess the amount of cultivable MOB in the pond, most probable number analysis was performed on the three pond samples. 9.3 x 10^4 MOB mL^{-1} [1.8 x 10^4 to 4.2 x 10^5 MOB mL^{-1}] were found for $sample_{5cm,\ visible\ algae}$ and $sample_{5cm,\ no\ visible\ algae}$, while $sample_{25cm,\ no\ algae}$ had a concentration of 2.3 x 10^4 MOB mL^{-1} [4.6 x 10^3 to 9.4 x 10^4 MOB mL^{-1}].

The CH_4 emissions of the primary facultative pond (PFP) were measured during late summer/early autumn with a floating gas capturing device. An average CH_4 emission of 8.6±1.9 m^3 CH_4 d^{-1} was found for the whole pond, indicating the presence of dissolved CH_4, a necessity for methanotrophic activity. dAMS was chosen as enrichment medium since this typical growth medium for MOB has a similar pH of 6.9 and a similar ammonium content of 25 mg NH_4^+-N L^{-1} as the pond water. Presence of MOB was confirmed by enrichment in dAMS as well as in filtered pond water. No significant differences in CH_4 oxidation rates were observed between both growth media, indicating that the use of dAMS had no influence on the methanotrophic activity of the enriched community. After a lag-phase of about 24h, significant CH_4 oxidation rates were observed for both media and the maximal daily CH_4 oxidation rates of 190±5 and (dAMS) and 179±24 mg CH_4 L^{-1}_{liquid} d^{-1} (pond water) were found during the third day of enrichment. At the same time, respectively 441±48 and 412±90 mg O_2 L^{-1}_{medium} d^{-1} were consumed,

indicating that there was no significant difference in apparent O_2 consumption. This led to apparent molar ratios of 1.16±0.13 (dAMS) and 1.15±0.19 (pond water) $O_{2\ consumed}$ over $CH_{4\ oxidized}$ respectively, for the whole microbial community.

Isolation and identification of cultivable MOB

In total, 16 MOB pure cultures were isolated out of the three pond samples, enriched on dAMS. Five type II MOB were isolated, identified as *Methylocystis* sp. (closest related type strain of *Methylocystis parvus* with 99.4-99.5% 16S rRNA gene sequence similarity) and dereplicated into at least three different strains based on rep-PCR fingerprinting. Seven type Ib MOB were isolated; these had identical rep-PCR profiles and were identified as *Methylococcaceae* sp., very distantly related to any of the known type Ib MOB (closest related type strain of *Methylocaldum gracile* with 94.2% 16S rRNA gene sequence similarity). Four type Ia MOB were isolated and identified as *Methylomonas* sp. (closest related type strain of *Methylomonas koyamae* with 100% 16S rRNA gene sequence similarity). The rep-PCR profile of these four isolates were identical but distinct from the type strain of *Methylomonas koyamae* (Figure II-3).

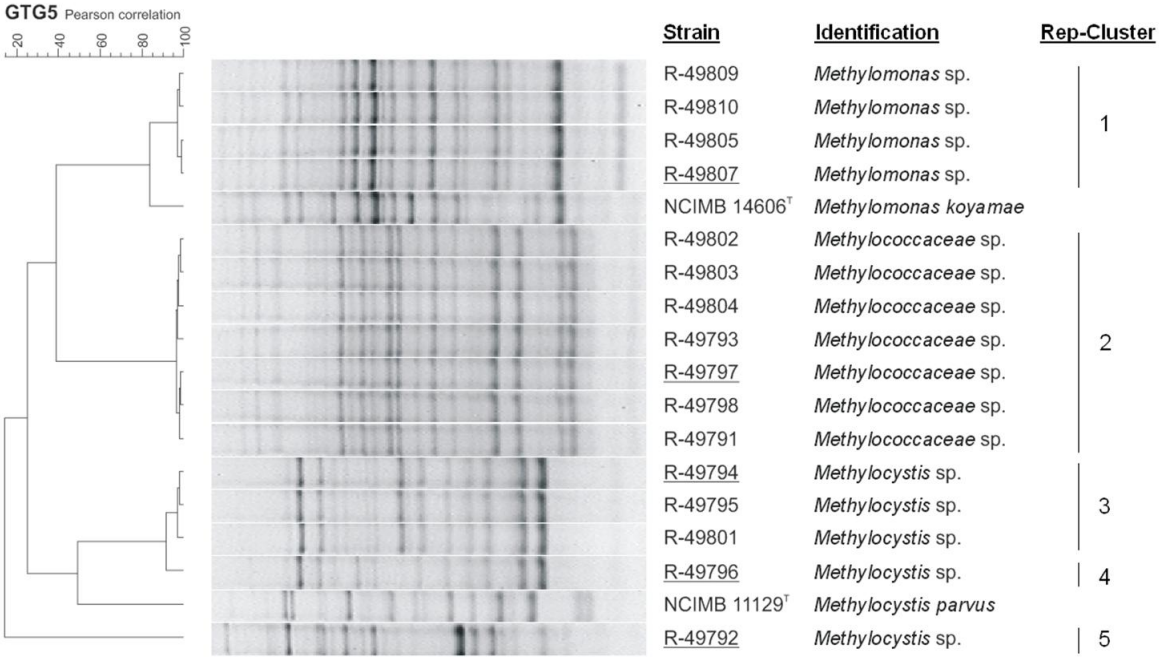

Figure II-3. GTG_5 rep-PCR fingerprinting using Pearson product moment correlation coefficient and UPGMA. MOB isolates were divided into five rep-clusters. Four isolates identified as *Methylomonas* sp. by 16S rRNA gene sequencing had a similar rep-profile (cluster 1), distinct from the most closely related type strain *Methylomonas koyamae* NCIMB 14606^T. Seven *Methylococcaceae* isolates shared a similar rep-profile (cluster 2). The five *Methylocystis* isolates could be divided into three groups (cluster 3, 4 and 5) based on their rep-PCR fingerprint, distinct from the most closely related type strain *Methylocystis parvus* NCIMB 11129^T. A representative of each cluster was selected randomly (underlined) for accession number assignment.

Algal assisted methane oxidation

To evaluate how the *in situ* algal O_2 production influenced the CH_4 oxidizing activity, fresh pond water with a DO concentration of only 0.6 mg O_2 L^{-1} was added to bottles and inoculation occurred with enrichments of MOB and microalgae originating from the same facultative pond. Under an atmosphere of 20% CH_4 (v/v) in air, the CH_4 oxidation rate was evaluated under stagnant conditions, simulating a water column open to the air. Three dark bottles were shielded from the light, while three illuminated bottles allowed the indigenous algae to produce O_2. During the first day of incubation, a lag-phase without significant CH_4 oxidation was observed in both test series. From the second day on, CH_4 oxidation started in the darkened as well as in the illuminated bottles. However, a significantly higher CH_4 oxidation rate was observed in the illuminated bottles (Table II-2).

Table II-2. Comparison of activity parameters between three illuminated bottles and three darkened bottles: the total CH_4 oxidation (mg CH_4 L^{-1}_{liquid}), methane oxidation rate (MOR, mg CH_4 L^{-1}_{liquid} d^{-1}) for the second and third day, total O_2 consumption (mg O_2 L^{-1}_{liquid}), molar ratio of apparent consumption of O_2 over CH_4 and concentration of dissolved O_2 in the liquid phase after the test period of 72h. Significant differences between darkened and illuminated bottles are marked with an asterisk (*) (p<0.05).

	Illuminated bottles	Darkened bottles
Total CH_4 oxidation	318±27*	202±25*
MOR 24-48h	135±33*	64±28*
MOR 48-72h	172±25	141±29
Total O_2 consumption	616±49	596±25
Molar O_2:CH_4 ratio	0.97±0.14*	1.48±0.26*
Dissolved oxygen	7.3±0.6*	3.1±0.4*

Also during the third day, a higher CH_4 oxidation rate was observed in the illuminated bottles. This led to a 1.6 times higher amount of CH_4 removed over the whole test period. At the same time, a similar apparent O_2 consumption was observed, resulting in a significantly higher apparent molar O_2:CH_4 consumption ratio in the dark bottles (Table II-2). The higher *in situ* O_2 production in the illuminated bottles was also observed at the end of the testing period, as a significantly higher DO concentration was present in the liquid phase of the illuminated bottles compared to the dark bottles (Table II-2).

DISCUSSION

Algae assist the methane oxidation

Without the photosynthetic activity of the algae, the whole pond would be anaerobic, except for a surface layer of a few millimetres, where wind and diffusion effects provide some aeration. Photosynthetic O_2 production is a necessity for methanotrophic activity as aerobic MOB can only use molecular O_2 as final electron acceptor (Hanson and Hanson, 1996). It has been reported that photosynthetic organisms like the aquatic grass *Calamogrostis canadensis* or submerged *Spaghnum* mosses can provide O_2 to MOB in anaerobic or microaerophilic conditions, thereby enhancing CH_4 oxidizing activity (Kip et al., 2010). This ability was also observed for algae in laboratory tests, where algae enhanced methanotrophic activity by the *in situ* production of O_2 (van der Ha et al., 2011). This interaction was in this study confirmed in a straightforward set-up, mimicking a shallow stagnant anaerobic water column under an atmosphere of CH_4 and air. In the darkened bottles, the CH_4 oxidizing activity was limited by the slow gas transfer of O_2 to the water column (Melse and Van der Werf, 2005), while in the illuminated bottles algal photosynthetic activity increased the concentration of the limiting dissolved O_2, resulting in a 57% higher CH_4 removal rate. The difference in the apparent O_2:CH_4 ratio can be explained by *de novo* O_2 production by the active algae, which led to a lower consumption of O_2 originating from the gas phase. It can also be concluded that the CH_4 oxidizing activity in these systems is limited by O_2 availability, rather than the amount of available CH_4. This beneficial effect of the algae on MOB is probably even more pronounced in the pond, as the water column there is much deeper, leading to an even more difficult gas transfer. Moreover, a stronger influence of the algae can be expected in the pond, as light intensities during the day are more than an order of magnitude higher than in the batch test, resulting in a zone of more than 50 cm with algae.

Facultative ponds harbor a robust and diverse methanotrophic community

The microbial community from the pond showed a high CH_4 oxidizing activity when incubated with CH_4, demonstrating the presence of CH_4 oxidizing organisms. The maximal observed CH_4 oxidation rate was quickly achieved and those rates were similar to those observed in comparable studies, where enrichments of various inocula were used (van der Ha et al., 2010; Hoefman et al., 2012a). As the activity of the enrichments with filtered pond water and typical dAMS growth medium was similar, no severe nutrient limitations

seemed to occur in the pond. Indeed, sufficient NH_4^+-N and PO_4^{3-}-P was present in the pond to support bacterial activity, while the observed pH and temperature ranges are suited for most in literature described MOB. These conditions explain why at least 10^4 cultivable MOB mL^{-1} were observed in pond samples from the upper 50 cm of the water column. This number is undoubtedly an underestimation of the total amount of active MOB as a relatively short incubation period was applied and not all MOB are cultivable in one mineral growth medium (Hoefman et al., 2012a). Still, these numbers show that, in contrast to results by van der Steen et al. (2003), a large pool of MOB is present in the facultative pond. The isolation campaign showed that MOB indeed were responsible for CH_4 oxidation, as species were isolated and identified from all three major types of proteobacterial MOB, i.e. type Ia, Ib and II. This reveals a diverse methanotrophic seed bank and implies that there was no strong selection towards one specialized species. Moreover, a *Methylococcaceae* sp. was isolated that is only very distantly related to all known MOB type strains. This finding could be the result of the specific conditions of the pond and deserves further investigation.

During the incubation period of pond samples with CH_4, short lag-phases were observed compared to similar batch tests with different types of CH_4 oxidizing inocula, showing that many MOB were active or able to resuscitate quickly from dormancy (van der Ha et al., 2011; Hoefman et al., 2012a; Hoefman et al., 2012b). Indeed, fast phenotypic adaptation and formation of resting structures are common characteristics of MOB and would create a major advantage for the MOB in facultative ponds, where drastic changes in growth conditions take place (Whittenbury et al., 1970a; Hanson and Hanson, 1996). Due to the non-sheltered nature of the facultative pond and the algal activity, extreme changes of DO, pH and temperature occurred over time and created temporarily harsh growth conditions. Moreover, the combination of high pH with strong light intensities and O_2 oversaturation could result in an increased production of harmful radicals, which are known to negatively influence MOB and induce dormancy (Murase and Sugimoto, 2005). Also, the presence of toxic H_2S, originating from anaerobic processes in the sludge blanket, was observed regularly. Probably the MOB form resting structures and/or have mechanisms to protect themselves to a certain extent against such negative influences (Roslev and King, 1994; Hanson and Hanson, 1996). These characteristics could be of importance for biotechnological applications where robustness and a fast response towards negative stimuli are required.

Importance within the framework of global warming

It can be concluded that algal O_2 production is the driving force for the CH_4 oxidizing activity in facultative ponds. As such ponds are important emission sources of CH_4, understanding and enhancing these interactions could help to decrease the C footprint of these wastewater treatment units. Indeed, although at least part of the CH_4 produced by methanogens in the anaerobic sludge blanket is consumed by the MOB before it reaches the atmosphere, elementary extrapolation of measured pond emissions still shows a total loss of about 3000 m^3 CH_4 y^{-1}, equalling a yearly contribution of 55 ton CO_2-equivalents y^{-1} or an emission of 0.98 kg CH_4 y^{-1} per inhabitant for this relatively small pond. The role of this algal driven CH_4 oxidation seems underestimated and therefore deserves further attention.

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REFERENCES

Aiyuk, S., Amoako, J., Raskin, L., van Haandel, A.,Verstraete, W., 2004. Removal of carbon and nutrients from domestic wastewater using a low investment, integrated treatment concept. Water Res. 38, 3031-3042.

Anonymous 2008 Most probable number procedure and tables. Washington D.C., USA: USDA-FSIS.

Bahr, M., Stams, A.J.M., De la Rosa, F., Garcia-Encina, P.A.,Munoz, R., 2011. Assessing the influence of the carbon oxidation-reduction state on organic pollutant biodegradation in algal-bacterial photobioreactors. Appl. Microbiol. Biot. 90, 1527-1536.

Bowman, J.P. (2006) The methanotrophs - the families *Methylococcaceae* and *Methylocystacea*. In *The Prokaryotes: Vol. 5: Protebacteria: Alpha and Beta Subclasses*. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K., and Stackebrandt, E. (eds). New York, NY, USA: Springer Science, pp. 266-289.

Caldwell, D.H., 1946. Sewage oxidation ponds: performance, operation and design. Sewage Works J. 18, 433-458.

Craggs, R., Park, J.,Heubeck, S., 2008. Methane emissions from anaerobic ponds on a piggery and a dairy farm in New Zealand. Aust. J. Exp. Agric. 48, 142-146.

De Almeida, P.G.S., Chernicharo, C.A.L.,Souza, C.L., 2009. Development of compact UASB/trickling filter systems for treating domestic wastewater in small communities in Brazil. Water Sci. Technol. 59, 1431-1439.

DeGarie, C.J., Crapper, T., Howe, B.M.,McCarthy, P.J., 2000. Floating geomembrane covers for odour control and biogas collection and utilization in municipal lagoons. Water Sci. Technol. 42, 291-298.

Eaton, A.D., Clesceri, L.S., Greenberg, A.E.,Franson, M.A. 1998 Standard methods for the examination of water and wastewater. Washington D.C., USA: American Public Health Association.

Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., Fahey, D.W. et al. (2007) Changes in atmospheric constituents and in radiative forcing. In *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M.Tignor and H.L. Miller (eds.) (ed). Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

Greenberg, A., Clesceri, L.,Eaton, A. 1992 Standard methods for the examination of water and wastewater. Washington D.C., USA: American Public Health Association Publications.

Hanson, R.S., Bratina, B.J.,Brusseau, G.A. 1993 Phylogeny and ecology of methylotrophic bacteria. Andover, UK: Intercept Press, Ltd.

Hanson, R.S.,Hanson, T.E., 1996. Methanotrophic bacteria. Microbiological Reviews 60, 439-+.

Hoefman, S., Van Hoorde, K., Boon, N., Vandamme, P., De Vos, P.,Heylen, K., 2012. Survival or revival: long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria. PLoS One 7, e34196.

Hoefman, S., van der Ha, D., De Vos, P., Boon, N.,Heylen, K., 2012a. Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria. Microbiol Biotechnol 5, 368-378.

Hoefman, S., Van Hoorde, K., Boon, N., Vandamme, P., De Vos, P.,Heylen, K., 2012b. Survival or revival: Long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria. Plos One 7, e34196.

Kip, N., van Winden, J.F., Pan, Y., Bodrossy, L., Reichart, G.J., Smolders, A.J.P. et al., 2010. Global prevalence of methane oxidation by symbiotic bacteria in peat-moss ecosystems. Nat. Geosci. 3, 617-621.

Mara, D. 2004 Domestic wastewater treatment in developing countries. London, UK: Earthscan.

Mara, D. (2005) Pond process design - a practical guide. In *Pond Treatment Technology*. Shilton, A. (ed). London, UK: IWA Publishing, pp. 172-174.

Marais, G.V.R., 1966. New factors in design operation and performance of waste-stabilization ponds. Bull. W.H.O. 34, 737-744.

Melse, R.W.,Van der Werf, A.W., 2005. Biofiltration for mitigation of methane emission from animal husbandry. Environ. Sci. Technol. 39, 5460-5468.

Murase, J.,Sugimoto, A., 2005. Inhibitory effect of light on methane oxidation in the pelagic water column of a mesotrophic lake (Lake Biwa, Japan). Limnol. Oceanogr. 50, 1339-1343.

Oswald, W.J. 1976 Experiences with new pond designs in California. Austin, TX, USA: University of Texas Press.

Pearson, H.W., Mara, D.D., Mills, S.W.,Smallman, D.J., 1987. Factors determining algal populations in waste stabilization ponds and the influence of algae on pond performance. Water Sci. Technol. 19, 131-140.

Picot, B., Paing, J., Sambuco, J.P., Costa, R.H.R.,Rambaud, A., 2003. Biogas production, sludge accumulation and mass balance of carbon in anaerobic ponds. Water Sci. Technol. 48, 243-250.

Reed, R.A. 1995 Sustainable sewerage: Guidelines for community schemes. London, UK: Intermediate Technology Publications: WEDC.

Roslev, P.,King, G.M., 1994. Survival and recovery of methanotrophic bacteria starved under oxic and anoxic conditions. Appl. Environ. Microbiol. 60, 2602-2608.

van der Ha, D., Hoefman, S., Boeckx, P., Verstraete, W.,Boon, N., 2010. Copper enhances the activity and salt resistance of mixed methane-oxidizing communities. Appl Microbiol Biot 87, 2355-2363.

van der Ha, D., Bundervoet, B., Verstraete, W.,Boon, N., 2011. A sustainable, carbon neutral methane oxidation by a partnership of methane oxidizing communities and microalgae. Water Research 45, 2845-2854.

van der Steen, N.P., Nakiboneka, P., Mangalika, L., Ferrer, A.V.M.,Gijzen, H.J., 2003. Effect of duckweed cover on greenhouse gas emissions and odour release from waste stabilisation ponds. Water Sci. Technol. 48, 341-348.

Verstraete, W., van de Caveye, P.,Diamantis, V., 2009. Maximum use of resources present in domestic "used water". Bioresour. Technol. 100, 5537-5545.

Whittenbury, R., Davies, S.L.,Davey, J.F., 1970a. Exospores and cysts formed by methane-utilizing bacteria. J Gen Microbiol 61, 219-228.

Reflection & Discussion

Isolation

In Chapter I and II miniaturized extinction culturing was used as fast isolation procedure to retrieve MOB from a variety of methanotrophic enrichments. In addition, the method was applied to isolate a methanotroph from a high ammonium enrichment, which is discussed more thoroughly in Chapter IV. In total, 17 genetically distinct MOB clusters were obtained by dereplication using GTG_5 rep-PCR. From each group, one representative isolate was randomly selected, some of which are used in the experiments described in Chapter III, IV, V, VI and VII. As a result, MOB were isolated from the three major types of proteobacterial methanotrophs: Type Ia, Type Ib and Type II.

The use of miniaturized extinction culturing originated out of necessity. Indeed, our initial attempts to isolate MOB from a variety of methanotrophic enrichments showing methane oxidation activity had failed. In order to isolate MOB, these enrichments were serially diluted onto NMS, AMS and dNMS agar plates with or without 10 µM copper amended to the media and incubated under a methane air atmosphere. Following incubation, approximately 50 colonies that differed morphologically were picked up and subcultured to purity. Unfortunately, none of the cultures appeared to be methanotrophic since they (i) grouped among heterotrophic lineages based on 16S rRNA gene sequence analysis (ii) grew well on nutrient rich media and (iii) grew, albeit poorly, on mineral media regardless of methane addition. Such "cryptic growth", i.e. poor growth of microorganisms on supposedly carbon-free mineral media, perhaps originates from dissolved organic materials from the solidifying agent as suggested by Whittenbury and colleagues (1970). Alternatively, growth could have been supported by traces of carbon in the liquid medium, perhaps enhanced by volatile non-methane carbon compounds when supplying methane, although the methane used throughout the thesis was 99.95% pure. In contrast, type strains from eight different MOB genera, obtained from culture collections, grew well in the exact same setup.

We concluded that we were unlucky and had to enlarge our isolation campaign, motivated by the general idea that isolating MOB is known to be a challenge, and that numerous

isolation attempts by other researchers yielded only a few methanotrophic pure cultures, although it is hardly ever mentioned how much effort it took to obtain these strains. The results of this isolation campaign are presented in Chapter I. In an attempt to reduce cryptic growth, we switched from agar to gellan gum as solidifying agent when performing conventional serial dilution plating from the methanotrophic enrichments. Unfortunately, again, none of the 200 cultures picked up in this way appeared to be methanotrophic. To date, we do not know why serial dilution plating, which proved successful many times for other researchers, was never successful for isolation of MOB in our lab.

In contrast, miniaturized extinction culturing allowed us to rapidly and efficiently isolate MOB from the exact same enrichments as used for serial dilution plating. This procedure has two main advantages over dilution plating, namely (i) the requirement of less laboratory equipment for an equally sized isolation campaign, (ii) almost effortless purification as multichannel liquid handlers allow to easily transfer cultures between microtiter plates and serially dilute cultures within one plate. Liquid handlers are compatible with 96-well microtiter plates and are available in 8-channel and up to 96-channel formats, either manually or automatically operated. Regarding the first advantage, gastight jars (Anaerobic Jar, Oxoid) are commonly used to cultivate MOB. In one jar, 16 agar plates can be incubated. In contrast, a jar can be filled with six 96-well microtiter plates, and thus allows the incubation of almost 600 independent liquid cultures. In hindsight, it would've been more productive if we had exclusively focused on miniaturized extinction culturing, as a lot more time and lab equipment was invested in analyzing the 200 colonies obtained by dilution plating than in analyzing the 48 extinction series that were tested. If we were to redo the isolation campaign, we would focus solely on miniaturized extinction culturing and use different enrichment media in parallel with dNMS for MOB isolation, by for example varying the copper concentration, nitrogen source and headspace composition (CH_4, O_2 and CO_2). As with all isolation campaigns that use an initial enrichment step, we only know that the isolates were present *in situ*, but cannot be certain whether these were dominant or even active in the environment. Nonetheless, it is useful to isolate as much MOB strains as possible, as this allows us to assess their physiological diversity which in return can offer opportunities for their application in biotechnology.

Therefore, we advocate that the use of conventional serial dilution plating should be replaced by miniaturized extinction culturing when attempting to isolate MOB from an initial enrichment. Although the tool itself is simple and we obviously do not claim the

invention of the microtiter plate, we showed that the method works, and that it allows a diverse set of MOB to be rapidly obtained from a variety of enrichments. Furthermore, we are convinced that the obtained diversity is driven by the initial enrichment (the choice of medium, headspace composition, headspace volume over medium volume and the extent of shaking), and not by the used miniaturized method itself. For example, dAMS (ammonium as sole nitrogen source) was used for MOB isolation from facultative pond water, explained in Chapter II. Following isolation, purification and dereplication, multiple attempts to grow the *Methylocystis* strains R-49792, R-49792 and R-49796 obtained from the facultative pond on NMS and dNMS (nitrate as sole nitrogen source) failed, while these strains grow easily with dAMS and AMS. Therefore, if we had chosen dNMS as enrichment medium, as we did in Chapter I, we would not have retrieved these *Methylocystis* cultures, but perhaps other MOB. To further show that the initial enrichment steers the recovered methanotrophic diversity, we isolated an MOB from a 70 mM ammonium enrichment, explained in Chapter IV. In subsequent experiments, this strain proved to grow easily with ammonium levels up to 100 mM, while such concentrations were inhibiting to most other strains, strains that were isolated with only 0 to 2 mM ammonium in the medium.

Dereplication

In isolation campaigns that yield a lot of isolates, for example when a researcher is interested in the total culturable heterotrophic diversity of a natural sample, it is common practice to perform a dereplication step on the obtained isolates for example by rep-PCR or MALDI-TOF MS analysis (Ghyselinck et al., 2011). Rep-PCR analysis is a genotypic fingerprinting technique based on repetitive elements in the whole genome, while MALDI-TOF MS is based on protein mass spectra of the cells. Dereplication allows to rapidly distinguish isolates at a fine taxonomic level (strain or subspecies). For example, two isolates with a different rep-PCR profile do not have an identical genome and thus belong to two different strains. However, depending on the dereplication tool used and the taxon under study, two isolates with the same profile might still belong to two different strains. The taxonomic resolution of rep-PCR and MALDI-TOF MS analysis is situated at the species to strain level. In comparison, the resolution of 16S rRNA gene sequence analysis is situated at the genus to species level. In this thesis, rep-PCR analysis was applied for the dereplication of all isolates. Furthermore, for the isolates obtained in Chapter I, rep-PCR analysis was compared with MALDI-TOF MS (Figure B-1). Both techniques yielded the

same clusters of isolates.

In contrast, we could not find any literature reports performing such a dereplication step following the isolation of methanotrophs. Perhaps this is a result of the fact that many studies only yield one or a few MOB isolates. In cases where isolates all had different 16S rRNA gene sequences, it is of course unnecessary to perform techniques with a higher taxonomic resolution. However, in other cases it is important to perform such tests. For example, if one methanotrophic strain becomes dominant in an enrichment procedure, and subsequent isolation attempts yield 100 separate colonies of this strain, one could claim the isolation of 100 MOB upon culturing the 100 colonies separately, although in fact only one strain was obtained. Subsequent genus assignment and physiological tests on these 100 isolates would falsely give the impression that certain physiological traits are robust within the genus. A dereplication tool can be applied to avoid this kind of conclusions, and we showed that both rep-PCR and MALDI-TOF MS analysis can be useful additions to an MOB isolation campaign to rapidly group highly similar isolates which also allows more efficient downstream analysis.

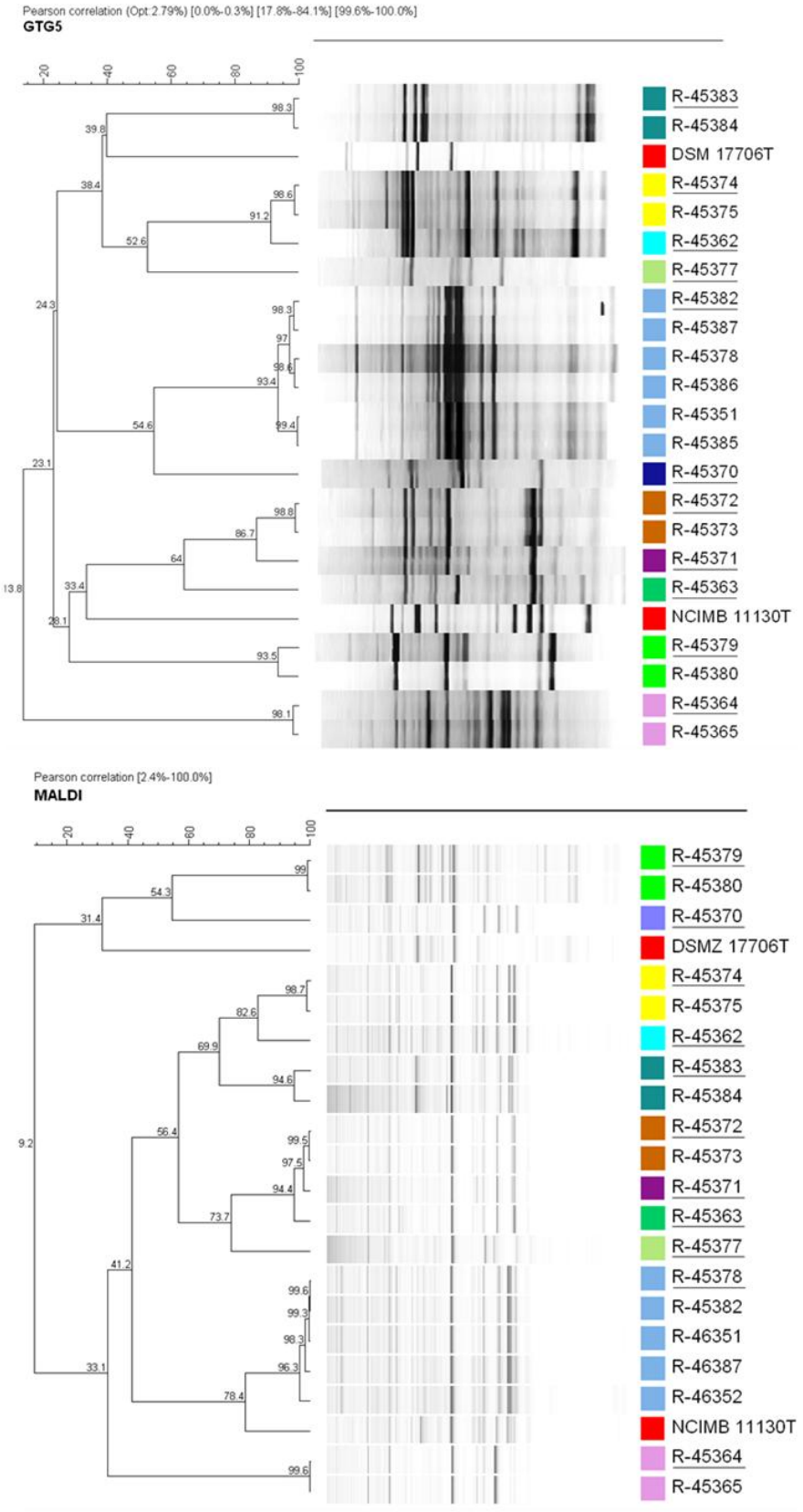

Figure B-1: UPGMA/Pearson clustering of the GTG_5 rep-PCR profiles (top) and MALDI-TOF MS profiles (bottom) of 22 MOB isolates isolated in Chapter I and the type strains *Methylomonas methanica* NCIMB 11130^T and *Methylosinus sporium* DSMZ 17706^T. The cultures are grouped in identical clusters according to both methods. Underlined strains were chosen as representatives for each cluster.

REFERENCES

Ghyselinck, J., Van Hoorde, K., Hoste, B., Heylen, K.,De Vos, P., 2011. Evaluation of MALDI-TOF MS as a tool for high-throughput dereplication. J Microbiol Methods.

Whittenbury, R., Phillips, K.C.,Wilkinson, J.F., 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61, 205-217.

Part C

Physiology of methane-oxidizing bacteria

Chapter III

Customized media based on miniaturized screening improve growth rate and cell yield of *Methylomonas* strains

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Redrafted from:

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Author Contributions:

SH designed and performed the experiments, analyzed the data and wrote the manuscript. KH, DvdH, PDV, PV and NB commented on the manuscript.

SUMMARY

The growth of twelve methanotrophic strains within the genus *Methylomonas*, including the type strains of *M. methanica* and *M. koyamae*, was evaluated in 40 different variations of standard diluted nitrate mineral salts medium in 96-well microtiter plates. Unique profiles of growth preferences were observed for each strain, showing a strong strain dependency for optimal growth conditions, especially with regards to the preferred concentration and nature of nitrate or ammonium as nitrogen source. Based on the miniaturized screening, customized media were designed for each strain and the growth parameters improvements were confirmed in batch setups for several strains, either by a reduction in the lag phase or by a faster biomass accumulation. As such, the maintenance of fastidious strains could be facilitated while the growth of fast-growing *Methylomonas* strains could be further improved. *Methylomonas* sp. R-45378 displayed a 50% increasing in cell dry weight when grown in its customized medium and showed the lowest observed nitrogen and oxygen requirement compared to other strains. We demonstrated that the presented miniaturized approach for medium optimization is a simple tool to quickly generate strain-specific growth preference data that can be applied downstream of an isolation campaign, as a first step in the search for strains with biotechnological potential, to facilitate cultivation of fastidious strains or to steer future isolation campaigns.

INTRODUCTION

Methanotrophs are a physiologically and genetically diverse functional guild grouped by their ability to oxidize the greenhouse gas methane and utilize it as sole carbon and energy source (Hanson and Hanson, 1996). Aerobic methane-oxidizing bacteria (MOB) convert methane to methanol in the first step of their methane oxidation pathway by means of particulate or soluble methane monooxygenase (pMMO or sMMO), enzymes with a broad substrate specificity (Semrau et al., 2010). As a consequence, MOB are potentially useful for many industrial applications such as (i) mitigation of greenhouse gas emissions, (ii) bioremediation of pollutants by co-metabolic activity and (iii) production of valuable bio-compounds, such as single cell protein or poly-β-hydroxybutyrate (Anthony, 1982; Zhang et al., 2008; Scheutz et al., 2009; Jiang et al., 2010; Wendlandt et al., 2010; Semrau, 2011). However, their application in biotechnology has so far remained limited in part due to the relative slow growth rate and low cell density of the available strains (Jiang et al., 2010). Growth in optimal media is essential to assess whether strains are suitable candidates for any potential biotechnological application. Moreover, improved growth parameters will make it less likely for strains to become lost over time, a common problem for some of the more fastidious methanotrophs (Bowman et al., 1993; Hanson and Hanson, 1996) and knowledge of medium composition preference of methanotrophs in general could steer future isolation campaigns. However, optimizing growth conditions becomes laborious very rapidly when numerous conditions are assessed in parallel batch or continuous reactors. Although such attempts have been made, the obtained data are usually limited to a few conditions tested on one or several strains (Park et al., 1991; Park et al., 1992; Shah et al., 1992; Kolesnikov et al., 2004; Doronina et al., 2008). MOB are usually grown in Nitrate Mineral Salts (NMS) medium as described by Whittenbury and co-workers (Whittenbury et al., 1970) or in an adaptation thereof depending on the strain and application. For example, the copper-to-biomass ratio determines which MMO is expressed (Semrau et al., 2010), and therefore NMS without copper addition can be used if sMMO expression is desired (Park et al., 1991) due to e.g. its broader substrate specificity (Semrau et al., 2010), while growth is more rapid when NMS is amended with copper due to exclusive pMMO expression (Park et al., 1992; Doronina et al., 2008). The nature and concentration of nitrogen source is also an important medium parameter. Although all described MOB can grow with ammonium instead of nitrate (Anthony, 1982), direct ammonia inhibition due to competitive MMO inhibition or indirectly due to nitrite and/or

hydroxylamine toxicity produced through ammonia oxidation has been observed (Whittenbury et al., 1970; Bodelier and Laanbroek, 2004). However, growth yields with ammonium have been reported to be higher than with nitrate (Whittenbury et al., 1970; Doronina et al., 2008), while some strains are not capable of growing with nitrate as sole nitrogen source (Whittenbury et al., 1970; Anthony, 1982), and nitrate inhibition has also been observed at elevated concentrations (Reay and Nedwell, 2004; Lee et al., 2009), although potentially due to osmotic effects (Bodelier and Laanbroek, 2004). Therefore, depending on the situation, ammonium can be an inhibitor as well as a stimulator for growth. Besides being an obvious nitrogen source for growth, these nitrogen compounds also play a poorly understood regulatory role on methane oxidation (Bodelier and Laanbroek, 2004). Besides copper and nitrogen, many other medium compounds have been found crucial in cultivating MOB, such as iron (Whittenbury et al., 1970; Park et al., 1991), phosphates (Whittenbury et al., 1970; Bowman, 2006), trace elements (Whittenbury et al., 1970), calcium (Whittenbury et al., 1970; Tsubota et al., 2005), chlorides (Kolesnikov et al., 2004; Tsubota et al., 2005), magnesium (Doronina et al., 2008) and other minerals (Kolesnikov et al., 2004), which gave rise to other NMS adapted media such as 5-fold diluted NMS [dNMS; (Wise et al., 1999; Dunfield et al., 2003)], ANMS (Tsubota et al., 2005), vitamin and copper rich NMS (Bodrossy et al., 1997) or media more adapted to the natural conditions of the MOB (Dedysh et al., 1998). The growth of two model MOB strains have been well-characterized (Jiang et al., 2010), namely *Methylococcus capsulatus* Bath (Type I; Gammaproteobacterial MOB) and *Methylosinus trichosporium* OB3b (Type II; Alphaproteobacterial MOB). However, it is unclear whether these chosen model organisms are representative for other MOB. Moreover, it is doubtful that these strains would be the best-suited candidates for all types of applications, each with a specific set of conditions.

In the present study, the genus *Methylomonas* (Type I) was chosen to perform a high throughput assessment of medium composition to optimize growth rate and/or cell yield, as a first step towards potential assessment for biotechnology. This genus is particularly well suited since its members are among the fastest growing mesophilic MOB (Whittenbury et al., 1970), are physiologically diverse (e.g. strain dependency of sMMO and nitrogenase possession) and have been found in a wide variety of environments (Koh et al., 1993; Hanson and Hanson, 1996; Bowman, 2006). The growth of twelve genetically different *Methylomonas* strains, including two type strains, was evaluated in 40 different dNMS medium variations using 96-well microtiter plates. Using customized media for each strain,

enhanced growth parameters were subsequently confirmed in batch setups.

EXPERIMENTAL PROCEDURES

Bacterial strains and standard growth conditions

Twelve methane-oxidizing pure cultures belonging to the genus *Methylomonas* were used in this study. Ten strains with distinct GTG_5 rep-PCR patterns were selected (Hoefman et al., 2012): *Methylomonas* spp. R-45362, R-45363, R-45364, R-45370, R-45371, R-45372, R-45374, R-45377, R-45378, R-45383 [original isolation numbers H1, E1, K1, B1, D1, C1, G1, I1, A6 and J1, respectively; (Hoefman et al., 2012)]. In addition, the two most closely related extant type strains to the ten novel strains were included: *Methylomonas methanica* NCIMB 11130^T and *Methylomonas koyamae* NCIMB 14606^T, both obtained from the NCIMB culture collection (www.ncimb.com). Based on 16S rRNA gene identification, strains R-45362, R-45363, R-45364, R-45371, R-45372 and R-45374 share *M. methanica* NCIMB 11130^T as closest related type strain (98.3-98.6% sequence similarity), while *M. koyamae* is the closest related type strain for R-45378 (99.5%) and R-45383 (97.9%). *Methylomonas* sp. R-45370 and R-45377 cluster separately from any of the known *Methylomonas* type strains with *M. scandinavica* $SR5^T$ as closest related type strain with 97.5% sequence similarity (data not shown). Strains were routinely cultivated under a methane : air (1 : 1) atmosphere in gastight jars (Oxoid, UK) on diluted Nitrate Mineral Salts (dNMS) medium (Dunfield et al., 2003) with 2 mM KNO_3, 4 mM $MgSO_4$, 0.9 mM $CaCl_2$, 2 mM Na_2HPO_4/KH_2PO_4 buffer (pH 6.8), 14 µM ferric-sodium-EDTA and a trace element solution (Bowman, 2006) with Cu^{2+} concentration adjusted to 10 µM.

Miniaturized medium screening

Key medium components of dNMS were varied in concentration or type of compound. The following 40 dNMS variations could be evaluated for growth compared to standard dNMS: 1, 5, 10, 20 and 50 mM nitrate; 1, 5 and 10 mM buffer; 1, 5 and 50 µM Cu^{2+}; 70, 140 and 700 µM Fe^{3+}; dNMS at pH 4, 5, 5.8, 6.3, 7.3, 7.8, 9 and 10; dNMS supplemented with 5x, 10x and 50x trace element solution (Bowman, 2006), and dNMS supplemented with 1%, 5% and 10% (w/v) NaCl. Additionally, the nitrogen source was switched from nitrate to ammonium in the following concentrations of NH_4^+/buffer: 2 mM / 2 mM, 2 mM / 10 mM, 10 mM / 2 mM, 10 mM / 40 mM, 40 mM / 10 mM and 40 mM / 40 mM. Buffer concentrations were linked to ammonium, since methantrophs rapidly acidify their medium

when grown with ammonium instead of nitrate (Whittenbury et al., 1970). In addition, variations to dNMS were prepared by 5-fold lower and 5, 10 and 20-fold higher concentration of all dNMS medium components (i.e. KNO_3, $MgSO_4$, $CaCl_2$, buffer, ferric-sodium-EDTA and trace elements solution).

Fresh colonies from one-week grown cultures on standard dNMS plates were used as inoculum (OD_{600nm} 0.01, final concentration). To be able to reach identical cell densities, two-week grown cultures were used for the two slower-growing strains *Methylomonas* sp. R-45370 and R-45377. All strains were inoculated in liquid dNMS and in each of the above mentioned dNMS variations in sterile polystyrene 96-well microtiter plates in duplicate (300 µL, final volume per well). Cultures were incubated under a CH_4 : air (1 : 1) atmosphere at isolation temperature (20°C) for the ten recently isolated strains and at 28°C for the two type strains. Growth was determined through OD_{600nm} measurements and verified by visual inspection at the start of incubation and after 1, 2, 3, 7 and 14 days of incubation.

For each strain, the positive, negative or neutral effect on growth measured as cell yield in each medium composition was evaluated compared to standard dNMS. The impact of a medium adaptation was scored positive when for the majority of time-points (i) the average growth (OD_{600nm}) in the adapted medium was larger than the average growth in standard dNMS increased with 3-times the standard deviation of growth in standard dNMS. Conversely, a negative score was obtained when for the majority of time-points the average growth in an adapted medium was lower than the average growth of dNMS decreased with 3-times its standard deviation and supported visually. When neither of the two above cases were true, it was interpreted that the medium adaptation did not influence growth compared to standard dNMS. This evaluation allowed to assign positive scores to adapted media which (i) improved cell yield, (ii) improved growth rate or (iii) improved both cell yield and growth rate, since higher OD_{600nm} values at final time-points will be observed for adapted media which improve cell yield and since higher OD_{600nm} values at earlier time-points will be observed for adapted media that allow faster growth.

In parallel, cultures were grown in standard dNMS and incubated at 5°C, 15°C, 20°C, 25°C, 28°C, 33°C, 37°C, 45°C and 52°C to determine their optimal growth temperature based on the above mentioned evaluation method.

Composition of customized media per strain

Based on the miniaturized medium screening, one or more dNMS variations had a positive

effect on the growth of several strains. Customized media were composed for these strains by adjusting the concentration of the following key dNMS compound-categories: nitrogen source, copper, iron, trace elements and/or buffer of the original dNMS medium, to the concentration of the best-scoring positive medium adaptation [highest overall average OD_{600nm} values considering both earlier (growth rate) and final (cell yield) time-points] in each category. When no improvement was observed in a specific compound-category, the initial dNMS concentration was used (e.g. when the copper concentration did not positively influence growth, the original dNMS copper concentration of 10 µM was used for the customized medium). For some strains, there was no clear growth improvement for any of the adapted media compared to standard dNMS, for which no customized medium was designed and no further batch experiments were set up.

Evaluation of customized media in batch cultures

For strains for which customized media could be composed based on the miniaturized evaluation, batch tests were set up in duplicate whereby the growth parameters in customized media were compared to those in standard dNMS. Cultures were inoculated as explained above in standard dNMS and customized dNMS medium in gastight bottles and incubated for 10 days on a shaker (90 rpm) at optimal temperature (based on the evaluation at above mentioned temperatures) with 20% CH_4 added to the headspace (30 mL culture, 100 mL air, 25 mL methane). The CH_4, O_2 and CO_2 concentration was measured daily with a Compact GC (Global Analyzer Solutions, Belgium), equipped with one channel connected to a flame ionization detector and two channels connected to a thermal conductivity detector. Values were converted to mmol CH_4, O_2 and CO_2 L_{liquid}^{-1} by compensating for change in gas pressure (measured with an Infield 7 pressure meter, UMS, Germany) and taking the solubility of the gases into account. Methane oxidation activity correlated with an expected drop in gas pressure and an increase in turbidity due to growth of the culture. Growth was monitored by OD_{600nm} and protein content (BCA protein assay kit, Thermo Scientific) measurements. Colorimetric methods were used to determine the nitrate (Cataldo et al., 1975) or ammonium (Taylor et al., 1974) consumption. Several parameters were extracted from the dataset such as the assimilated carbon (total CH_4 consumption subtracted by the total CO_2 production at the end of the batch experiment; expressed in mmol assimilated carbon L_{liquid}^{-1}), the molar O_2 : CH_4 ratio (total O_2 consumption divided by the total CH_4 consumption at the end of the batch experiment) and the molar N : C ratio (the total N consumption divided by the assimilated carbon at the end

of the batch experiment). The maximum Methane-Oxidation Rate (MOR) was defined as the maximum observed drop in CH_4 concentration between time-points, expressed in mmol CH_4 L_{liquid}^{-1} day $^{-1}$. The time at which this maximum MOR was achieved is also shown: e.g. when the highest drop in CH_4 concentration was observed between one and two days of cultivation, the time to maximum MOR is expressed as Day 1-2 (Table III-2).

Evaluation of customized media in semi-continuously fed cultures

Based on the results from the batch experiment which mainly evaluated growth rate improvement, four strains were selected (see results section) to evaluate cell yield improvement in customized dNMS compared to standard dNMS in semi-continuously fed cultures in an identical set up as mentioned above, but where both the headspace and the medium was refreshed daily from two to three days (strain dependant, see results section) of incubation onward. Upon refreshing, cells were retained by centrifugation for 15 min at 6,000 g and the total liquid phase was exchanged with an equal volume of fresh medium, while the headspace, 20% methane in air, was also refreshed. In addition to daily measurements of (i) the CH_4, O_2 and CO_2 concentrations, (ii) protein content and (iii) OD_{600nm}, the cell dry weight content was determined after seven refreshment/incubation cycles.

RESULTS

Miniaturized medium screening

Growth of twelve methane-oxidizing strains from the genus *Methylomonas* was evaluated in 40 variations of standard diluted nitrate mineral salts medium (dNMS) in 96-well microtiter plates. Since growth rate improvements result in higher OD_{600nm} values at earlier time-points of the growth curve and cell yield improvements resulted in higher OD_{600nm} values at final time-points, both parameters were automatically assessed in the evaluation. *Methylomonas* sp. R-45371 grew mostly attached to the polystyrene sides of the wells and not in solution, which resulted in a large discrepancy between visual interpretations and OD_{600nm} measurements and therefore this strain was excluded from further evaluation. For the remaining eleven strains, increase, decrease or no change in OD was observed in adapted media compared to standard dNMS (Table III-1). Fourteen medium variations showed lower growth for all strains: dNMS with 0 mM and 50 mM KNO_3, 2 and 10 mM NH_4Cl with 2 mM buffer, 1 µM $CuSO_4$, 50-fold concentrated trace elements solution, 5-fold diluted dNMS, dNMS supplemented with 5 and 10 % NaCl and dNMS with pH below 6.3 and above 7.8. For the remaining 26 conditions, the impact of each medium adaptation on growth was strain dependent.

Table III-1 (Next Page): The positive (dark grey; +), unchanged (grey; =) or negative (white; -) impact of the 40 medium variations compared to dNMS on the growth of strains NCIMB11130^T, R-45363, R-45362, R-45364, R-45372, R-45374, NCIMB 14606^T, R-45378, R-45383, R-45370 and R-45377. The medium variations were obtained by modifying the initial dNMS concentration of nitrogen source (KNO_3 or NH_4Cl), buffer, trace elements, $CuSO_4$ and FeNaEDTA. Additionally, all dNMS components were diluted 5-times (dNMS /5) or concentrated 5- to 50-times (dNMS 5x, 10x, 50x), dNMS was tested at different pH and NaCl was supplemented to dNMS (1%, 5%, 10% NaCl). As a reference, for standard dNMS these varying compounds are 2 mM KNO_3, 2 mM buffer, 1-time trace elements, 10 µM $CuSO_4$, 14 µM FeNaEDTA and pH 6.8 without salt addition. R-45371 was excluded from the evaluation since this strain only grew attached to the polystyrene microtiter plates for which reliable OD_{600nm} measurements could not be obtained.

Medium adaptation	NCIMB 11130^T	R-45363	R-45362	R-45364	R-45372	R-45374	NCIMB 14606^T	R-45378	R-45383	R-45370	R-45377
0 mM KNO_3	-	-	-	-	-	-	-	-	-	-	-
1 mM KNO_3	-	=	-	=	-	-	=	=	-	-	-
5 mM KNO_3	+	=	=	=	-	-	=	=	=	=	-
10 mM KNO_3	+[a]	=	=	+	-	-	=	=	=	=	-
20 mM KNO_3	-	=	=	+[a]	-	-	=	=	=	=	-
50 mM KNO_3	-	-	-	-	-	-	-	-	-	-	-
2 mM KNO_3 / 1 mM Buffer	=	=	=	=	=	=	=	-	-	-	=
2 mM KNO_3 / 5 mM Buffer	+[a]	=	=	=	=	=	=	=	=	=	=
2 mM KNO_3 / 10 mM Buffer	+	-	=	=	=	=	=	=	=	-	=
2 mM NH_4Cl / 2 mM Buffer	-	-	-	-	-	-	-	-	-	-	-
10 mM NH_4Cl / 2 mM Buffer	-	-	-	-	-	-	-	-	-	-	-
2 mM NH_4Cl / 10 mM Buffer	=	=	-	+	-	+	=	=	=	=	=
10 mM NH_4Cl / 10 mM Buffer	=	=	-	-	+[a]	+[a]	=	+[a]	=	=	=
10 mM NH_4Cl / 40 mM Buffer	+	=	-	-	=	+	-	+	=	=	=
40 mM NH_4Cl / 10 mM Buffer	=	=	-	-	=	+	=	=	=	=	-
40 mM NH_4Cl / 40 mM Buffer	=	=	-	-	-	+	-	=	-	=	-
1µM $CuSO_4$	-	-	-	-	-	-	-	-	-	-	-
5µM $CuSO_4$	-	=	=	=	=	=	=	=	=	=	+[a]
50µM $CuSO_4$	-	=	=	=	=	=	-	=	=	-	-
70 µM FeNaEDTA	=	=	=	=	+[a]	=	=	=	=	=	-
140 µM FeNaEDTA	=	=	=	=	=	-	=	+[a]	=	-	-
700 µM FeNaEDTA	-	=	=	-	-	-	=	-	-	-	-
trace elements 5x	=	-	-	=	-	-	-	-	-	-	-
trace elements 10x	-	-	-	=	-	-	-	-	-	-	-
trace elements 50x	-	-	-	-	-	-	-	-	-	-	-
dNMS 5x	=	=	=	=	=	=	=	-	+	=	=
dNMS 10x	=	=	=	=	=	+	-	-	+[a]	-	-
dNMS 20x	-	-	-	-	=	-	-	-	=	-	-
dNMS /5	-	-	-	-	-	-	-	-	-	-	-
dNMS pH 4	-	-	-	-	-	-	-	-	-	-	-
dNMS pH 5	-	-	-	-	-	-	-	-	-	-	-
dNMS pH 5.8	-	-	-	-	-	-	-	-	-	-	-
dNMS pH 6.3	-	=	-	-	=	=	=	-	-	-	=
dNMS pH 7.3	=	=	=	=	=	-	=	=	=	=	=
dNMS pH 7.8	=	=	=	=	=	-	-	-	=	-	=
dNMS pH 9	-	-	-	-	-	-	-	-	-	-	-
dNMS pH 10	-	-	-	-	-	-	-	-	-	-	-
1% NaCl	-	-	-	-	-	-	-	-	-	=	=
5% NaCl	-	-	-	-	-	-	-	-	-	-	-
10% NaCl	-	-	-	-	-	-	-	-	-	-	-

[a] Condition selected for optimized medium

Upon increasing the nitrate concentration from 2 to 20 mM KNO_3, strains NCIMB 11130^T (up to 10 mM KNO_3) and R-45364 (up to 20 mM KNO_3) showed improved growth, while for other strains such an increase either did not affect or negatively affected growth. No effect on growth was observed for all strains when the buffer concentration of dNMS was increased from 2 to 5 mM, except for NCIMB 11130^T exhibiting improved growth. No change or increased growth was observed for all strains for at least one condition where nitrate was replaced with ammonium as sole nitrogen source, except for *Methylomonas* sp. R-45362 (although growth as such was also observed for R-45362 with ammonium, data not shown). Generally, growth with ammonium improved with increasing buffer concentrations from 2 mM to 10-40 mM. Such an impact of the buffer concentration was not apparent for conditions with nitrate as sole nitrogen source. Interestingly, strains R-45372, R-45374 and R-45378 showed a preference for growth with ammonium as nitrogen source over nitrate, with 10 mM NH_4Cl complemented with 10 mM buffer as the best medium adaptation. Other strains, such as NCIMB 11130^T, R-45362 and R-45364 seemed to prefer nitrate over ammonium as nitrogen source, while such a preference was not observed for the remaining strains.

A two-fold reduction of the copper concentration had an improved effect on the growth of R-45377, while this did not affect the other strains, except for NCIMB 11130^T which was negatively affected by such a reduction. R-45372 and R-45378 showed improved growth with a 5 to 10 fold iron increase, while this was not apparent for the other strains. Reduced growth was observed when the concentration of trace elements was increased 5 or 10-fold, except for NCIMB 11130^T (5-fold) and R-45364 (10-fold).

Overall, the results of the miniaturized medium screening experiments showed that the growth of each strain was influenced uniquely by the 40 adapted media resulting in a different profile of medium composition preference per strain (Table III-1).

Customized media per strain and their assessment in batch cultures

For seven out of eleven strains, improved growth was observed for at least one adapted medium compared to standard dNMS. Based on these observations (Table III-1), the following customized media were composed: dNMS with 20 mM KNO_3 for R-45364, dNMS with 10 mM NH_4Cl (without nitrate), 10 mM buffer and 70 µM FeNaEDTA for R-45372, dNMS with 10 mM NH_4Cl (without nitrate) and 10 mM buffer for R-45374, dNMS with 5 µM $CuSO_4$ for R-45377, dNMS with 10 mM NH_4Cl (without nitrate), 40 mM buffer and 140 µM FeNaEDTA for R-45378, dNMS with all compounds concentrated 10-

times except for trace elements for R-45383 and dNMS with 10 mM KNO_3 and 5 mM buffer for NCIMB 11130^T. For other strains, none of the adapted media appeared more suitable than dNMS in this setup and according to the applied growth criteria.

Growth of the remaining seven strains in their customized strain-dependent media was compared to dNMS in a batch setup. The results of these experiments are shown in Figure III-1 and summarized in Table III-2. In customized dNMS, a three day shorter lag phase was observed for R-45383. As a result, the period to achieve the maximum MOR decreased from 4-5 days in dNMS to 1-2 days, even though other parameters such as the maximum MOR itself or the assimilated carbon were not affected (Table III-2). Similarly, R-45374 seemed to grow faster in optimized dNMS compared with standard dNMS (Figure III-1 and Table III-2) as the time to maximum MOR decreased from between day 1 and 2 to day 0 and 1. The slow-growing *Methylomonas* sp. R-45377 (growth on dNMS plates and in microtiter wells was generally apparent after two weeks), appeared to grow faster in customized dNMS, while the strain was not active in standard dNMS during the tested time frame of 10 days. Although R-45372 and R-45378 did not seem to grow faster in customized dNMS, both strains showed significantly higher assimilated carbon in customized dNMS (p-value < 0.05, two-sample t-test), which was defined as the total CH_4 consumption subtracted by the total CO_2 production, expressed in mmol assimilated carbon L_{liquid}^{-1}. In customized dNMS, Strain R-45378 displayed the highest assimilated carbon (16.0 ± 0.4 mmol L_{liquid}^{-1}) obtained in this study, higher than the type strain of *M. methanica* in dNMS (10.4 ± 0.6 mmol L_{liquid}^{-1}) and in its customized dNMS (12.5 ± 1.0 mmol L_{liquid}^{-1}) and higher than the type strain of *M. koyamae* in dNMS (8.50 ± 0.15 mmol L_{liquid}^{-1}, data not shown since for this strain no customized medium could be designed).

Compared with the other strains, the two strains for which the time to reach maximum MOR was the longest, R-45377 and R-45383, also appeared to have lower MOR values (9.04 to 10.9 mmol L_{liquid}^{-1} Day^{-1}), indicating that these strains generally grow slower than the other *Methylomonas* strains tested. For strains R-45374 and R-45378, a lag phase in activity was not apparent (Figure III-1). Interestingly, in both standard dNMS and customized dNMS these strains also showed lowest observed molar O_2 : CH_4 ratios (1.10 to 1.19; Table III-2) and lowest observed molar N : C ratios (0.089 to 0.118; Table III-2), indicating a lower oxygen and nitrogen requirement.

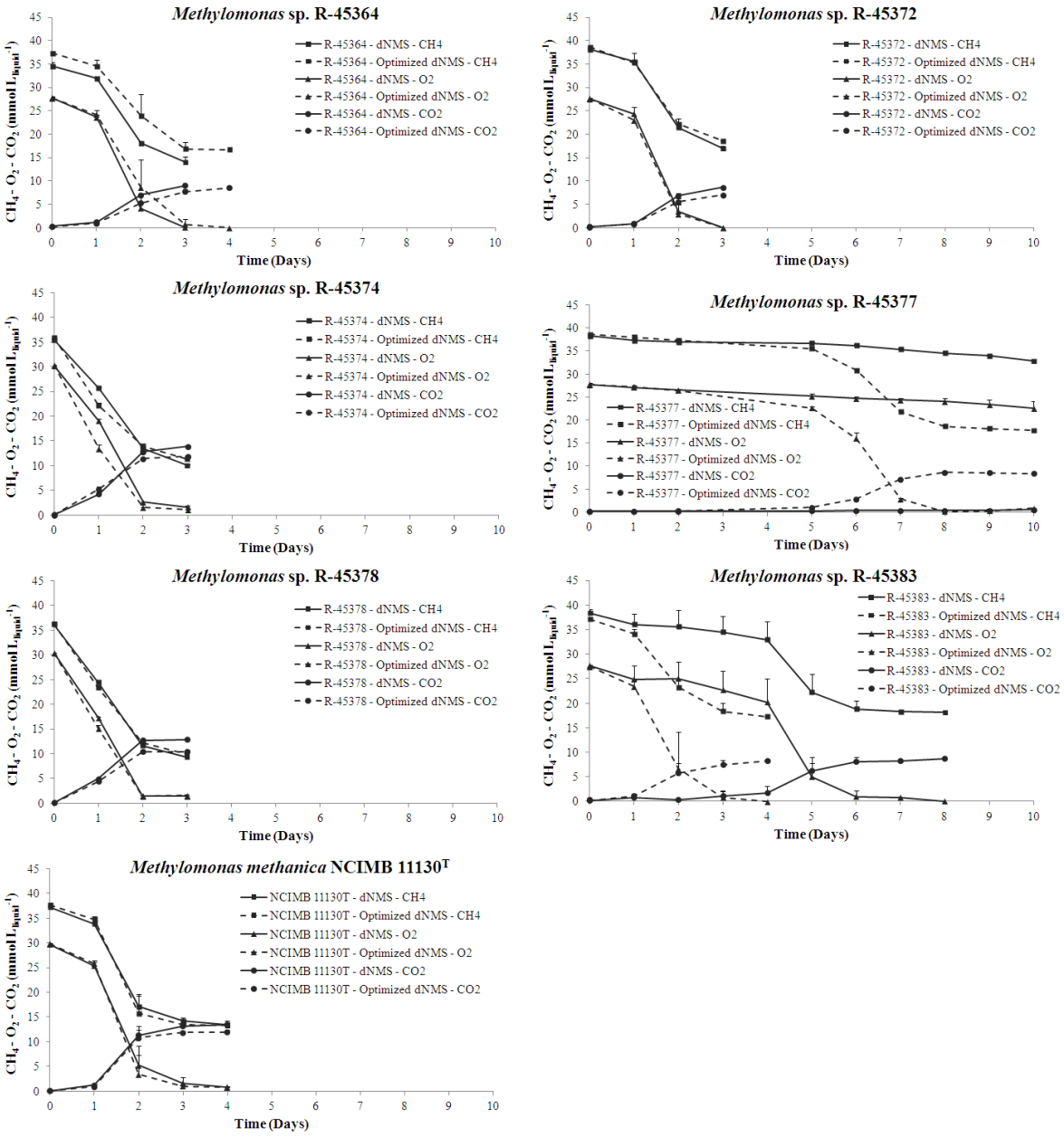

Figure III-1: Average (n = 2) total CH_4 (squares), O_2 (triangles) and CO_2 (circles) concentration (mmol L^{-1}_{liquid}) over time (days) in dNMS (solid line) and optimized dNMS (dashed line) of *Methylomonas* spp. R-45364, R-45372, R-45374, R-45377, R-45378, R-45383 and *M. methanica* NCIMB 11130^T cultivated in batch cultures at optimal temperature (20°C for R-45377 and 28°C for the other strains). The customized dNMS used for each strain are listed in the results section. When approximately all O_2 was consumed, cultures were not sampled further. For *Methylomonas* sp. R-45377 GC samples were not taken at day 3 and 4, since the gas pressure (as indicator for methane oxidation) did not drop and cultures were not turbid (growth as indicator for activity), but final time-points were taken to obtain complete profiles. One-sided error bars are shown. For several time-points, error bars are not clearly visible due to the limited variation between duplicates.

Table III-2: Average (n = 2) assimilated carbon, molar O_2 : CH_4 ratio, N : C ratio, the maximum measured methane oxidation rate (maximum MOR, mmol L^{-1}_{liquid} day^{-1}) and time at which this rate was achieved in dNMS and optimized dNMS (listed in the results section per strain) of *Methylomonas* spp. R-45364, R-45372, R-45374, R-45377, R-45378 and R-45383 and *M. methanica* NCIMB 11130^T cultivated in batch cultures. Standard deviations are listed between brackets. For R-45377 cultivated in dNMS, no activity or growth was observed within the measured time-frame (10 days incubation).

	R-45364		R-45372		R-45374		R-45377	
	dNMS	Optimized	dNMS	Optimized	dNMS	Optimized	dNMS	Optimized
Assimilated carbon (mmol L_{liquid}^{-1})	11.8 (0.4)	12.9 (0.4)	12.8 (0.0)	13.4 (0.1)	11.7 (0.2)	12.9 (0.5)	no growth	12.1 (1.3)
Molar O_2 : CH_4 ratio	1.35 (0.01)	1.32 (0.01)	1.30 (0.00)	1.37 (0.00)	1.12 (0.01)	1.19 (0.01)	no growth	1.34 (0.10)
Molar N : C ratio	0.159 (0.020)	0.171 (0.098)	0.161 (0.007)	0.129 (0.047)	0.104 (0.019)	0.092 (0.014)	no growth	0.127 (0.037)
Max. MOR (mmol L_{liquid}^{-1} Day^{-1})	13.8 (0.3)	10.6 (3.3)	14.1 (0.1)	13.1 (0.8)	12.3 (0.4)	13.8 (0.7)	no growth	9.04 (0.61)
Time to maximum MOR	Day 1-2	Day 1-2	Day 1-2	Day 1-2	Day 1-2	Day 0-1	no growth	Day 6-7

	R-45378		R-45383		NCIMB 11130^T	
	dNMS	Optimized	dNMS	Optimized	dNMS	Optimized
Assimilated carbon (mmol L_{liquid}^{-1})	13.9 (0.2)	16.0 (0.4)	12.0 (0.4)	11.9 (0.2)	10.4 (0.6)	12.5 (1.0)
Molar O_2 : CH_4 ratio	1.1 (0.0)	1.10 (0.01)	1.35 (0.03)	1.38 (0.01)	1.21 (0.01)	1.19 (0.03)
Molar N : C ratio	0.118 (0.006)	0.089 (0.007)	0.122 (0.005)	0.205 (0.046)	0.185 (0.034)	0.135 (0.017)
Max. MOR (mmol L_{liquid}^{-1} Day^{-1})	12.9 (0.3)	12.9 (0.5)	10.7 (0.0)	10.9 (4.2)	16.7 (2.5)	19.0 (3.4)
Time to maximum MOR	Day 1-2	Day 0-1	Day 4-5	Day 1-2	Day 1-2	Day 1-2

An improvement of growth characteristics in customized dNMS was not apparent for R-45364 and NCIMB 11130^T. Their customized dNMS consisted of an increase in KNO_3 from 2 mM in standard dNMS to 10 mM KNO_3 for NCIMB 11130^T (including an extra increase in buffer concentration from 2 to 5 mM) and to 20 mM KNO_3 for R-45364. In our batch setup oxygen was rate-limiting (Figure III-1) and upon oxygen depletion approximately 1.5 to 2 mM nitrogen was consumed depending on the strain, while in the miniaturized setup oxygen depletion was unlikely due to daily atmosphere refreshment upon OD_{600nm} measurement. Therefore, it is assumed that for these two strains nitrogen became limiting in the miniaturized setup and not in the batch setup (due to oxygen limitation) in standard dNMS, which would explain why the addition of extra KNO_3 enhanced growth in the miniaturized setup but not in the batch setup.

Evaluation of customized media in semi-continuously fed cultures

An increase in assimilated carbon for strains R-45372 and R-45378 in customized dNMS (see above) suggests that more CH_4 was used for the production of biomass. To confirm this hypothesis, an additional batch experiment was set up comparing standard dNMS and customized dNMS whereby the headspace and medium were refreshed daily. Next to R-45372 and R-45378, *Methylomonas* sp. R-45364 and R-45374 were also included as negative controls since the assimilated carbon for these strains in customized dNMS did not increase significantly. Cultures were refreshed daily from two (R-45378 and R-45374) to three (R-45372 and R-45364) days onward for seven refreshment/incubation cycles. The results are shown in Figure III-2.

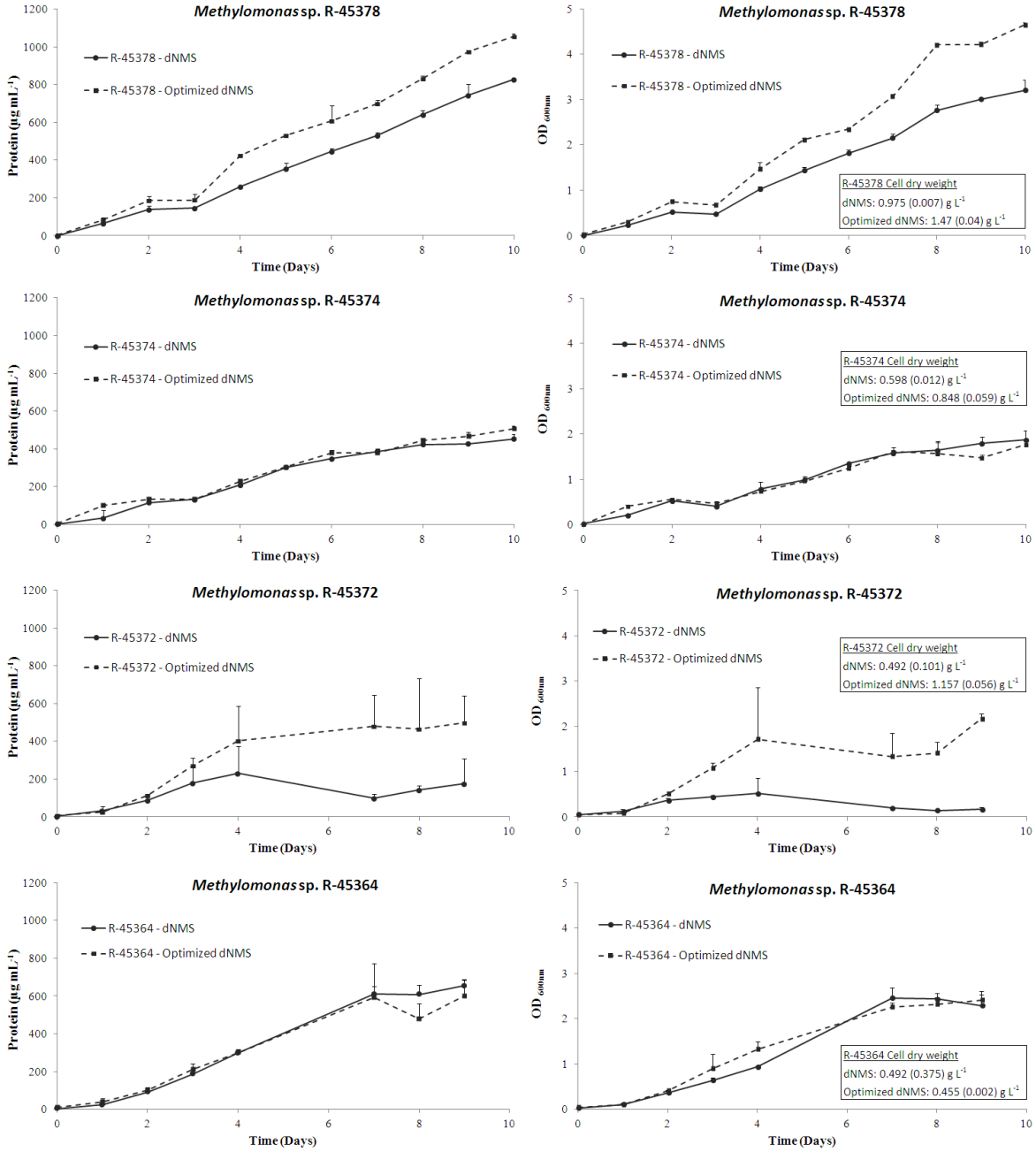

Figure III-2: Average (n = 2) protein content in µg mL^{-1} (left) and OD_{600nm} value (right) of *Methylomonas* sp. R-45378, R-45374, R-45372 and R-45364 cultured at optimal temperature (28°C) in dNMS (solid line) and optimized dNMS (dashed line) which were refreshed (both headspace and medium) daily for seven days from the third day onward for R-45378 and R-45374 and from the second day onward for R-45372 and R-45364. The cell dry weight content was determined after seven refreshment/incubation cycles of which the average values in dNMS and customized dNMS are shown with standard deviations between brackets. The customized dNMS used for each strain are listed in the results section. On day 5 and 6, cultures of R-45372 and R-45364 were refreshed, but not sampled for protein or OD_{600nm} measurement. One-sided error bars are shown. For several time-points, error bars are not clearly visible due to the limited variation between duplicates.

Strains R-45378 and R-45372 exhibiting a significantly increased assimilated carbon in customized dNMS in the batch setup (see previous section), also showed higher protein, OD_{600nm} and cell dry weight values in customized dNMS compared to standard dNMS upon daily refreshment, while this was not apparent for R-45364 and the control strain R-45374, confirming the results of the statistical analysis performed for the batch setup experiments. Overall, *Methylomonas* sp. R-45378 showed the highest observed protein, OD_{600nm} and cell dry weight values (as well as the highest observed assimilated carbon in the batch experiment; Table III-2) between strains, even more so in customized dNMS than in standard dNMS. In relation to other strains, R-45364 appeared to have low cell dry weight values compared to the rather high protein and OD_{600nm} levels of this strain (Figure III-2). After 2 refreshment cycles, a stationary phase (stagnant protein and OD_{600nm} levels) was reached in both customized dNMS as well as standard dNMS for R-45372. Such a stationary phase was reached after five refreshment cycles for R-45364, but was not yet reached for R-45378 and R-45374 (less apparent) at the end of the experiment after seven refreshment cycles. As opposed to the other strains, R-45372 was a rigorous floc former when grown in standard dNMS which resulted in either low or highly variable (large error bars) protein and OD_{600nm} values due to the difficulty in accurately measuring these flocs. This explains the rather high cell dry weight values (the measurement of which is independent of floc formation) compared to the lower protein and OD_{600nm} levels, especially in standard dNMS (Figure III-2). Although R-45372 still formed flocs in customized dNMS, the floc formation was less apparent than in standard dNMS and the strain also clearly grew planktonically. As a result, both protein, OD_{600nm} as well as cell dry weight levels were higher in customized dNMS (Figure III-2).

DISCUSSION

Detailed knowledge on growth requirements of any functional group of bacteria is highly relevant for unraveling their ecophysiology, improving isolation efforts, facilitating long-term maintenance and not in the least identifying their biotechnological applicability, for which high growth rates and/or cell yields are crucial parameters (Jiang et al., 2010). We used a miniaturized medium screening approach allowing the evaluation of 40 different medium variations compared with standard dNMS regarding the improvement of growth rate or cell yield of twelve methane-oxidizing strains from a single genus. *Methylomonas* was the obvious choice as several members of this genus are among the fastest growing MOB (Whittenbury et al., 1970) and are known to be physiologically diverse (Bowman et al., 1993). Using 96-well microtiter plates and multichannel pipetting, growth could be easily assessed by monitoring OD_{600nm} values over time and unique profiles of medium composition preference were obtained for each strain (Table III-1). Based on the results of the rapid screening approach, customized strain-dependent media were designed using a combination of the best-scoring medium parameters. The relevance of the data extracted from miniaturized screening was confirmed in batch experiments comparing the customized media with standard dNMS. As a result, five strains indeed showed enhanced growth: three strains grew more rapidly as their time to reach their maximum methane-oxidation rate was reduced, while two strains showed an increase in assimilated carbon, protein content, OD_{600nm} value and cell dry weight in their customized medium. Moreover, for one of these strains, R-45472, floc formation, as potential indicator of stress (Wilshusen et al., 2004), was visually reduced when grown in its customized medium. The remaining two strains for which a customized medium was designed, NCIMB 11130^T and R-45364, showed no enhanced growth parameters in this medium, although this could be attributed to technical differences in cultivation setups (nitrogen limitation occurring faster than oxygen limitation in the miniaturized setup versus the opposite in the batch setup) rather than a failed customization of medium composition. Overall, the miniaturized medium screening proved highly efficient in rapidly generating nutrient requirement data in order to design strain-dependent customized media resulting in enhanced growth of several *Methylomonas* strains.

Interesting trends were observed regarding the nitrogen preference of the *Methylomonas* strains. For example, R-45372, R-45374 and R-45378 showed a clear preference of growth with ammonium over nitrate both in the miniaturized and batch setup. Interestingly, R-

45372 and R-45374 suffered from increasing KNO_3 concentrations above the 2 mM KNO_3 of dNMS although these strains grew better with ammonium up to a concentration of 10-40 mM, indicating KNO_3 concentration dependent growth rate inhibition of these strains rather than just osmotic stress. In contrast, other strains such as R-45362 and R-45364 clearly preferred nitrate over ammonium, and suffered from increasing levels of ammonium addition. Generally, while a 2 mM buffer seems efficient for growth using nitrate, a stronger buffer (10-40 mM) was clearly needed when growing these strains with ammonium, as ammonium assimilation releases protons resulting in a pH decrease when not sufficiently buffered (Whittenbury et al., 1970; Molinuevo-Salces et al., 2010). The pH aspect should thus always be addressed when comparing nitrate versus ammonium amendments, since a drop below pH 6.3-6.8 already negatively affected growth of the strains in the present study.

Aside from nitrogen, strain-dependent preference for copper and other trace elements at different concentrations were observed. A ten-fold reduction in copper concentration negatively affected growth of all strains, as copper is required for pMMO expression. Although six *Methylomonas* strains in this study also possessed sMMO, which can be expressed once copper becomes limiting, growth can be enhanced when cells produce pMMO over sMMO (Park et al., 1991; Park et al., 1992; Dalton, 2005). A five-fold reduction of all major components of standard dNMS reduced growth for all strains most likely due to limitation of a specific nutrient, e.g. copper. Generally, growth was reduced when more trace elements solution was added to dNMS. As observed by other researchers (Park et al., 1992), this growth reduction might be caused by an increase in copper concentrations above 10 µM. However, since a 5-fold increase in the copper concentration did not lower growth for most strains, while a growth reduction occurred when increasing all trace elements including copper a 5-fold, this suggests that other trace elements also reduce growth upon a 5-fold increase. The exact concentrations of trace elements are often neglected and differ between researchers referring to the same medium, which might impact growth success for some strains. For example, trace element concentrations of NMS medium differ in the two reference books Bergey's Manual (Bowman, 2005) and The Prokaryotes (Bowman, 2006). In our study, NCIMB11130^T (up to 5-times trace increase) and R-45364 (up to 10-times trace increase) seem to be more resistant to fluctuations in concentration of trace elements. Interestingly, R-45383 had an optimal growth when the components of dNMS, including trace elements, were increased 10-fold,

while the strain already suffered from a sole 5-fold increase in trace elements, which suggests that for this strain the benefits of increasing other components outweighed the negative effects from an increase of concentration of trace elements. For the customized dNMS medium, we increased all components by 10-fold except for trace elements which were not increased. The customized medium drastically improved the time to reach maximum MOR for this strain (Figure III-1; Table III-2).

The miniaturized medium screening proved reliable in establishing several expected trends. Growth was lower compared to standard dNMS when no nitrogen was added to the medium (medium adaptation 0 mM KNO_3; Table III-1), as the *Methylomonas* strains either cannot fix nitrogen or possess an oxygen-sensitive nitrogenase (data not shown). dNMS amended with 50 mM KNO_3 also lowered growth for all strains, which is in agreement with similar findings from other research groups when KNO_3 levels exceeded 40 mM (Park et al., 1992; Reay and Nedwell, 2004; Lee et al., 2009), potentially due to osmotic stress (Bodelier and Laanbroek, 2004). As expected for members of *Methylomonas*, optimal growth parameters were obtained at pH 6.3 to 7.8 (Bowman et al., 1993), although recently some acid-tolerant members have also been described (Danilova et al., 2012; Kip et al., 2012). Strains R-45370 and R-45377 were more salt resistant (growth in dNMS with 1% NaCl), grew slower and shared a lower optimal growth temperature than other strains in this study, which fits with their close relatedness with *Methylomonas scandinavica* $SR5^T$ (Kalyuzhnaya et al., 1999).

Miniaturized medium screening proved to be a simple tool to rapidly gain insights into the growth preferences of a group of strains. Such knowledge can also guide the isolation of novel MOB or the maintenance of MOB in culture. The first step in isolating MOB is usually to perform an enrichment rendering MOB dominant. Since our study shows that small medium variations can greatly alter growth preference of strains, future isolation studies that focus on many enrichments using similar but different media could proof a useful strategy in isolating novel MOB by allowing dominance of different MOB in each enrichment. For future screening attempts, other parameters could be varied as well, such as the incubation temperature, magnesium and calcium content or the gas atmosphere (e.g. external addition of CO_2 and CH_4 in different concentrations) by incubating microtiter plates in different gastight jars, as these compounds have shown to have an impact on growth of MOB (Whittenbury et al., 1970; Park et al., 1992; Tsubota et al., 2005; Doronina et al., 2008). Alternatively, additional compounds such as citrate could be supplemented to

standard dNMS in a miniaturized setting to evaluate their impact on growth (Xing et al., 2006). We observed a positive impact on growth of R-45364 by adding vitamin B12 (0.1 µg L^{-1}) to dNMS (data not shown). Additionally, miniaturized medium screening could prove useful for maintenance purposes of strains. Some MOB are notoriously fastidious (Bowman, 2006) and their fitness can be reduced over time through sub-cultivation due to a sub-optimal growth medium. Indeed, several fully described *Methylomonas* strains are no longer extant (Bowman et al., 1993; Hanson and Hanson, 1996; Bowman, 2006). In our study, R-45370 and R-45377 proved more difficult to maintain than the other *Methylomonas* strains. However, our miniaturized setup showed that R-45377 grew better with a reduced copper concentration (5 µM), which was subsequently confirmed in batch experiments. As a direct result, this strain is now easily maintained in the lab.

From a biotechnological point of view, it is interesting to improve growth regardless of whether strains are difficult to maintain. Via rapid miniaturized medium screening, customized dNMS media could be designed resulting in higher growth rate or increased cell density of several strains. For R-45372, floc formation could be reduced in an customized medium with 10 mM NH_4Cl instead of nitrate, 10 mM buffer and a five-fold increase in FeNaEDTA, which resulted in higher protein content, OD_{600nm} and cell dry weight values in semi-continuously fed cultures. Strain R-45378 showed the highest observed assimilated carbon, protein, OD_{600nm} and cell dry weight values of all strains. For this strain, cell dry weight values increased by 50% in customized dNMS (10 mM NH_4Cl instead of nitrate, 10 mM buffer and a ten-fold increase in FeNaEDTA) in semi-continuously fed cultures. Interestingly, this strain appeared to have a low N : C of approximately 0.09 in customized medium, compared to most other strains in this study (approximately 0.12-0.20) and MOB in general [0.25 (Anthony, 1982)]. Also, this strain had the lowest oxygen requirement in the present study (O_2 : CH_4 ratio of 1.1, compared to approximately 1.3 for most other strains). As such, this strain grew significantly better than the available type strains of *Methylomonas*.

Our miniaturized medium screening approach resulted in unique profiles observed for all strains (Table III-1) suggesting a strong strain dependency towards medium composition preference, most notably with regards to nitrogen, even for very closely related strains within one genus. Significantly enhanced growth in customized media indicates the usefulness for rapid medium screening for assessment of biotechnological potential, facilitation of long-term maintenance or steering future isolation campaigns. In addition,

our results also demonstrate that extrapolating data on growth parameters of model organisms such as *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b to other, even closely related, methane-oxidizing bacteria is not straightforward.

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REFERENCES

Anthony, C. 1982 Biochemistry of methylotrophs. London: The Academic Press.

Bodelier, P.L.E.,Laanbroek, H.J., 2004. Nitrogen as a regulatory factor of methane oxidation in soils and sediments. FEMS Microbiol Ecol 47, 265-277.

Bodrossy, L., Holmes, E.M., Holmes, A.J., Kovacs, K.L.,Murrell, J.C., 1997. Analysis of 16S rRNA and methane monooxygenase gene sequences reveals a novel group of thermotolerant and thermophilic methanotrophs, Methylocaldum gen. nov. Arch Microbiol 168, 493-503.

Bowman, J. (2005) Family V. Methylocystaceae fam. nov. In *Bergey's Manual of Systematic Bacteriology, second ed*. Garrity, G.M., Brenner, D.J., Krieg, N.R., Staley, J.T., Boone, D.R., De Vos, P. et al. (eds). New York: Springer Verlag.

Bowman, J. (2006) The Methanotrophs - The Families *Methylococcaceae* and *Methylocystaceae*. Chapter 3.1.14. *Prokaryotes* **5**: 266-289, DOI: 10.1007/0-387-30745-1_15.

Bowman, J.P., Sly, L.I., Nichols, P.D.,Hayward, A.C., 1993. Revised taxonomy of the methanotrophs – Description of *Methylobacter* Gen Nov, emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. International Journal of Systematic Bacteriology 43, 735-753.

Cataldo, D.A., Maroon, M., Schrader, L.E.,Youngs, V.L., 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun Soil Sci Plant Anal 6, 71-80.

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

Danilova, O.V., Kulichevskaya, I.S., Rozova, O.N., Detkova, E.N., Bodelier, P.L., Trotsenko, Y.A.,Dedysh, S.N., 2012. Methylomonas paludis sp. nov., the first acid-tolerant member of the genus Methylomonas, from an acidic wetland. Int J Syst Evol Microbiol.

Dedysh, S.N., Panikov, N.S.,Tiedje, J.M., 1998. Acidophilic methanotrophic communities from Sphagnum peat bogs. Appl Environ Microbiol 64, 922-929.

Doronina, N.V., Ezhov, V.A.,Trotsenko, Y.A., 2008. Growth of Methylosinus trichosporium OB3b

on methane and poly-beta-hydroxybutyrate biosynthesis. Applied Biochemistry and Microbiology 44, 182-185.

Dunfield, P.F., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A.,Dedysh, S.N., 2003. *Methylocella silvestris* sp nov., a novel methanotroph isolated from an acidic forest cambisol. Int J Syst Evol Microbiol 53, 1231-1239.

Hanson, R.S.,Hanson, T.E., 1996. Methanotrophic bacteria. Microbiol. Rev 60, 439-471.

Hoefman, S., van der Ha, D., De Vos, P., Boon, N.,Heylen, K., 2012. Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria. Microb. Biotechnol 5, 368-378.

Jiang, H., Chen, Y., Jiang, P., Zhang, C., Smith, T.J., Murrell, J.C.,Xing, X.-H., 2010. Methanotrophs: Multifunctional bacteria with promising applications in environmental bioengineering. Biochem Eng J 49, 277-288.

Kalyuzhnaya, M.G., Khmelenina, V.N., Kotelnikova, S., Holmquist, L., Pedersen, K.,Trotsenko, Y.A., 1999. Methylomonas scandinavica sp. nov., a new methanotrophic psychrotrophic bacterium isolated from deep igneous rock ground water of Sweden. Syst Appl Microbiol 22, 565-572.

Kip, N., Ouyang, W., van Winden, J., Raghoebarsing, A., van Niftrik, L., Pol, A. et al., 2012. Detection, isolation, and characterization of acidophilic methanotrophs from Sphagnum mosses. Appl Environ Microbiol 77, 5643-5654.

Koh, S.C., Bowman, J.P.,Sayler, G.S., 1993. Soluble methane monooxygenase production and trichloroethylene degradation by a Type-I methanotroph, *Methylomons methanica*-68-1. Appl Environ Microbiol 59, 960-967.

Kolesnikov, O.M., Dedysh, S.N.,Panikov, N.S., 2004. Inhibition of growth and methane consumption in Methylocapsa acidiphila by mineral salts. Microbiology 73, 488-490.

Lee, S.W., Im, J., DiSpirito, A.A., Bodrossy, L., Barcelona, M.J.,Semrau, J.D., 2009. Effect of nutrient and selective inhibitor amendments on methane oxidation, nitrous oxide production, and key gene presence and expression in landfill cover soils: characterization of the role of methanotrophs, nitrifiers, and denitrifiers. Appl Microbiol Biotechnol 85, 389-403.

Molinuevo-Salces, B., García-González, M.C.,González-Fernández, C., 2010. Performance comparison of two photobioreactors configurations (open and closed to the atmosphere) treating anaerobically degraded swine slurry. Bioresource technology 101, 5144-5149.

Park, S., Hanna, M.L., Taylor, R.T.,Droege, M.W., 1991. Batch cultivation of *Methylosinus trichosporium* OB3b. 1. Production of soluble methane monooxygenase. Biotechnol Bioeng 38, 423-433.

Park, S.H., Shah, N.N., Taylor, R.T.,Droege, M.W., 1992. Batch cultivation of *Methylosinus trichosporium* OB3b. 2. Production of particulate methane monooxygenase. Biotechnol Bioeng 40, 151-157.

Reay, D.S.,Nedwell, D.B., 2004. Methane oxidation in temperate soils: effects of inorganic N. Soil Biol Biochem 36, 2059-2065.

Scheutz, C., Kjeldsen, P., Bogner, J.E., De Visscher, A., Gebert, J., Hilger, H.A. et al., 2009. Microbial methane oxidation processes and technologies for mitigation of landfill gas emissions. Waste Manag Res 27, 409-455.

Semrau, J.D., DiSpirito, A.A.,Yoon, S., 2010. Methanotrophs and copper. FEMS Microbiol Rev 34, 496-531.

Semrau, J.D., 2011. Bioremediation via Methanotrophy: Overview of Recent Findings and Suggestions for Future Research. Front Microbiol 2, 209.

Shah, N.N., Park, S., Taylor, R.T.,Droege, M.W., 1992. Cultivation of *Methylosinus trichosporium*

OB3b. 3. Production of particulate methane monooxygenase in continuous culture. Biotechnol Bioeng 40, 705-712.

Taylor, S., Ninjoor, V., Dowd, D.M.,Tappel, A.L., 1974. Cathepsin B2 measurement by sensitive fluorometric ammonia analysis. Anal. Biochem 60, 153-162.

Tsubota, J., Eshinimaev, B.T., Khmelenina, V.N.,Trotsenko, Y.A., 2005. *Methylothermus thermalis* gen. nov., sp nov., a novel moderately thermophilic obligate methanotroph from a hot spring in Japan. Int J Syst Evol Microbiol 55, 1877-1884.

Wendlandt, K.D., Stottmeister, U., Helm, J., Soltmann, B., Jechorek, M.,Beck, M., 2010. The potential of methane-oxidizing bacteria for applications in environmental biotechnology. Eng Life Sci 10, 87-102.

Whittenbury, R., Phillips, K.C.,Wilkinson, J.F., 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61, 205-217.

Wilshusen, J.H., Hettiaratchi, J.P., De Visscher, A.,Saint-Fort, R., 2004. Methane oxidation and formation of EPS in compost: effect of oxygen concentration. Environ Pollut 129, 305-314.

Wise, M.G., McArthur, J.V.,Shimkets, L.J., 1999. Methanotroph diversity in landfill soil: Isolation of novel type I and type II methanotrophs whose presence was suggested by culture-independent 16S ribosomal DNA analysis. Appl Environ Microbiol 65, 4887-4897.

Xing, X.H., Wu, H., Luo, M.F.,Wang, B.P., 2006. Effects of organic chemicals on growth of Methylosinus trichosporium OB3b. Biochem Eng J 31, 113-117.

Zhang, Y.X., Xin, J.Y., Chen, L.L., Song, H.,Xia, C.U., 2008. Biosynthesis of poly-3-hydroxybutyrate with a high molecular weight by methanotroph from methane and methanol. Journal of Natural Gas Chemistry 17, 103-109.

Chapter IV

Versatile and strain-dependent nitrogen metabolism within the genus *Methylomonas*

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SH and KH designed the experiments. SH performed the experiments and analyzed the data. SH and KH wrote the paper. DvdH, NB, PV and PDV commented on the manuscript.

SUMMARY

Both stimulating and inhibitory effects on the methane oxidation activity have been observed by nitrogenous fertilizer additions in a wide variety of environments. Different Type I methanotrophic genera, including *Methylomonas*, appear to be stimulated by an increase in nitrogen load depending on the environment or situation. However, it is unclear whether such preferential adaptations to changing environments are genus-specific, or if within one genus major physiological strain-dependent differences can occur. In this study, tolerance to ammonium, nitrate, nitrite and hydroxylamine, and high sodium chloride concentrations was assessed for fourteen genetically different *Methylomonas* strains (Type Ia), including the type strains of *M. methanica* and *M. koyamae*, and compared with a Type Ib (*Methylococcaceae* sp.) and a Type II (*Methylosinus* sp.) methanotroph. In addition, their ability to fix nitrogen, the presence of the *nifH* gene, and their ability to produce nitrite and/or nitrous oxide from media with nitrate or ammonium as sole nitrogen source was evaluated. Overall, *Methylomonas* strains showed a high physiological diversity in regard to all tested aspects of their nitrogen metabolism, even between strains which shared 100% 16S rRNA gene sequence relatedness. However, production of nitrous oxide from ammonium and from nitrate, reduction of nitrate to nitrite and fixation of dinitrogen gas appeared to be widespread within the genus. As a specific example, *Methylomonas* sp. R-49799 supported growth at high ammonium (100 mM) levels and lower nitrate (40 mM) levels. This strain could utilize nitrite as sole nitrogen source, fixed atmospheric nitrogen, tolerated hydroxylamine levels up to 1 mM and produced nitrous oxide from ammonium and nitrate. As such, this strain appears to possess a diverse set of tools which can provide a competitive advantage in different situations over other *Methylomonas* strains or methanotrophs in general.

INTRODUCTION

Methane-oxidizing bacteria (MOB) derive energy from the oxidation of methane to carbon dioxide, using their key enzyme, methane monooxygenase (MMO), in the first step of methane oxidation (Semrau et al., 2010). Most aerobic methanotrophic bacteria cultured so far are phylogenetically positioned within the *Gammaproteobacteria* (Type I methanotrophs, forming two clades, i.e. Type Ia and Ib), the *Alphaproteobacteria* (Type II methanotrophs) (Hanson and Hanson, 1996; Dedysh, 2009), and *Verrucomicrobia* (Op den Camp et al., 2009). Most MOB have a particulate methane monooxygenase (pMMO), except for members of the genera *Methylocella* and *Methyloferula* that only possess a soluble type (sMMO), and some contain both MMO types (Dedysh, 2009; Vorobev et al., 2010). These methanotrophs are physiologically diverse, especially in regard to their nitrogen metabolism (Stein and Klotz, 2011). Methanotrophic bacteria share similar traits with ammonia-oxidizing bacteria (Hanson and Hanson, 1996). They are able to cometabolically oxidize ammonia, although it is not coupled to a proton motive force (Stein and Klotz, 2011), making ammonia a competitive inhibitor for MMO. Toxic intermediates hydroxylamine and nitrite can be detoxified to nitrous oxide via various pathways differing greatly among methanotrophs (Nyerges and Stein, 2009; Nyerges et al., 2010; Campbell et al., 2011). Methanotrophs can also reduce nitrate to nitrite (Bowman et al., 1993), either for assimilation or removal via similar detoxification pathways. Furthermore, some methanotrophs can fix atmospheric nitrogen by an oxygen-sensitive nitrogenase. Although this trait was initially thought to be limited to mostly Type II and Type Ib MOB (Murrell and Dalton, 1983), some Type Ia and verrucomicrobial MOB can also fix nitrogen (Auman et al., 2001; Boulygina et al., 2002; Khadem et al., 2010).

Versatile metabolic features can provide methanotrophs with distinct advantages over those lacking them in changing environmental conditions or under acute environmental stress. This has already been extensively exemplified in soils in response to nitrogenous fertilization, resulting in changed or unaltered methanotrophic activity and community composition (Bodelier et al., 2000; Bodelier and Laanbroek, 2004; Mohanty et al., 2006; Noll et al., 2008). Type I methanotrophs were found to be preferentially stimulated over type II methanotrophs with use of both ammonium- and nitrate based fertilizers, which relieved nitrogen limitation preventing activity and growth. In contrast, such nitrogen amendments have also been shown to inhibit methane oxidation activity, most likely due to either non-specific ionic effects (King and Schnell, 1998), competitive inhibition of

methane oxidation by ammonia or the formation of toxic intermediates (Schnell and King, 1994; Nyerges and Stein, 2009). Taken together, thus far available data suggest niche partitioning among methanotrophs in regard to their nitrogen metabolism, with methane oxidation activity responses to changes in nitrogen content being dependent on the *in situ* methanotrophic community structure (Mohanty et al., 2006). Unfortunately, widely applied tools for microbial community assessment based on 16S rRNA gene sequencing or proxies thereof (such as denaturating gradient gel electrophoresis (DGGE) or terminal-restriction fragment length polymorphism (T-RFLP)) only have a limited phylogenetic resolution mostly restricted to genus level diversity (Vandamme et al., 1996). As a consequence, intragenus or even intraspecies metabolic versatility in nitrogen metabolism was never evaluated nor considered as a cause for the (contradictory) observations of nitrogen influence on methane oxidation. Nevertheless, we know from other organisms that ecological traits can be strain specific, and closely related bacteria can occupy distinct niches (Johnson et al., 2006; Hunt et al., 2008). This was elaborately demonstrated in *Prochlorococcus*, with cultured strains having distinct pigmentation, maximum growth rates, metal tolerances, nutrient utilizations and photophysiological characteristics (Johnson et al., 2006). Also among methanotrophic genera of the same type (I or II), ammonia co-metabolisation and product inhibition was found to be organism-specific, although in this study it is difficult to ascertain the taxonomic level at which differences occur as only two strains from different genera were tested for each type (Nyerges and Stein, 2009).

We hypothesize that strains within the same methanotrophic genus or even species can demonstrate a large physiological versatility in their nitrogen metabolism, making current microbial community structure analyses inadequate for evaluating methane-oxidation responses to nitrogen amendment. Therefore, we evaluated the strain-dependent nature of the nitrogen metabolism within members of the genus *Methylomonas* (Type Ia). Fourteen *Methylomonas* strains, including the type strains of *M. methanica* and *M. koyamae* were evaluated for their tolerance to high ammonium, nitrate, nitrite and hydroxylamine concentrations, their ability to fix nitrogen, and their capacity to produce nitrite and nitrous oxide when cultivated in nitrate or ammonium-based mineral media. *Methylomonas* is a relevant genus for such a comparison, since *Methylomonas* strains are ubiquitous in nature (Bowman et al., 1993; Hanson and Hanson, 1996; Bowman, 2006; Danilova et al., 2012; Ogiso et al., 2012) and nitrogenous fertilization has been found to stimulate some of its members in rice field soils (Mohanty et al., 2006). Furthermore, the type strain of *M.*

koyamae was isolated from a rice paddy field (Ogiso et al., 2012) and four strains in this study were most closely related to this type strain. As a reference, a type Ib (*Methylococcaceae* sp.) methantroph and a type II (*Methylosinus* sp.) methanotroph were included in the analyses.

EXPERIMENTAL PROCEDURES

Bacterial strains and standard growth conditions

Fourteen of the sixteen tested strains were members of the genus *Methylomonas*: the type strain *Methylomonas methanica* NCIMB 11130^T and six strains R-45362, R-45363, R-45364, R-45371, R-45372 and R-45374 most closely related to this type strain (98.3-98.6% 16S rRNA sequence similarity; further referred to as *M. methanica* strains); the type strain *Methylomonas koyamae* NCIMB 14606^T and four strains R-45378, R-45383, R-49799 and R-49807 most closely related to this type strain (97.9-100% 16S rRNA sequence similarity; further referred to as *M. koyamae* strains); two strains *Methylomonas* sp. R-45370 and R-45377 most closely related to the no-longer extant type strain of *M. scandinavica* (97.5% 16S rRNA sequence similarity). All *Methylomonas* strains were genetically different as determined by GTG_5 rep-PCR fingerprinting (Hoefman et al., 2012; Chapter II). In addition, two non-*Methylomonas* strains were included as a reference: *Methylococcaceae* sp. R-49797, a member of the *Methylococcus-Methylocaldum-Methylogaea* clade (Type Ib) and *Methylosinus* sp. R-45379 (Type II). Included strains were obtained from various origins: strains R-45362, R-45363, R-45364, R-45371, R-45372, R-45374 and R-45370 were isolated from the top layer of a denitrification tank of a waste water treatment plant, strain R-45377 was isolated from a slurry pit of a cow stable; strains R-45378, R-45383 and R-45379 were isolated from a wetland (Hoefman et al., 2012); strains R-49807 and R-49797 were isolated from a facultative waste stabilization pond (Chapter II). Strain R-49799 was isolated from a high ammonium (70 mM) methanotrophic enrichment from a mixture of inocula (compost heap, top soil with leaf litter, anaerobic sludge and a wastewater treatment plant). All strains were routinely subcultured on diluted nitrate mineral salts (dNMS) plates (Dunfield et al., 2003) and incubated in gastight jars (Oxoid, UK) under a CH_4 : air (1 : 1) atmosphere. The composition of dNMS in this study was the following: 2 mM KNO_3, 4 mM $MgSO_4$, 0.9 mM $CaCl_2$, 2 mM Na_2HPO_4/KH_2PO_4 buffer (pH 6.8), 14 µM ferric-sodium-EDTA and a trace element solution (Bowman, 2006) with Cu^{2+} concentration adjusted to 10 µM. Fresh

colonies from one-week grown cultures on dNMS plates were used as start inoculum (OD_{600nm} 0.01, final concentration) for the different growth experiments performed in this study. To be able to reach comparable cell densities, two-week grown cultures were used for the two slower-growing strains *Methylomonas* sp. R-45370 and R-45377.

Assessment of nitrogen assimilation and toxicity

The ability of the strains to grow with a range of concentrations (2, 10, 20, 40, 150 and 200 mM) of $NaNO_3$, KNO_3, NH_4Cl and $(NH_4)_2SO_4$-N and $NaNO_2$ (2, 5, 10 mM) was assessed. In order to evaluate general osmotic influences, sodium chloride tolerance was also tested in dNMS and dAMS (ammonium instead of nitrate), each supplemented with 40, 100, 150 and 200 mM NaCl. Hydroxylamine tolerance was evaluated in dAMS supplemented with 0.01, 1 and 2 % (w/v) hydroxylamine. Initial cultivation experiments showed that strains cultivated with ammonium consistently preferred a 10 mM buffer over a 2 mM buffer, while the opposite was observed with nitrate as nitrogen source. Therefore, in this study, dNMS (nitrate, nitrite or nitrogen-free medium, see below) media contained a 2 mM phosphate buffer, while dAMS (ammonium) media contained a 10 mM phosphate buffer. Besides nitrogen source and buffer concentration, all other components were identical between media.

All strains were inoculated in all of the above mentioned liquid media in sterile 96-well microtiter plates in duplicate (300 µL, final volume per well). Cultures were incubated under a CH_4 : air (1 : 1) atmosphere at optimal temperature (20°C for strains R-45370, R-45371 and R-45377 and 28°C for the other strains). Growth curves were determined through OD_{600nm} measurements and verified by visual inspection after 4, 7, 11, 14 and 21 days of incubation. For each strain cultivated in each condition, growth was scored positive when the average OD_{600nm} value became larger upon incubation than the average OD_{600nm} value added by 3-times its standard deviation at the start of incubation.

Dinitrogen fixation

Strains were inoculated in duplicate as explained above in liquid nitrogen-free dNMS in gastight flasks under a 20% CH_4 in air atmosphere (approximately 21% O_2, high oxygen tension condition) and under a 20% CH_4 in ten-fold diluted air (with helium) atmosphere (approximately 2.1% O_2, low oxygen tension condition). In addition to the Type Ib strain for which *nifH* gene amplification was negative, four extra methanotrophic strains without the *nifH* gene were included as negative controls (data not shown). Growth was determined

through OD_{600nm} measurements and verified by visual inspection after 4, 7, 11, 14 and 21 days of incubation. In addition, the methane oxidation activity was assessed by gas chromatography (see below). Absence of nitrate (below 0.15 mM), ammonium (below 0.15 mM) and nitrite (below 10 µM) in nitrogen-free dNMS was confirmed via colorimetry (see below).

Assessment of production of nitrite and nitrous oxide

Initial experiments on a limited number of *Methylomonas* strains showed that nitrous oxide was produced in cultures grown with ammonium or nitrate as sole nitrogen source once a stationary phase was induced via oxygen depletion, while nitrogen was not depleted. Therefore, all strains were cultivated in such a setup in dAMS with 10 mM NH_4Cl and in dNMS with 10 mM KNO_3 in gastight flasks under a 20% CH_4 in air atmosphere and time points were selected to include only the stationary phase (expected to start before or around 6 days, except for several slower growing cultures such as *Methylomonas* sp. R-45370 and R-45377), namely after 6, 9, 14 and 21 days of incubation; At each time point growth (OD_{600nm} measurements; see above), methane oxidation activity (gas chromatography see below), either nitrate or ammonium consumption, and nitrite and nitrous oxide production were assessed (colorimetry or gas chromatography see below).

Gas chromatography and colorimetric analyses

Methane oxidation activity was assessed by monitoring of CH_4, O_2 and CO_2 levels via gas chromatography using a Compact GC (Global Analyzer Solutions, Belgium) equipped with two columns (O_2/N_2 and CO_2/N_2O separation) connected to a thermal conductivity detector and one column (CH_4 levels) connected to an flame ionization detector. The change in gas pressure due to methane oxidation was monitored with an Infield 7 pressure meter (UMS, Germany). Values measured by gas chromatography were converted to µmol gas L_{liquid}^{-1} by compensating for change in gas pressure (measured with an Infield 7 pressure meter) and taking the solubility of the gases into account. Colorimetric assays were performed to determine the nitrite (Griess, 1879), nitrate (Cataldo et al., 1975) and ammonium (Taylor et al., 1974) concentration.

***NifH* gene detection**

DNA was extracted by alkaline lysis (Coenye et al., 2002). Amplification of the *nifH* gene encoding the highly conserved Fe protein of nitrogenase was performed using the primer

set F1/nifH439R, the PCR mix and temperature program as described in De Meyer et al., (2011). When amplification was positive, *nifH* sequences were generated and sequences were assembled with the BioNumerics 5.1 software (Applied Maths, Belgium). Protein translation analysis using Transeq (www.ebi.ac.uk/tools/emboss/transeq) and pBLAST (Altschul et al., 1990) confirmed that the sequences encoded for part of the nitrogenase enzyme.

Sequences were aligned using the MEGA 5 software based on translated protein sequences (Tamura et al., 2011). Based on the alignment, a DNA-based Maximum Likelihood phylogenetic tree was constructed using RAxML v7.3.5 based on the General Time Reversible substitution model with gamma-distributed rate variation (GTR+Gamma). Bootstrap analysis based on 1000 replicates was performed. The best scoring ML tree was exported in newick format and analyzed using MEGA 5.

Nucleotide sequence accession numbers

The *nifH* gene sequence data generated in this study has been deposited in GenBank/EMBL/DDBJ with accession numbers HF954366 to HF954376.

RESULTS & DISCUSSION

Assimilation of ammonium and nitrate and tolerance to high levels

All cultured MOB can grow with ammonium as sole nitrogen source via ammonium assimilation (Whittenbury et al., 1970), while most, but not all MOB, can grow with nitrate as sole nitrogen source (Anthony, 1982). Next to support of growth via assimilation, both nitrogen species can also inhibit methanotrophic growth when present at high levels, as non-specific ionic effects might occur (King and Schnell, 1998). In addition, ammonium amendments introduce a fraction of ammonia to the culture depending on the pH (NH_3/NH_4^+; pKa = 9.23). Ammonia can be a competitive inhibitor of MMO for methane oxidation or lead to accumulation of hydroxylamine and nitrite, toxic products of its oxidation (Hanson and Hanson, 1996). Also, high nitrate levels can result in nitrite built up via nitrate reduction. Nitrite is toxic at elevated levels (mM range) in a strain-dependent manner (Bowman et al., 1993; Hanson and Hanson, 1996). However, methanotrophs can possess various pathways to detoxify nitrite and hydroxylamine, differing largely between methanotrophic genera (Nyerges and Stein, 2009; Nyerges et al., 2010; Campbell et al., 2011). Unfortunately, data on nitrogen assimilation and tolerance in pure culture methanotrophs is very scarce, the pathways responsible for detoxification and the versatility to be expected between closely related strains is unknown. Therefore, we screened a strain set of fourteen *Methylomonas* strains, one Type Ib (*Methylococcaceae* sp. R-49797) strain and one Type II (*Methylosinus* sp. R-45379) strain for their ability to assimilate and/or tolerate high levels of ammonium, nitrate, nitrite and hydroxylamine (Table IV-1). In order to evaluate the influence of general osmotic effects, strains were also cultivated in equimolar concentrations of sodium chloride.

Table IV-1: Overview of the results of nitrogen assimilation experiments performed on the methanotrophic strains

	***Methylomonas methanica* strains**							***Methylomonas koyamae* strains**					***Methylomonas* sp.**		**Type Ib**	**Type II**
	NCIMB 11130^T	**R-45362**	**R-45363**	**R-45364**	**R-45371**	**R-45372**	**R-45374**	**NCIMB 14606^T**	**R-45378**	**R-45383**	**R-49799**	**R-49807**	**R-45370**	**R-45377**	**R-49797**	**R-45379**
NaCl (+NO_3^-)	100 mM	100 mM	100 mM	40 mM	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM	200 mM	200 mM	100 mM	40 mM
NaCl (+NH_4^+)	40 mM	100 mM	100 mM	40 mM	100 mM	40 mM	100 mM	100 mM	100 mM	100 mM	150 mM	100 mM	200 mM	200 mM	100 mM	40 mM
$NaNO_3$ & KNO_3[a]	40 mM	40 mM	40 mM	40 mM	40 mM	40 mM	40 mM	40 mM	40 mM	40 mM	40 mM	40 mM	100 mM	100 mM	40 mM	20 mM
NH_4Cl	40 mM	40 mM	40 mM	40 mM	40 mM	40 mM	40 mM	100 mM	40 mM	40 mM	100 mM	40 mM	40 mM	40 mM	100 mM	40 mM
$(NH_4)_2SO_4$-N	40 mM	40 mM	40 mM	40 mM	40 mM	40 mM	40 mM	40-100 mM[b]	40 mM	40 mM	100 mM	40 mM	40 mM	40 mM	100 mM	40 mM
$NaNO_2$	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM	5 mM	-
Hydroxylamine[c]	1 mM	-	1 mM	1 mM	1 mM	1 mM	-	1 mM	-	-	1 mM	-	-	-	-	1 mM
nifH presence	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
N_2 fixation[d]	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+

Values represent maximum concentration supporting growth of the culture; +, positive for growth based on OD_{600nm} values; -, negative for growth based on OD_{600nm} values

[a] Due to a technical error, strains R-49799, R-49807, R-45370, R-45377, R-49797 and R-45379 were not tested with KNO_3 concentrations above 10 mM

[b] Different outcome for duplicates; [c] Hydroxylamine tolerance, with 2 mM NH_4Cl as nitrogen source; [d] Under low O_2 tension only: +, positive and -, negative for growth based on OD_{600nm} values in nitrogen-free medium

The complete strain panel was able to use both ammonium and nitrate for assimilation and strains could cope in general with a maximum of 40 mM amended nitrogen, except the type II strain which only tolerated a maximum of 20 mM NO_3^--N (Table IV-1). Two *M. koyamae* strains, R-49799 and NCIMB 14606^T, showed a higher tolerance for NH_4^+-N, up to 100 mM, although growth for the latter strain was delayed (data not shown). Notably, growth at these high ammonium concentrations was not supported for *M. koyamae* R-49807, sharing a 100% 16S rRNA gene sequence similarity with *M. koyamae* NCIMB 14606^T and *M. koyamae* R-49799. In addition, out of these three strains only R-49799 exhibited a higher NaCl tolerance when grown with ammonium as sole nitrogen source over nitrate (150 mM compared with 100 mM). So, although these three strains are phylogenetically very closely related, strain-dependent tolerances to ammonium and sodium chloride were observed.

For *M. methanica* cultures, strains NCIMB 11130^T and R-45372 exhibited a higher salt tolerance when cultivated with NH_4^+-N. *M. methanica* R-45364 was salt sensitive regardless of the nitrogen source and demonstrated equal tolerance to sodium chloride, ammonium and nitrate (≤40 mM). The other *M. methanica* strains tolerated higher NaCl concentrations than equimolar nitrate and ammonium concentrations, most likely due to compound-specific osmotic stress (King and Schnell, 1998).

Only the two *Methylomonas* sp. strains (R-45370 and R-45377) grew in the presence of 100 mM nitrate and tolerated higher concentrations of nitrate than ammonium (maximum of 40 mM), while also showing the highest salt tolerance regardless of their nitrogen source (200 mM NaCl). The other strains tolerated higher NaCl additions than equimolar nitrate additions, again suggesting concentration-dependent nitrate inhibition. This confirms previous reports of methanotrophic growth inhibition above 40 mM nitrate by other research groups (Park et al., 1992; Lee et al., 2009) and is in agreement with our previous results showing a steep decline in the growth of *Methylomonas* strains with 50 mM KNO_3 compared to 20 mM KNO_3 amended to the medium (Chapter III).

Assimilation and toxicity of potential intermediates nitrite and hydroxylamine

Tolerance of methanotrophs to ammonia can be dependent of intrinsic toxicity levels of hydroxylamine and nitrite as well as their ability to use either of them for assimilation. For our strain panel, preliminary experiments showed that none of the strains could grow with 1 mM or 2 mM hydroxylamine as sole nitrogen source. Therefore, tolerance of hydroxylamine was tested by providing 2 mM ammonium as nitrogen source for growth

and spiking 0.01 mM, 1 mM and 2 mM hydroxylamine. None of the tested strains supported growth in the presence of 2 mM hydroxylamine. Eight strains exhibited growth when 0.01 mM and 1 mM hydroxylamine was added, while eight other strains did not even show growth at a concentration of only 0.01 mM hydroxylamine (Table IV-1). Interestingly, *M. koyamae* NCIMB 14606^T and R-49799, both demonstrating a high ammonium tolerance (up to 100 mM) were among the strains that tolerated 1 mM hydroxylamine amendments.

Although nitrite can be toxic at low levels depending on the methantroph (Bowman et al., 1993; Hanson and Hanson, 1996), all *Methylomonas* strains in this study were able to grow with up to 2 mM nitrite as sole nitrogen source. These results demonstrated not only nitrite tolerance but also nitrite assimilation, which contrasted previous reports that nitrite utilization is rare for *Methylomonas methanica* members (Bowman et al., 1993). The type Ib strain R-49797 could use nitrite for assimilation and tolerated the highest nitrite concentration (up to 5 mM). However, this strain was sensitive to hydroxylamine, even at concentrations of 0.01 mM, suggesting that potential ammonia oxidation would allow only a low transient hydroxylamine peak and necessitate a quick reduction further to either nitrite or nitric oxide. *Methylosinus* strain R-45379 (Type II) was not able to assimilate nitrite, which agreed with reports for its most closely related type strain, *Methylosinus sporium* 5^T (Bowman et al., 1993).

Nitrite and nitrous oxide production as detoxification mechanisms

A low tolerance to ammonium and nitrate can be caused by compound-specific ionic effects (King and Schnell, 1998), competitive inhibition of methane by ammonia, or hydroxylamine and/or nitrite toxicity (Schnell and King, 1994; Nyerges and Stein, 2009). From genomic studies of other methanotrophs, it was observed that the gene inventory dealing with toxic intermediates can differ significantly (Stein and Klotz, 2011). The conversion of hydroxylamine directly to nitric oxide or indirectly via nitrite is well studied and limited to the activity of hydroxylamine oxidoreductase, encoded by the genes *haoAB* (Nyerges and Stein, 2009; Nyerges et al., 2010; Campbell et al., 2011) and to a much lesser extent cytochrome P460 encoded by the gene *cytL* (Stein and Klotz, 2011). In contrast, nitrate metabolism is underexplored in methanotrophs and little is known except that nitrite from nitrate can be produced by both assimilatory and dissimilatory nitrate reductases (Anthony, 1982; Stein and Klotz, 2011). To our knowledge no literature reports explicitly state methanotrophs' ability of nitrate reduction for dissimilation but available genome

data show the presence of the genes encoding the cytoplasmic nitrate reductase *narGHI* in *Methylobacter tundripaludum SV96*, and the periplasmic nitrate reductase *napA*, which can play a role in both assimilation and dissimilation, in several other genomes of aerobic methanotrophs including *Methylomonas methanica* MC09. High levels of nitrite produced from nitrate also need to be converted to nitric oxide for further detoxification. One major source of this nitric oxide in non-denitrifying *Enterobacteriaceae* has been suggested to be the reduction by the membrane-bound nitrate reductase NarG (Smith and Zimmerman, 1981; Bleakley and Tiedje, 1982; Rowley et al., 2012). Alternatively, this step can also be performed by the copper-containing dissimilatory nitrite reductase, as the *nirK* gene has already been found in the genomes of *Methylocella silvestris* BL2 and *Methylocystis* sp. ATCC 49242, although it is unclear if this can be performed aerobically. Upon production, the cytotoxin nitric oxide needs to be immediately detoxified to the, at least for the cell, harmless nitrous oxide. This step can be performed by a multitude of nitric oxide reductase enzymes, which have either been described in detail (Zumft et al., 1994; Zumft, 2005; Hino et al., 2011; Stein, 2011; Vine and Cole, 2011) or just deduced from the genomes of both methanotrophic and other bacteria. Here, it is interesting to note that a novel type of nitric oxide reductase (menaquinol/cytochrome *c* dependent qCuANor, also denoted sNor) (Suharti et al., 2001), for which only recently the encoding genes were described (Heylen and Keltjens, 2012), is also found in the genome of *M. methanica* MC09 as well as the genes *norBC* encoding the widely occurring cytochrome *c* dependent nitric oxide reductase.

Although genome data for our strain panel is lacking, it is clear from other methanotroph genomes that methanotrophs can possess a variety of detoxification pathways that can potentially explain high or low tolerance levels to ammonium and nitrate. Here, we experimentally tested the ability of our strain panel to produce nitrite or nitrous oxide from moderate levels (10 mM) of ammonium and nitrate (due to technical limitations hydroxylamine production was not assessed). The data was used to demonstrate the variability of detoxification mechanisms of strains within the genus *Methylomonas*, as well as to formulate hypotheses on the detoxification mechanisms behind our above-mentioned observations of their tolerance levels. Cultures were initially cultivated at high oxygen tension (O_2 levels in air) that was gradually decreased by methanotrophic activity to a final point where oxygen levels became too low to support further methane oxidation. As was also found for tolerance levels to nitrogen species, high diversity among the strain panel was observed for nitrite and nitrous oxide production (Table IV-2; Figure IV-1). Most

strains could clearly produce nitrite (15 out of 16) and nitrous oxide (13 out of 16) in the presence of nitrate, while with ammonium only nitrous oxide production was a general feature (14 out of 16 strains). Nitrite from ammonium was only clearly produced by *Methylosinus* sp. R-45379 (Type II) and only very low nitrite levels near detection limit (10 µM) for three other strains. Taking these results together, the strain panel could be divided into eight different dissimilatory nitrogen patterns (Table IV-2); an example of each pattern is displayed in Figure IV-1.

In nitrate-amended batch experiments, nitrate was converted to nitrite once oxygen concentration became low in all strains except for *M. methanica* R-45363, which probably lacks a nitrate reductase (Table IV-2; Figure IV-1; pattern III). When methane oxidation activity ceased due to oxygen limitation, no additional nitrite was produced but several strains showed a slow decrease in NO_2^- levels with a corresponding rise in N_2O over time (Column 3; Table IV-2). This was observed for strains exhibiting patterns II and VI, but was most clear for *M. koyamae* R-49807 (Table IV-2; Figure IV-1; pattern VI). From the start of the incubation at high oxygen tension (O_2 levels in air) until day six (approximately 1.7% v/v O_2), methane was oxidized but no nitrite or nitrous oxide could be measured. At day nine, NO_2^- levels of approximately 400 µM were measured at oxygen levels below 0.3% v/v, and from that point onwards, until the end of the incubation at day 21, NO_2^- levels dropped with a corresponding rise in N_2O levels, while no more methane oxidation occurred. These observations nicely correspond with activities of an oxygen-sensitive nitrate reductase in actively growing cells, with subsequent detoxification by a membrane-bound nitrite reductase and a nitric oxide reductase mostly during the stationary phase, as previously described for non-denitrifiers (Smith and Zimmerman, 1981; Smith, 1983). *Methylomonas* sp. R-45383 produced nitrite but did not produce nitrous oxide (Table IV-2; Figure IV-1; pattern V), probably because it lacks a nitric oxide reductase. *M. methanica* NCIMB 11130^T and R-45364 and *Methylosinus* sp. R-45379 produced both NO_2^- and N_2O, but did not show a subsequent drop in NO_2^- and corresponding rise in N_2O (Table IV-2; Figure IV-1 patterns I, IV and VIII). Maybe these observations can be attributed to the presence of an active nitrite reductase (e.g. NirK) that can only convert nitrite to NO during growth, thus at high enough oxygen tension. *M. methanica* R-45371 produced N_2O and small amounts of NO_2^-, approximating the detection limit of the assay (10 µM).

Table IV-2: Overview of the production of nitrite and nitrous oxide of the methanotrophic strains cultivated in nitrate and ammonium medium

		dNMS (10 mM KNO_3) cultivation			dAMS (10 mM NH_4Cl) cultivation		Pattern
		NO_2 production	N_2O production	$NO_2\searrow$ & $N_2O\nearrow$[a]	NO_2 production	N_2O production	
M. methanica	NCIMB 11130^T	+	+	-	-	+	I
	R-45362	+	+	+	-	+	II
	R-45363	-	-	-	-	+	III
	R-45364	+	+	-	-	+	I
	R-45371	+/-[b]	+	-	+/-[b]	+	IV
	R-45372	+	+	+	-	+	II
	R-45374	+	+	+	-	+	II
M. koyamae	NCIMB 14606^T	+	+	+	-	+	II
	R-45378	+	+	+	-	+	II
	R-45383	+	-	-	-	-	V
	R-49799	+	+	+	-	+	II
	R-49807	+	+	+	-	-	VI
Methylomonas sp.	R-45370	+	+	Nt[c]	+/-[b]	+	VII
	R-45377	+	Nt[c]	Nt[c]	+/-[b]	+	VII
Type Ib	R-49797	+	+	+	-	+	II
Type II	R-45379	+	+	-	+	+	VIII

+, positive result (>10 µM NO_2 or N_2O-N); -, negative result (<10 µM NO_2 or N_2O-N)

[a] Observed decline in NO_2 levels and corresponding rise in N_2O levels over time

[b] +/-, Values around the detection limit of the assay (10 µM) [c] Nt, Not tested due to slow growth of the strain

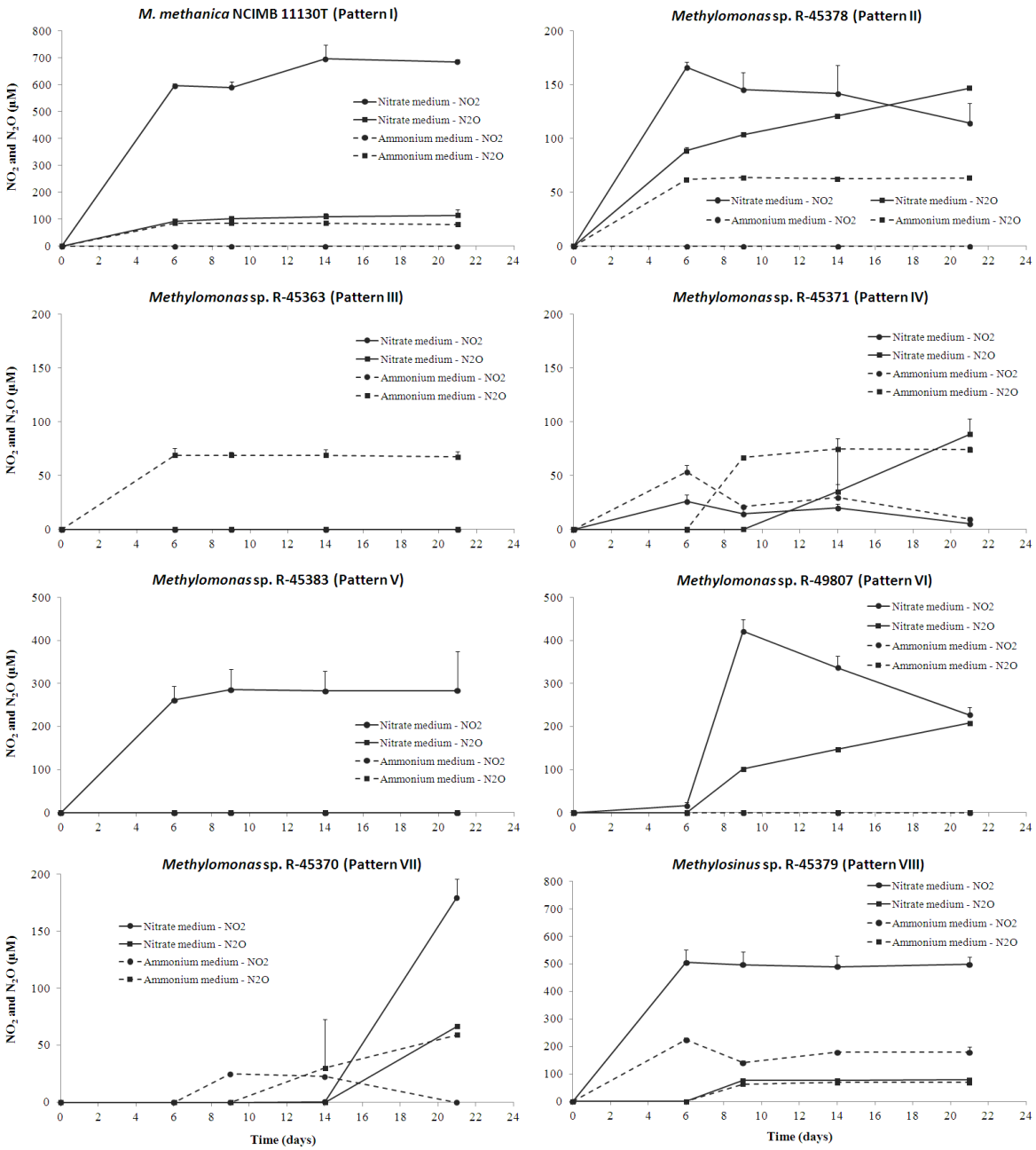

Figure IV-1: Nitrite (circles) and nitrous oxide (squares) production of selected methanotrophic strains based on different dissimilatory nitrogen profiles cultivated in nitrate medium (dNMS, solid line) and ammonium medium (dAMS, dashed line). N_2O values were corrected to N_2O-N to allow equimolar nitrogen comparison with NO_2. The Y-axis could not be made uniform for all strains for clarity, care should be taken when interpreting the data between strains. CH_4, O_2, CO_2 NO_3^-, NH_4^+ and OD_{600nm} levels of these cultures were also measured to support the NO_2 and N_2O observations, but are not shown for clarity.

In ammonium-amended batch experiments, *M. methanica* R-45371, *Methylomonas* sp. R-45370 and R-45377, and *Methylosinus* sp. R-45379 (Table IV-2; Figure IV-1 patterns IV, VII and VIII respectively) produced both measurable levels of nitrite and nitrous oxide. Interestingly, between day 6 and 9, a fraction of the produced nitrite seems to be converted into N_2O, after which both NO_2^- and N_2O levels remained stable over time [Table IV-2;

Figure IV-1; patterns IV and VIII (pattern VII deviates because of slow growth)]. Here, activity of hydroxylamine reductase is hypothesized to produce both nitrite and nitric oxide that is immediately detoxified via nitric oxide reductase. However, most strains were able to produce nitrous oxide without preceding measureable levels of nitrite. This is probably attributed to a small transient nitrite peak below detection levels or an immediate conversion of hydroxylamine to nitric oxide by hydroxylamine oxidoreductase. This hypothesis is supported by the nearly identical N_2O profiles observed for *M. methanica* NCIMB 11130^T and *Methylosinus* sp. R-45379, regardless of detectable nitrite peak (Figure IV-1, patterns I and VIII), suggesting the same detoxification mechanism.

Overall, observed patterns for nitrite and nitrous oxide production were very diverse over the various *Methylomonas* strains tested. *M. koyamae* R-45383 was the only strain not able to produce nitrous oxide from either nitrate or ammonium. *M. koyamae* strain R-49807 on the other hand did produce N_2O from nitrate, but did not produce N_2O from ammonium. The three other *M. koyamae* strains showed the same pattern of nitrite and nitrous oxide production from nitrate and only nitrous oxide production from ammonium. Within *M. methanica*, all strains produced nitrous oxide from both nitrate and ammonium, except for R-45363. This strain did not produce nitrous oxide from nitrate, probably because of its inability to reduce nitrate to nitrite. Also variation in linkage between nitrite removal and nitrous oxide production was clear. Due to the slower growth of *Methylomonas* sp. R-45370 and R-45377, it was difficult to formulate conclusions for these strains, although the strains did produce nitrite and nitrous oxide (not confirmed for R-45377 with nitrate as nitrogen source).

N_2 fixation

Several methanotrophs are known to fix atmospheric nitrogen, an ability that provides a competitive advantage in nitrogen-limited environments. Although traditionally, mostly type II and type Ib methanotrophs were thought to possess this trait (Murrell and Dalton, 1983), verrucomicrobial methanotrophs (Khadem et al., 2010) and several type Ia members, including *Methylomonas* strains, have been found expressing nitrogenase activity (Auman et al., 2001; Bulygina et al., 2002). Here, we found that dinitrogen gas fixation is widespread among *Methylomonas* strains. The genetic potential for nitrogen fixation was first confirmed by the presence of the *nifH* gene encoding the Fe protein of nitrogenase in all strains of the test panel except for *Methylococcaceae* sp. R-49797. The

nifH gene phylogeny compared with selected reference strains is presented in Figure IV-2. Actual dinitrogen gas fixation was demonstrated via parallel growth experiments in nitrogen-free medium under high and low oxygen tension (approximately 21% and 2.1% respectively), with four additional negative controls besides *Methylococcaceae* sp. R-49797, and *M. methanica* NCIMB 11130^T (Auman et al., 2001; Boulygina et al., 2002) as well as the type II strain *Methylosinus* sp. R-45379 as positive controls. As expected, only at low oxygen tension, both positive controls along with ten out of thirteen additional tested *Methylomonas* strains could growth without any nitrogen added except for N_2 in the headspace (Table IV-1). Since oxygen became rapidly limiting in this setup, volumes of air equal to the initial volume were injected (up to eight times) to allow further activity and growth of the cultures. Maximum OD_{600nm} values averaged at 0.332 ± 0.122 (start OD_{600nm} values of 0.017 ± 0.019) across strains, while activity was clearly demonstrated with drops in methane and oxygen levels and subsequent increase in CO_2 levels (data not shown). No growth or activity was observed for the strains cultivated in parallel in nitrogen-free medium under high oxygen tension, which again confirmed the absence of nitrogenous compounds in the growth medium (in addition to colorimetric analysis). Furthermore, all strains that did not possess *nifH* were also negative for growth and activity in nitrogen-free medium at high and low oxygen tension.

Three tested *Methylomonas* strains were not active and did not grow in nitrogen-free medium during the 21-days experiment. Strain R-49807 unexpectedly did not exhibit N_2 fixation, although this strain shares a 100% 16S rRNA and a >99% *nifH* sequence similarity with strains NCIMB 14606^T and R-49799, both positive for N_2 fixation under low oxygen tension. This can possibly be explained by a higher oxygen sensitivity of R-49807, requiring oxygen concentrations lower than the 2% of our experimental set-up, since variations in the oxygen sensitivity of nitrogenase in different methanotrophs have been reported (Vorob'ev and Dedysh, 2008; Khadem et al., 2010).

Strains R-45370 and R-45377 typically grew slower than the other *Methylomonas* strains (one to two-week lag phase in liquid media and doubling time of approximately 12 hours under optimal growth conditions). Therefore, it is possible that these strains would fix N_2 after longer incubation periods.

The presence of the *nifH* gene and the ability to fix nitrogen with an oxygen-sensitive nitrogenase appears to be widespread within the genus *Methylomonas*, although both traits appear to be strain-dependent when combining the results of this study with other reports

(Auman et al., 2001). Since the *Methylomonas methanica* strains R-45362, R-45363, R-45364, R-45371, R-45372 and R-45374 were positive for N_2 fixation and, unlike the type strain of the species, can also express sMMO next to pMMO, these strains could prove particularly interesting for certain applications such as trichloroethylene degradation, for which these features are advantageous (Auman et al., 2001), especially bearing in mind the inherent fast growth of members of this species.

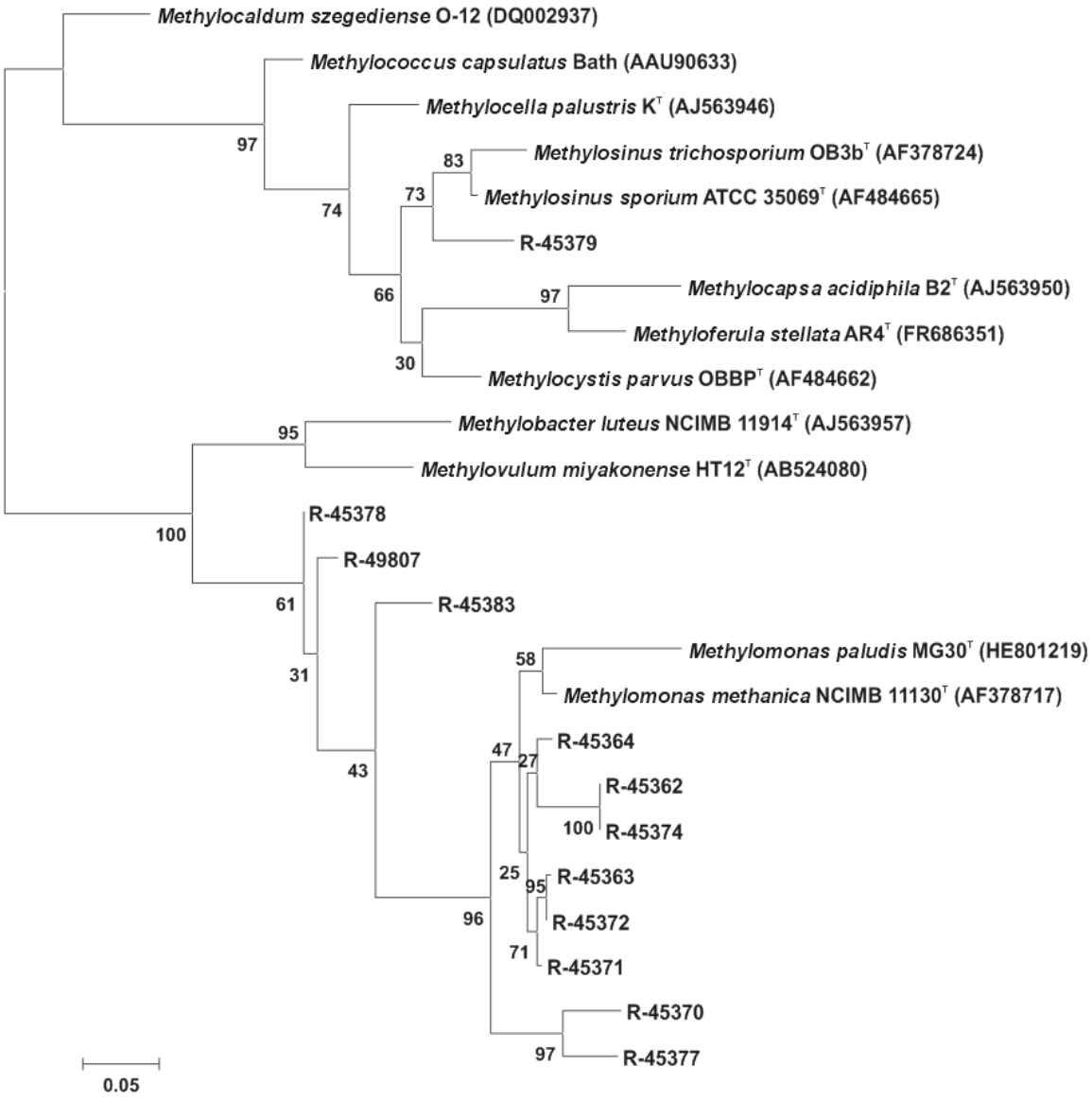

Figure IV-2: *nifH* gene phylogenetic maximum likelihood tree (285 nucleotide alignment; GTR+Gamma substitution model; 1000-replicate bootstrapping) of strains used in this study with the available *nifH* sequences of selected type strains. Bar: 0.05 substitutions per nucleotide position. Since short *nifH* sequences were obtained for strains *Methylomonas koyamae* NCIMB 14606^T and R-49799, these were not included in the final *nifH* alignment.

Conclusion

There have been contradicting reports on the effect of *in situ* ammonium addition, either stimulating or inhibiting the methane oxidation activity in different environments (Bodelier and Laanbroek, 2004). In nitrogen-limited environments, ammonium amendments are expected to stimulate methane oxidation activity of the methanotrophs due to relief of nitrogen limitation (Bodelier and Laanbroek, 2004; Stein and Klotz, 2011). However, in environments where ammonium and methane are available in excess, the community will shift, if possible, towards the enrichment of those methanotrophs that tolerate or flourish in such conditions. In our study, we showed that even very closely related strains within one genus possess a different tolerance to high nitrogen amendments and possess different tools to cope with such situations, perhaps offering a simple explanation for some contradictory reports: when the natural methanotrophic seed bank of a given environment possesses a strain with a high ammonium tolerance and with the tools to tolerate and/or detoxify hydroxylamine and nitrite, the addition of high ammonium loads will favor growth of such a strain and methane oxidation activity will either remain indifferent or become enhanced upon its enrichment. When such a strain is not available in the environment, methane oxidation activity will decline. In such situations it could perhaps prove a useful strategy to add strains like *Methylomonas* sp. R-49799 to enhance the biological mitigation of the greenhouse gas methane, since this strain grew at 100 mM ammonium levels, utilized 2 mM nitrite as sole nitrogen source, tolerated hydroxylamine levels up to 1 mM, produced nitrous oxide most likely as a detoxification process, and can fix N_2 should nitrogen become limiting.

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REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W.,Lipman, D.J., 1990. BASIC LOCAL ALIGNMENT SEARCH TOOL. J Mol Biol 215, 403-410.

Anthony, C. 1982 Biochemistry of methylotrophs. London: The Academic Press.

Auman, A.J., Speake, C.C.,Lidstrom, M.E., 2001. nifH sequences and nitrogen fixation in type I and type II methanotrophs. Appl Environ Microbiol 67, 4009-4016.

Bleakley, B.H.,Tiedje, J.M., 1982. Nitrous oxide production by organisms other than nitrifiers or denitrifiers. Appl. Environ. Microbiol. 44, 1342-1348.

Bodelier, P.L., Roslev, P., Henckel, T.,Frenzel, P., 2000. Stimulation by ammonium-based fertilizers of methane oxidation in soil around rice roots. Nature 403, 421-424.

Bodelier, P.L.E.,Laanbroek, H.J., 2004. Nitrogen as a regulatory factor of methane oxidation in soils and sediments. FEMS Microbiol Ecol 47, 265-277.

Boulygina, E.S., Kuznetsov, B.B., Marusina, A.I., Turova, T.P., Kravchenko, I.K., Bykova, S.A. et al., 2002. [Study of nucleotide sequences of nifH genes in methanotrophic bacteria]. Mikrobiologiia 71, 500-508.

Bowman, J. (2006) The Methanotrophs - The Families *Methylococcaceae* and *Methylocystaceae*. Chapter 3.1.14. *Prokaryotes* **5**: 266-289, DOI: 10.1007/0-387-30745-1_15.

Bowman, J.P., Sly, L.I., Nichols, P.D.,Hayward, A.C., 1993. Revised taxonomy of the methanotrophs – Description of *Methylobacter* Gen Nov, emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. International Journal of Systematic Bacteriology 43, 735-753.

Bulygina, E.S., Kuznetsov, B.B., Marusina, A.I., Turova, T.P., Kravchenko, I.K., Bykova, S.A. et al., 2002. [Study of nucleotide sequences of nifH genes in methanotrophic bacteria]. Mikrobiologiia 71, 500-508.

Campbell, M.A., Nyerges, G., Kozlowski, J.A., Poret-Peterson, A.T., Stein, L.Y.,Klotz, M.G., 2011. Model of the molecular basis for hydroxylamine oxidation and nitrous oxide production in methanotrophic bacteria. FEMS Microbiol Lett 322, 82-89.

Cataldo, D.A., Maroon, M., Schrader, L.E.,Youngs, V.L., 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun Soil Sci Plant Anal 6, 71-80.

Coenye, T., Spilker, T., Martin, A.,LiPuma, J.J., 2002. Comparative assessment of genotyping methods for epidemiologic study of Burkholderia cepacia genomovar III. Journal of Clinical Microbiology 40, 3300-3307.

Danilova, O.V., Kulichevskaya, I.S., Rozova, O.N., Detkova, E.N., Bodelier, P.L., Trotsenko, Y.A.,Dedysh, S.N., 2012. Methylomonas paludis sp. nov., the first acid-tolerant member of the genus Methylomonas, from an acidic wetland. Int J Syst Evol Microbiol.

De Meyer, S.E., van Hoorde, K., Vekeman, B., Braeckman, T.,Willems, A., 2011. Genetic diversity of rhizobia associated with indigenous legumes in different regions of Flanders (Belgium). Soil Biol Biochem 43, 2384-2396.

Dedysh, S.N., 2009. Exploring methanotroph diversity in acidic northern wetlands: Molecular and cultivation-based studies. Microbiology 78, 655-669.

Dunfield, P.F., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A.,Dedysh, S.N., 2003. *Methylocella silvestris* sp nov., a novel methanotroph isolated from an acidic forest cambisol. Int J Syst Evol Microbiol 53, 1231-1239.

Griess, P., 1879. Bemerkungen zu der abhandlung der H.H. Weselsky und Benedikt "Ueber einige azoverbindungen." Chem. Ber 12, 426-428.

Hanson, R.S.,Hanson, T.E., 1996. Methanotrophic bacteria. Microbiol. Rev 60, 439-471.

Heylen, K.,Keltjens, J., 2012. Redundancy and modularity in membrane-associated dissimilatory nitrate reduction in Bacillus. Frontiers in Microbiology 3.

Hino, T., Nagano, S., Sugimoto, H., Tosha, T.,Shiro, Y., 2011. Molecular structure and function of bacterial nitric oxide reductase. Biochim Biophys Acta 1817, 680-687.

Hoefman, S., van der Ha, D., De Vos, P., Boon, N.,Heylen, K., 2012. Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria. Microb. Biotechnol 5, 368-378.

Hunt, D.E., David, L.A., Gevers, D., Preheim, S.P., Alm, E.J.,Polz, M.F., 2008. Resource Partitioning and Sympatric Differentiation Among Closely Related Bacterioplankton. Science 320, 1081-1085.

Johnson, Z.I., Zinser, E.R., Coe, A., McNulty, N.P., Woodward, E.M.S.,Chisholm, S.W., 2006. Niche Partitioning Among Prochlorococcus Ecotypes Along Ocean-Scale Environmental Gradients. Science 311, 1737-1740.

Khadem, A.F., Pol, A., Jetten, M.S.,Op den Camp, H.J., 2010. Nitrogen fixation by the verrucomicrobial methanotroph 'Methylacidiphilum fumariolicum' SolV. Microbiology 156, 1052-1059.

King, G.M.,Schnell, S., 1998. Effects of ammonium and non-ammonium salt additions on methane oxidation by Methylosinus trichosporium OB3b and Maine forest soils. Appl Environ Microbiol 64, 253-257.

Lee, S.W., Im, J., DiSpirito, A.A., Bodrossy, L., Barcelona, M.J.,Semrau, J.D., 2009. Effect of nutrient and selective inhibitor amendments on methane oxidation, nitrous oxide production, and key gene presence and expression in landfill cover soils: characterization of the role of methanotrophs, nitrifiers, and denitrifiers. Appl Microbiol Biotechnol 85, 389-403.

Mohanty, S.R., Bodelier, P.L.E., Floris, V.,Conrad, R., 2006. Differential effects of nitrogenous fertilizers on methane-consuming microbes in rice field and forest soils. Appl Environ Microbiol 72, 1346-1354.

Murrell, J.C.,Dalton, H., 1983. NITROGEN-FIXATION IN OBLIGATE METHANOTROPHS. Journal of General Microbiology 129, 3481-3486.

Noll, M., Frenzel, P.,Conrad, R., 2008. Selective stimulation of type I methanotrophs in a rice paddy soil by urea fertilization revealed by RNA-based stable isotope probing. FEMS Microbiol Ecol 65, 125-132.

Nyerges, G.,Stein, L.Y., 2009. Ammonia cometabolism and product inhibition vary considerably among species of methanotrophic bacteria. FEMS Microbiol Lett 297, 131-136.

Nyerges, G., Han, S.K.,Stein, L.Y., 2010. Effects of Ammonium and Nitrite on Growth and Competitive Fitness of Cultivated Methanotrophic Bacteria. Appl Environ Microbiol 76, 5648-5651.

Ogiso, T., Ueno, C., Dianou, D., Huy, T.V., Katayama, A., Kimura, M.,Asakawa, S., 2012. Methylomonas koyamae sp nov., a type I methane-oxidizing bacterium from floodwater of a rice paddy field. Int J Syst Evol Microbiol 62, 1832-1837.

Op den Camp, H.J.M., Islam, T., Stott, M.B., Harhangi, H.R., Hynes, A., Schouten, S. et al., 2009. Environmental, genomic and taxonomic perspectives on methanotrophic Verrucomicrobia. Environmental Microbiology Reports 1, 293-306.

Park, S.H., Shah, N.N., Taylor, R.T.,Droege, M.W., 1992. Batch cultivation of *Methylosinus*

trichosporium OB3b. 2. Production of particulate methane monooxygenase. Biotechnol Bioeng 40, 151-157.

Rowley, G., Hensen, D., Felgate, H., Arkenberg, A., Appia-Ayme, C., Prior, K. et al., 2012. Resolving the contributions of the membrane-bound and periplasmic nitrate reductase systems to nitric oxide and nitrous oxide production in Salmonella enterica serovar Typhimurium. Biochem J 441, 755-762.

Schnell, S.,King, G.M., 1994. Mechanistic analysis of ammonium inhibition of atmospheric methane consumption in forest soils. Appl Environ Microbiol 60, 3514-3521.

Semrau, J.D., DiSpirito, A.A.,Yoon, S., 2010. Methanotrophs and copper. FEMS Microbiol Rev 34, 496-531.

Smith, M.S.,Zimmerman, K., 1981. Nitrous oxide production by nondenitrifying soil nitrate reducers. Soil Sci. Soc. Am. J. 45, 865-871.

Smith, M.S., 1983. Nitrous oxide production by *Escherichia coli* is correlated with nitrate reductase activity. Appl Environ Microbiol 45, 1545-1547.

Stein, L.Y. (2011) Surveying N_2O-producing pathways in bacteria. In *Methods in Enzymology: research on nitrification and related processes*. Klotz, M.G. (ed). San Diego: Elsevier Academic Press, pp. 131-152.

Stein, L.Y.,Klotz, M.G., 2011. Nitrifying and denitrifying pathways of methanotrophic bacteria. Biochem Soc Trans 39, 1826-1831.

Suharti, Strampaard, M.J.F., Schröder, I.,de Vries, S., 2001. A novel copper A containing menaquinol NO reductase from *Bacillus azotoformans*. Biochemistry 40, 2632-2639.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M.,Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28, 2731-2739.

Taylor, S., Ninjoor, V., Dowd, D.M.,Tappel, A.L., 1974. Cathepsin B2 measurement by sensitive fluorometric ammonia analysis. Anal. Biochem 60, 153-162.

Vandamme, P., Pot, B., Gillis, M., DeVos, P., Kersters, K.,Swings, J., 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiological Reviews 60, 407-+.

Vine, C.E.,Cole, J.A., 2011. Nitrosative stress in *Escherichia coli*: reduction of nitric oxide. Biochem Soc Trans 39, 313-315.

Vorob'ev, A.V.,Dedysh, S.N., 2008. Inadequacy of enrichment culture technique for assessing the structure of methanotrophic communities in peat soil. Microbiology 77, 504-507.

Vorobev, A.V., Baani, M., Doronina, N.V., Brady, A.L., Liesack, W., Dunfield, P.F.,Dedysh, S.N., 2010. Methyloferula stellata gen. nov., sp. nov., an acidophilic, obligately methanotrophic bacterium possessing only a soluble methane monooxygenase. Int J Syst Evol Microbiol.

Whittenbury, R., Phillips, K.C.,Wilkinson, J.F., 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61, 205-217.

Zumft, W.G., Braun, C.,Cuypers, H., 1994. Nitric oxide reductase from *Pseudomonas stutzeri*. Primary structure and gene organization of a novel bacterial cytochrome bc complex. Eur. J. Biochem. 219, 418-490.

Zumft, W.G., 2005. Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme–copper oxidase type. J. Inorg. Biochem. 99, 194–215.

Chapter V

Exploration and prediction of interactions between methanotrophs and heterotrophs

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SH and MS contributed equally. SH, MS, KH, WW and FMK designed the experiments. SH and MS performed the experiments. MS analyzed the data. SH and MS wrote the manuscript. KH, NB, BDB, WW and PDV commented on the manuscript.

SUMMARY

Methanotrophs form the basis of a methane-driven food web on which heterotrophic microorganisms can feed. In return, these heterotrophs can stimulate the growth of methanotrophs in co-culture by providing growth additives. However, only a few specific interactions are currently known. We have incubated nine methanotrophs with 25 heterotrophic strains in a pairwise miniaturized co-cultivation setup. Through PCA analysis and *k*-means clustering, methanotrophs and heterotrophs could be grouped according to their interaction behavior, suggesting strain-dependent methanotroph-heterotroph complementarity. Co-cultivation significantly enhanced the growth parameters of three methanotrophs. This was most pronounced for *Methylomonas* sp. M5, with a three-fold increase in maximum density and a four-fold increase in maximum increase in density in co-culture with *Cupriavidus taiwanensis* LMG 19424. In contrast, co-cultivation with *Methylobacterium radiotolerans* LMG 2269 and *Pseudomonas aeruginosa* LMG 12228 inhibited the growth of most methanotrophs. Whole genome data suggested the importance of vitamin metabolism for co-cultivation success. The generated data set was then successfully exploited as a proof-of-principle for predictive modeling of co-culture responses based on other interactions of the same heterotrophs and methanotrophs, yielding ROC area under the curve values of 0.73 upon 50% missing values for the maximum increase in density parameter. As such, these modeling-based tools were shown to hold great promise in reducing the amount of data that needs to be generated when conducting large co-cultivation studies.

INTRODUCTION

Microbial processes are very important, both from an environmental and a biotechnological point of view. Hence, many techniques have been developed to model microbial systems. For example, spatio-temporal models for the formation of colonies and biofilms can successfully match wet lab observations (Picioreanu et al., 1998; Nishiyama et al., 2010). Also, constraint-based metabolic models proved useful for the metabolic engineering of industrially relevant microorganisms (Patil et al., 2004; Park et al., 2009). However, most microbiological processes in nature are not performed by individual strains, but by a consortium of many functionally distinct microorganisms (Read et al., 2011). In order to quantitatively model such processes, these interactions have to be accounted for.

Therefore, similar to the developments in systems biology, microbial ecology is increasingly dealing with the reconstruction of global microbial networks from large data sets (Fuhrman, 2009; Faust and Raes, 2012). Such approaches fit nicely with the ecoinformatics point of view, in which ecology in general is seen as a data-driven science (Michener and Jones, 2012). The standard way of inferring these microbial networks is by using the so-called guilt by association principle, similar to how gene regulatory networks are commonly discovered. Typically, one searches for patterns in tables of species abundances by looking at correlations or more complex measures such as the maximal information coefficient (Reshef et al., 2011). The present study focused on a smaller scale and more controlled setup, whereby only two types of microorganisms were considered, methanotrophs and heterotrophs, which were investigated through pairwise co-cultivation.

Methanotrophs are a physiologically and phylogenetically diverse set of organisms grouped by their ability to oxidize the greenhouse gas methane and utilize it as sole carbon and energy source (Hanson and Hanson, 1996). Methanotrophic bacteria have been found to form close interactions with other organisms, such as marine invertebrates (Childress et al., 1986; Cavanaugh et al., 1987; Petersen and Dubilier, 2009), plants (King, 1994; Raghoebarsing et al., 2005; Kip et al., 2010), algae (van der Ha et al., 2011) and other bacteria such as denitrifying (Modin et al., 2007) or heterotrophic bacteria in general (Hrsak and Begonja, 1998, 2000; van der Ha et al., 2012). In such consortia, methanotrophs form the basis of a methane-driven food web, providing carbon compounds derived from methane to other organisms (Hutchens et al., 2004; Murase and Frenzel, 2007; Qiu et al., 2009; Dumont et al., 2011). Interactions can be mutually beneficial, by

providing a stable environment and optimal gas composition in the case of endosymbiotic interactions (Raghoebarsing et al., 2005; Petersen and Dubilier, 2009) or by the removal of toxic waste products of methane oxidation, e.g. formaldehyde (Hanson and Hanson, 1996). Hence, methanol-utilizing hyphomicrobia are often found to copurify during the isolation of methanotrophic bacteria (Bowman, 2006). In addition, cobalamin produced by rhizobia was shown to stimulate the growth of some methanotrophs (Iguchi et al., 2011). However, interactions that negatively influence growth also exist, such as competition (e.g. oxygen competition negatively influencing both partners), predation (positive for one partner and negative for the other) and amensalism (negative for one partner and indifferent for the other) (Faust and Raes, 2012).

To explore the methanotroph-heterotroph interactions, we evaluated the use of data mining and predictive modeling based on growth parameters generated in a miniaturized setup of co-cultures with methane as sole carbon source, by combining nine methanotrophic strains (from the genera *Methylomonas*, *Methylosarcina*, *Methylocystis* and *Methylosinus*) with 25 heterotrophs (24 heterotrophic bacteria and one yeast strain). Heterotrophic bacteria from fourteen genera were selected based on the availability of their whole genome. This allowed us to also search for determining genomic features for beneficial or negative interactions.

EXPERIMENTAL PROCEDURES

Methanotrophic and heterotrophic strains and growth conditions

Nine methanotrophic strains were used in this study of which six environmental strains (Hoefman et al., 2012): *Methylomonas* spp. R-45363 (M1), R-45364 (M2), R-45374 (M3), R-45378 (M4) and R-45383 (M5) and *Methylosinus* sp. R-45379 (M6) and three type strains, *Methylosarcina fibrata* DSM 13736^T (M9), *Methylocystis hirsuta* DSM 18500^T (M7) and *Methylocystis parvus* NCIMB 11129^T (M8). For clarity in interpreting the results of this study, the methanotrophic strains were assigned new numbers (M1 up to M9), which are listed in brackets behind the original strain number. Nitrate Mineral Salts [NMS; (Bowman, 2006)] medium with Cu^{2+} adjusted to 10 µM was used for the cultivation of methanotrophic bacteria. Methanotrophic strains were cultivated on NMS plates under a CH_4:air (1:1) atmosphere in gastight jars (Oxoid, UK) at 28°C.

Twenty four heterotrophic bacteria for which whole genome data was available were selected: *Acinetobacter baumannii* LMG 1025 (H1), *Bacillus azotoformans* LMG 9581^T (H2), *Bacillus bataviensis* LMG 21833^T (H3), *Bacillus licheniformis* LMG 6933 (H4), *Bacillus vireti* LMG 21834^T (H5), *Cupriavidus taiwanensis* LMG 19424 (H6), *Escherichia coli* R-23895 (H7), *Escherichia coli* R-23891 (H 8), *Escherichia coli* R-23894 (H9), *Escherichia fergusonii* LMG 7866 (H10), *Flavobacterium johnsoniae* LMG 1341 (H11), *Methylobacterium nodulans* R-7055 (H12), *Methylobacterium radiotolerans* LMG 2269 (H13), *Ochrobactrum anthropi* LMG 2134 (H14), *Pseudomonas aeruginosa* LMG 12228 (H15), *Pseudomonas putida* LMG 24210 (H16), *Pseudomonas putida* R-17801 (H17), *Rhizobium radiobacter* LMG 287 (H18), *Ensifer meliloti* R-20688 (H19), *Rhodobacter sphaeroides* LMG 2827 (H20), *Roseobacter denitricans* LMG 19751 (H21), *Shigella exneri* R-23896 (H22), *Staphylococcus aureus* R-23700 (H23), *Staphylococcus aureus* R-23902 (H24). Strains were obtained from the in-house research collection of LM-UGent, if indicated by an R-number, or from the BCCM/LMG Bacteria Collection (www.bccm.belspo.be), if indicated by an LMG number. In addition, the yeast *Pichia pastoris* GS115 (H25) was included in this study and treated as an additional heterotroph. For clarity in interpreting the results of this study, the heterotrophic strains were assigned new numbers (H1 up to H25), which are listed in brackets behind the original strain number. Heterotrophic strains were cultivated at 28°C on nutrient agar (Oxoid, UK), except for *Methylobacterium nodulans* R-7055 (H12), cultivated on R2A agar (BD, France) and *Roseobacter denitricans* LMG 19751 (H21), cultivated on marine agar (BD,

France).

Co- and pure culture cultivation in miniaturized setup and extraction of growth parameters

Freshly-grown colonies were suspended in liquid NMS and used as inoculum (OD_{600nm} 0.005, final concentration). All possible pairs of methanotrophic and heterotrophic co-cultures, as well as all separate methanotrophic and heterotrophic strains, were inoculated in triplicate in liquid NMS in sterile 96-well microtiter plates (300 µL, final volume per well). Cultures were incubated under a CH_4:air (1:1) atmosphere at 28°C. Growth was determined by OD_{600nm} measurements (verified by visual inspection) at the start of incubation and after 2, 4, 6, 8, 10, 12 and 14 days of incubation. As such, growth curves were established for each co-culture and pure culture. From these growth curves, three growth parameters were extracted: (i) the maximum density (MD), (ii) the maximum increase in density (MI) and (iii) the time to reach the maximum increase in density (TMI). To establish the effect on growth by co-cultivation, the value of each growth parameter of a co-culture with a given methanotroph was normalized by the value of this methanotroph grown in pure culture, which will be further denoted as the reference of the co-culture. The maximum density (MD) of a co-culture is defined as the maximum measured average (n = 3) $\log_2$ normalized OD_{600nm} value of its growth curve subtracted by the $\log_2$ normalized maximum density value calculated for the reference growth curve. As such, an MD value of 1 indicates that a two-fold increase in maximum density was observed for the co-culture compared to the reference (methanotroph in pure culture), while an MD of 2 would indicate a four-fold increase. The maximum increase in density between two successive measurements (MI) of a co-culture is defined as the maximum measured average (n = 3) slope, defined at day t as: $(OD_{(t)}-OD_{(t-2)})/2$ with units [OD_{600nm} day^{-1}]. Similarly as explained for the growth parameter MD, normalization of MI was performed by subtraction of $\log_2$ values with the largest measured slope of the reference. The relative time of maximum increase in density (TMI) was defined as the difference in days between the MI of a co-culture and the MI of its reference. These growth parameters were selected to allow for fully automated extraction of relevant data from the growth curves using the statistical software environment R, which reduced human bias of the results and allowed high-throughput interpretation of relatively large data sets.

Co-culture interaction (dis)similarities

Several methods were explored to assess the similarities or dissimilarities between the methanotrophs in regard to the impact on their growth parameters by the addition of the heterotrophs in co-culture experiments.

(i) Significant dissimilarities between methanotrophs in their interaction with heterotrophs were appraised by a non-parametric paired Wilcoxon signed-rank test, since a normal distribution could not be guaranteed. A Holm-Bonferroni correction to counteract multiplicity was applied for the 36 tests (all pairwise combinations of the nine methanotrophs) and for each growth parameter.

(ii) Similarities between methanotrophs in their interaction with heterotrophs were explored using principal component analysis of the covariance matrix.

(iii) The heterotrophs were grouped according to how they influenced the methanotrophs through co-culturing for the three growth parameters using *k*-means clustering. The MI growth parameter was used to study the genomes of the heterotrophic bacteria (excluding the yeast strain H25, for which the genome was not available), using the SEED-Viewer annotations (Overbeek et al., 2005). As such, differences and similarities in genes between heterotrophic groups defined by *k*-means clustering could be explored. Firstly, only the second and third principal components were withheld from the interaction matrix, as these showed the most meaningful contrasts between methanotrophs (see Results section). Secondly, H13 and H15 were excluded, since these showed an atypical pattern in interaction (growth inhibition). From the reduced data set, three well-separated groups of heterotrophs could be distinguished using *k*-means clustering, from which collective gene sets were constructed for genes of the carbohydrate and secondary metabolism. The genes in a collective gene set for a group are, by definition, present in all heterotrophs of a particular group.

In addition, for each gene *g* and each group *c* we calculated the posterior probability $P(c|g)$ of a heterotroph belonging to group *c* if it possesses gene *g*:

$$P(c|g) = \frac{P(g|c)P(c)}{P(g)}.$$

Here, $P(g|c)$ is the likelihood of a heterotroph having gene g, if it belongs to group *c*, $P(c)$ is the prior probability of belonging to group *c* and $P(g)$ denoting the marginal likelihood of having gene *g*. This approach is similar to the naive Bayes classifier in which each feature (*in casu,* gene) is treated as an independent piece of evidence for or against belonging to a particular class (*in casu,* group of heterotrophs). Genes with a posterior

probability equal to 1 for a group are denoted as determining genes. Thus, determining genes occur in some or all members of a group, but never in another group.
Note that a determining gene for a group is not necessarily in the collective gene set and vice versa.

Predictive modeling of missing values of co-cultivation experiments

The use of predictive modeling was explored in order to assess the possibility to make predictions on the growth parameters of certain co-culture combinations of methanotrophs and heterotrophs that were not measured, based on other combinations of the same methanotrophs and heterotrophs. To fill missing values in such an interaction matrix, probabilistic principal component analysis (PPCA) was applied (Tipping and Bishop, 1997). PPCA is a reformulation of classical PCA in terms of a maximum likelihood solution of a linear probabilistic latent variable model. In our setting, it is assumed that each heterotroph can be described by an unobserved latent feature vector **z**. This vector **z** is drawn from a multivariate Gaussian distribution with unit covariance matrix. A vector **y** containing the observed interaction values with the nine methanotrophs can subsequently be obtained by performing a linear transformation (a rotation followed by a translation) on **z** and adding white noise. The parameters of this generative model can be estimated using the Expectation Maximization (EM) algorithm. This algorithm iteratively estimates the latent variables and subsequently the parameters of the model until convergence. In this formulation, missing values are treated as another kind of latent features. In the training phase of the PPCA, the non-observed combinations and latent features are jointly estimated with the parameters. The experiments in this section were performed using the Bioconductor package 'pcaMethods' (Stacklies et al., 2007).
The PPCA method was applied to predict 10, 25 and 50 % of the interaction matrix for the three growth parameters. Combinations were arbitrarily removed with the restriction that there was always at least one observation for each of the methanotrophs and heterotrophs and that both the missing as well as the remaining elements contained both positive and negative values. In this way, sampling of the missing values is strictly speaking not random, although only a minimal influence on the evaluation and use of the model is assumed (these restrictions would definitely be less stringent in larger scale experiments). The missing values were inferred using latent features with a dimension of 3, which was found to be representative for this data set based on silhouette plots for two to six clusters. Generated predictions were subsequently compared to the true measured values to assess

the quality of the predictions. The whole process was repeated 250 times in order to obtain reliable estimates of the performance measure.

Evaluation of prediction quality was approached as a classification problem, i.e. the model had to be able to make the distinction between a methanotroph-heterotroph pair that showed positive interaction and a pair that showed negative interaction. Therefore, we applied a cutoff to the continuous values, placing the strictly positive values in one class and the negative and zero values in the other class. The performance of this classification was visualized with a receiver operating characteristic (ROC) curve, where the true positive rate of a predictor is plotted in function of the false positive rate (Fawcett, 2004). The true positive rate is defined as the number of positive interacting combinations selected by the algorithm, divided by the total number of true positive combinations. Likewise, the false positive rate is the number of negatively interacting combinations that were predicted positive, divided by the total number of negative combinations in the test set. The ROC curve has the attractive property that it is independent of the unbalancedness of the class distribution, in contrast to the classification accuracy. Subsequently, the area under the ROC curve (AUC) was measured to obtain a scalar performance measure. This measure denotes the chance that a combination of a positively interacting pair is given a higher predicted value than a negative pair. An ideal classifier has an AUC equal to 1, while an AUC of 0.5 indicates a completely random classifier. Values from 0.7 onwards are a sign that the algorithm performs clearly better than random.

RESULTS

The impact of co-cultivation on growth parameters

Growth curves of pure cultures and pairwisely combined co-cultures of nine methanotrophic and 25 heterotrophic strains were established in a miniaturized setup in 96-well microtiter plates in nitrate mineral salts medium with methane added to the atmosphere as sole carbon source. As expected, in pure cultures, methanotrophic strains grew well in this setup, while the heterotrophs were unable to grow (OD_{600nm} values did not increase upon incubation) because of carbon deprivation (data not shown). For all co-cultures and pure cultures of methanotrophs, three growth parameters were automatically extracted via an R script: (i) the maximum density (MD), (ii) the maximum increase in density (MI) and (iii) the time to reach maximum increase in density (TMI). These parameters for co-cultures were normalized with those obtained from the respective methanotrophs in pure culture, to allow for interpretation of the impact on the growth of the addition of the heterotroph (Figure V-1). For all three growth parameters, specific combinations of methanotrophs with heterotrophs that either enhanced (positive MD and MI values and negative TMI values) or inhibited growth (negative MD and MI values and positive TMI values) were observed. The MD and MI showed a strong correlation (Pearson correlation = 0.970), while only a minor correlation was found for TMI with the other two parameters. About 50 % and 46 % of the co-cultures showed an increase in MD and MI, respectively, while only 34.6 % of the co-cultures showed an improved TMI. The MI varied over a greater range (from approximately -7.4 to 4.1) than the MD (from approximately -4.6 to 2.7). The TMI could be improved or delayed by a maximum of ten days by co-cultivation.

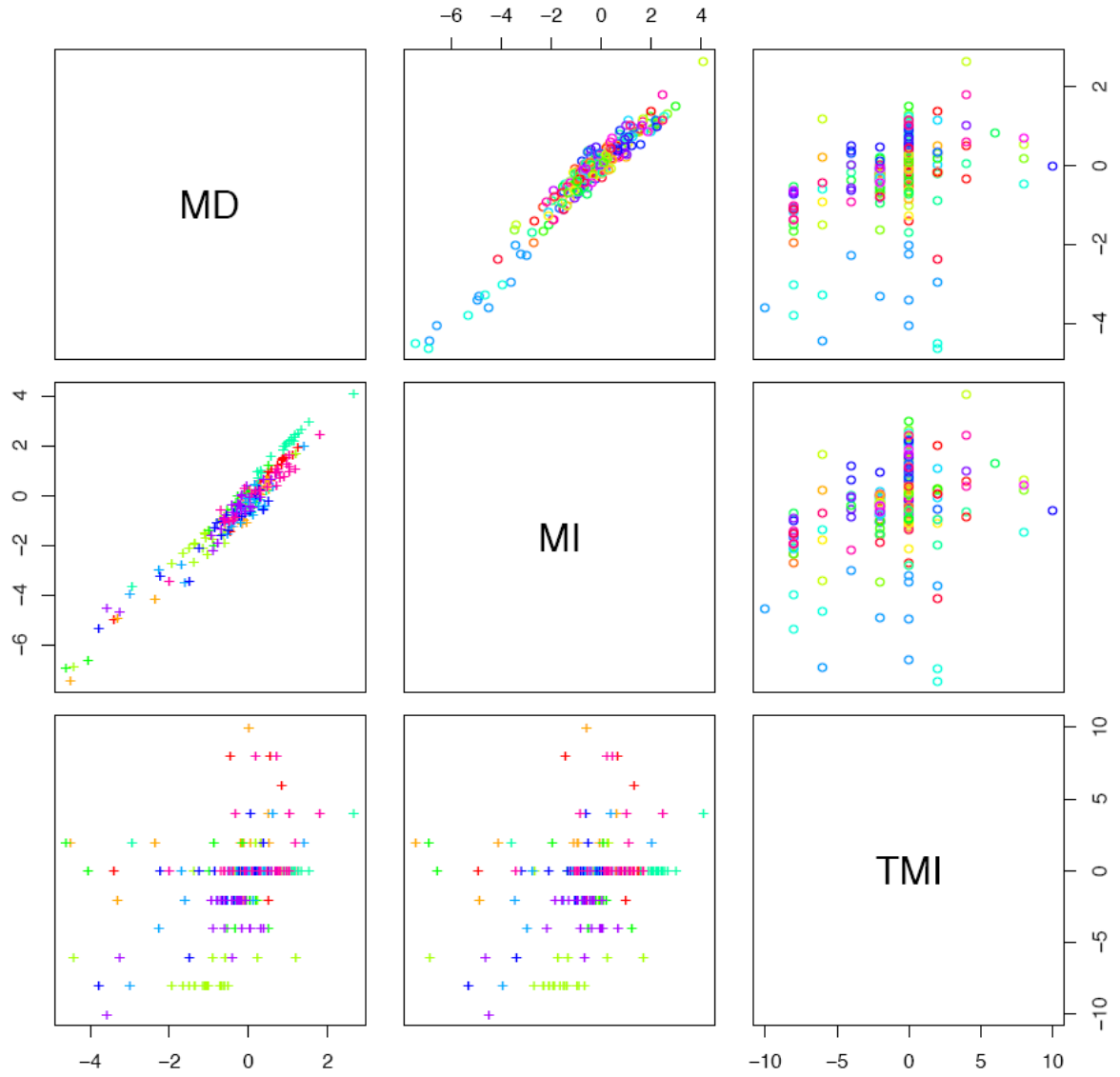

Figure V-1: Scatter plot matrices of the maximum density (MD), maximum increase in density (MI) and time of maximum increase in density (TMI) of co-cultures for all the combinations of methanotrophs and heterotrophs. Each box is a plot of a combination of two growth parameters, indicated on the diagonals. The three boxes above the diagonal are colored according to the 25 heterotrophs (indicated by o) and the three boxes below the diagonal are colored according to the nine methanotrophs (indicated by +). Combinations that have a strain in common often clustered together, while a strong correlation between MD and MI was observed.

Co-culture interaction (dis)similarities between methanotrophs

The impact on the three growth parameters of co-cultivation of a specific methanotroph with each heterotroph is represented in more detail in Figure V-2. Co-culturing stimulated growth measured as MD and MI mostly for *Methylomonas* spp. M1 and M5 and *Methylosarcina fibrata* DSM 13736^{T} (M9), almost regardless of the added heterotroph [except for *Pseudomonas aeruginosa* LMG 12228 (H15)]. The opposite was observed for

Methylomonas sp. (M3), although its TMI increased significantly when grown in co-culture. However, it should be noted that the TMI of M3 was achieved after an unusually long incubation period (12 days), which affected all TMI values of M3 co-cultures, since these are normalized for the M3 values in pure culture. To a lesser extent, co-culturing with most heterotrophs also improved the TMI of *Methylomonas* sp. M4 and *Methylocystis parvus* NCIMB 11129^T (M8) without positively impacting the two other growth parameters. For 52 % of the co-cultures, no impact was observed on the TMI compared to their respective pure methanotrophic cultures, although this could partially be attributed to the limited resolution of 2 days of this growth parameter.

We evaluated whether some methanotrophs reacted similarly or not to the addition of specific heterotrophs. For this, the medians of the co-culture responses for all combinations of methanotrophs were compared using a non-parametric paired Wilcoxon signed-rank test with Holm-Bonferroni correction (data not shown). For MD and MI, significantly higher values ($p < 0.05$) were found for *Methylomonas* spp. M1 and M5 and *Methylosarcina fibrata* DSM 13736^T (M9) in co-culture compared to the other methanotrophs. The median of TMI for *Methylocystis parvus* NCIMB 11129^T (M8) in co-culture is also significantly improved compared to the other methanotrophs, except for *Methylomonas* sp. (M3). *Methylomonas* spp. M1, M2, M5 and *Methylosarcina fibrata* DSM 13736^T (M9) in co-culture formed a group with a statistically indistinguishable TMI, which was mostly better than for *Methylomonas* sp. (M4), *Methylosinus* sp. (M6) and *Methylocystis hirsuta* DSM 18500^T (M7) in co-culture.

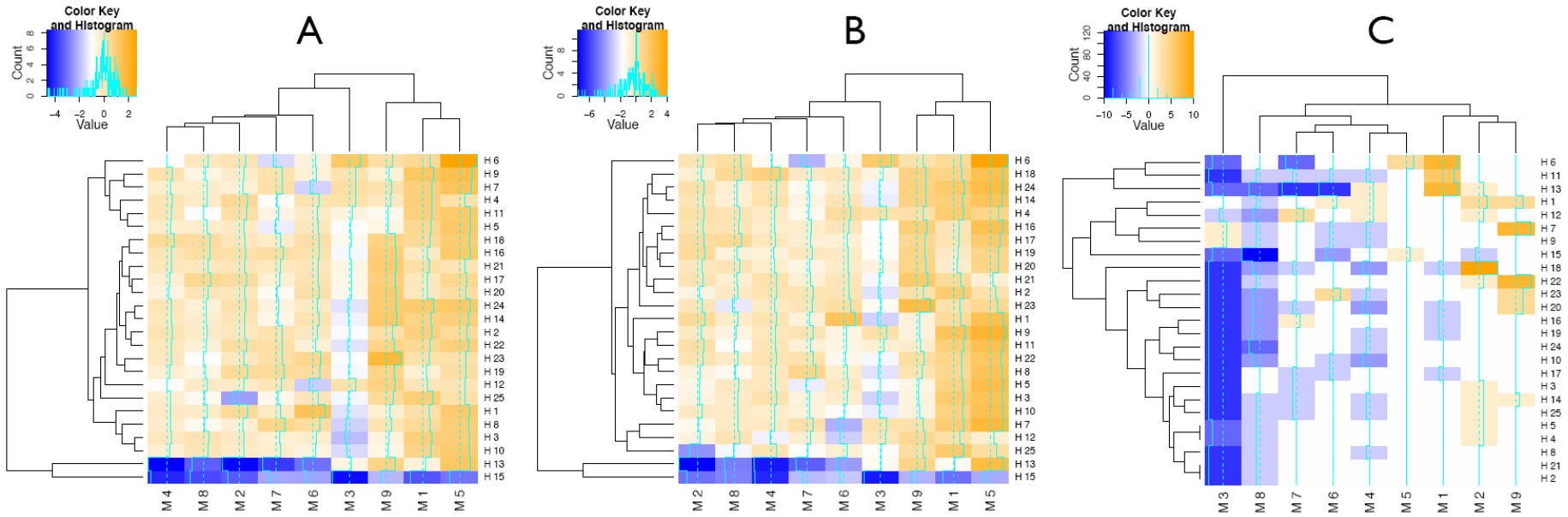

Figure V-2: Heat maps of (A) the maximum density (MD), (B) maximum increase in density (MI) and (C) the time of the maximum increase in density (TMI), assessed through co-cultivation of all combinations of methanotrophs and heterotrophs. The rows and columns of each interaction matrix are clustered using complete hierarchical clustering. The following methanotrophs were included: *Methylomonas* strains R-45363 (M1), R-45364 (M2), R-45374 (M3), R-45378 (M4) and R-45383 (M5), *Methylosinus* sp. R-45379 (M6), *Methylocystis hirsuta* DSM 18500^{T} (M7), *Methylocystis parvus* NCIMB 11129^{T} (M8) and *Methylosarcina fibrata* DSM 13736^{T} (M-9). The following heterotrophs were included: *Acinetobacter baumannii* LMG 1025 (H1), *Bacillus azotoformans* LMG 9581T (H2), *Bacillus bataviensis* LMG 21833T (H3), *Bacillus licheniformis* LMG6933 (H4), *Bacillus vireti* LMG 21834T (H5), *Cupriavidus taiwanensis* LMG 19424 (H6), *Escherichia coli* R-23895 (H7), *Escherichia coli* R-23891 (H 8), *Escherichia coli* R-23894 (H9), *Escherichia fergusonii* LMG 7866 (H10), *Flavobacterium johnsoniae* LMG 1341 (H11), *Methylobacterium nodulans* R-7055 (H12), *Methylobacterium radiotolerans* LMG 2269 (H13), *Ochrobactrum anthropi* LMG 2134 (H14), *Pseudomonas aeruginosa* LMG 12228 (H15), *Pseudomonas putida* LMG 24210 (H16), *Pseudomonas putida* R-17801 (H17), *Rhizobium radiobacter* LMG 287 (H18), *Ensifer meliloti* R-20688 (H19), *Rhodobacter sphaeroides* LMG 2827 (H20), *Roseobacter denitricans* LMG 19751 (H21), *Shigella exneri* R-23896 (H22), *Staphylococcus aureus* R-23700 (H23), *Staphylococcus aureus* R-23902 (H24) and *Pichia pastoris* GS115 (H25).

When the data was analyzed with principal component analysis (PCA), more than 50 % of the variance was explained by the first principal component for the growth parameters MD and MI. This component did not show contrasts between methanotrophs, but rather implied that the lion's share of variance is due to natural differences in growth between methanotrophs only. The second largest principal component (about 18 % of the variance) indicated a contrast between *Methylomonas* spp. M1, M3 and M5 and *Methylomonas* spp. M2 and M4, *Methylosinus* sp. M6 and *Methylocystis hirsuta* DSM 18500^T (M7). Thus, a heterotroph that stimulates the growth of the former group of methanotrophs is likely to have a negative effect on the latter group and vice versa. For the TMI growth parameter, the first principal component (28 % of the variance) did not account for overall change induced by a heterotroph, but showed a contrast between *Methylomonas* spp. M1 and M5 and methanotrophs M3, M6, M7 and M9. Apart from *Methylomonas* sp. M3, this result was comparable to the second principal component of MD and MI.

Determining genomic features of heterotrophic strains

The impact of a specific heterotroph on the three growth parameters of co-cultivation with each methanotroph can be viewed in more detail in Figure V-2. Most striking was the particularly good score for both MD and MI of co-cultures *Cupriavidus taiwanensis* LMG 19424 (H6) with *Methylomonas* sp. M5 and *Staphylococcus aureus* R-23700 (H23) with *Methylosarcina fibrata* DSM 13736^T (M9).

Using *k*-means clustering all heterotrophs could be clustered into four groups according to their influence on methanotrophs (data not shown). For the MD and MI growth parameters, heterotrophs *Methylobacterium radiotolerans* LMG 2269 (H13) and *Pseudomonas aeruginosa* LMG 12228 (H15) were clear outgroups, characterized by their strong negative influence on most methanotrophs (Figure V-2). The other heterotrophs formed two clear groups for MD and MI and four groups for TMI. Here, *Methylomonas* sp. M3 appeared to be key to separate the major groups, for all growth parameters. Thus, the way a heterotroph influenced methanotroph M3 provided strong clues for how the heterotroph would interact with the other methanotrophs.

For the MI parameter, three heterotrophs could be distinguished by *k*-means clustering using a reduced dataset, excluding heterotrophs *Methylobacterium radiotolerans* LMG 2269 (H13), *Pseudomonas aeruginosa* LMG 12228 (H15) and *Pichia pastoris* GS115 (H25). The first two were removed due to their abnormal growth pattern and the latter because no meaningful genetic comparison was possible as it was the sole Eukaryote in the

data set. Figure V-3 shows this reduced set in a cluster plot. Group I contained heterotrophs H6, H7 and H9 and was characterized by their increase in the MI in co-culture with methanotrophs M1, M3 and M5 while reducing growth in co-culture with methanotrophs M2, M4, M6 and M7. Group II clustered heterotrophs H1, H3, H5, H8, H10, H11, H14 and H24 and was characterized by their positive influence on the MI in co-culture with methanotrophs M4, M6 and to a lesser extent with M1 and M4. These heterotrophs also reduced the MI when co-cultivated with methanotrophs M3 and M9. Group III contained the remainder of heterotrophs, which mainly stimulated the growth of methanotrophs M2, M7 and M9.

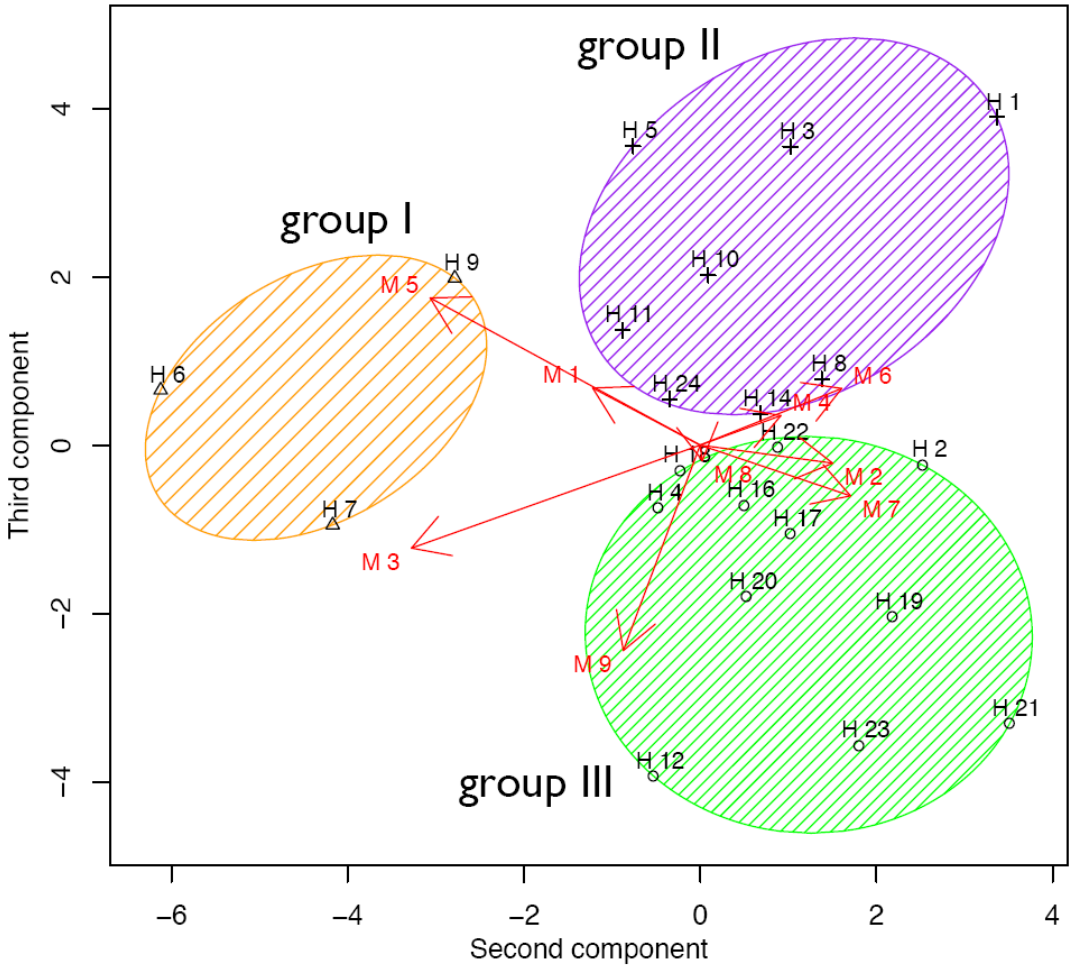

Figure V-3: Plot of the clustering of the reduced MI data. A *k*-means clustering performed in the space spanned by the second and third principal components of the MI growth parameter. The three obtained groups are characterized by different interaction patterns with the set of methanotrophs. The red arrows in the plot indicate the direction and relative magnitude of the loadings of the methanotrophs obtained in this subspace.

We attempted to link the occurrence of particular genes of the carbohydrate (1,029 genes over all heterotrophs) and the secondary (1,712 genes over all heterotrophs) metabolism to these three groups of heterotrophs. If all members of a group possessed a particular gene, this gene was assigned to the collective gene set of that group. Figure V-4 shows a Venn diagram of the overlap of the collective gene sets for the three groups of heterotrophs. The distributions of the different intersections are roughly the same for the two types of genes. Group I, due to only having three members, had the largest gene set: 120 of the 1,029 genes in the carbohydrate set and 150 of the 1,712 genes of the secondary metabolism set. Group II had no unique genes that did not appear in the first two groups, despite not being the smallest group nor having a significant smaller collective gene set. In addition to these collective gene sets, we also sought for determining genes, e.g., genes for which presence in a bacterium always indicated membership of a particular group. Group I had 10 and 5 determining genes for carbohydrate and secondary metabolism respectively. For group II, 65 and 1,299 determining genes were found for the two systems while group III had 115 and 27 determining genes.

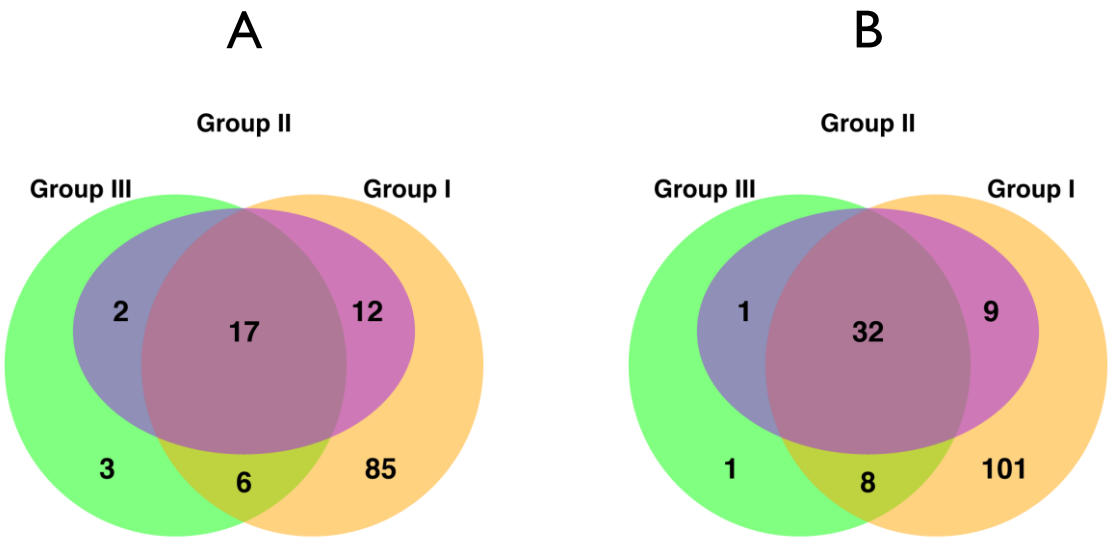

Figure V-4: Venn diagram of the collective gene sets of the different groups of heterotrophic strains (determined by their interaction with the methanotrophs) for (A) the carbohydrate and (B) secondary metabolism. A gene is considered part of a collective gene set for a group if it occurs in all the members of that group. Groups with fewer members have a larger gene set. There are no genes that occur in Group II that are not possessed by all the member of Group I or III.

It is striking that even though group II has no unique collective genes, there are many determining genes, especially for the secondary metabolism. Most determining genes for

the carbohydrate metabolism code for either enzymes involved in catabolism or transport, with the substrate varying with the different groups. For the secondary metabolism, many determining gene functions were linked with vitamin metabolism. In group I, two determining genes were involved in quinone metabolism (PqqC-like protein and glutamate-cysteine ligase) as well as genes related to vitamins B_6 (pyridoxine 4-oxidase) and B_{12} (cob(II)alamin reductase) metabolism. Group III is especially enriched with determining genes coding for transporters of cobalamin and cobalt.

Predictive modeling of missing values of co-cultivation experiments: proof-of-principle

A probabilistic principal component analysis (PPCA) was applied to predict the outcome of untested methanotroph-heterotroph combinations using available data of other co-cultivation experiments of the same strains. The ROC curves for the MI growth parameter with a fraction of 10, 25 and 50 % of the observations absent are shown (Figure V-5).

All curves lie above the diagonal, indicating that we succeeded in modeling part of the patterns of the microbial interactions. Typically, for ROC curves, the curve starts very steep, indicating that there is a part of the data set for which the model can make fairly accurate predictions (increasing the cutoff results in relatively more true positives than false positives). At a false positive rate of around 20 %, the curve showed an inflection point. From this point onwards, the model becomes increasingly unreliable in detecting positively interacting co-cultures. The area under the curve (AUC) was similar for the three parameters: for 10, 25 and 50 % missing values, AUC values were 0.751, 0.734, 0.729 for MI, 0.728, 0.711 and 0.692 for MD and 0.862, 0.782 and 0.801 for TMI, respectively. Thus, even with 50 % of the pairs missing, meaningful predictions could still be made.

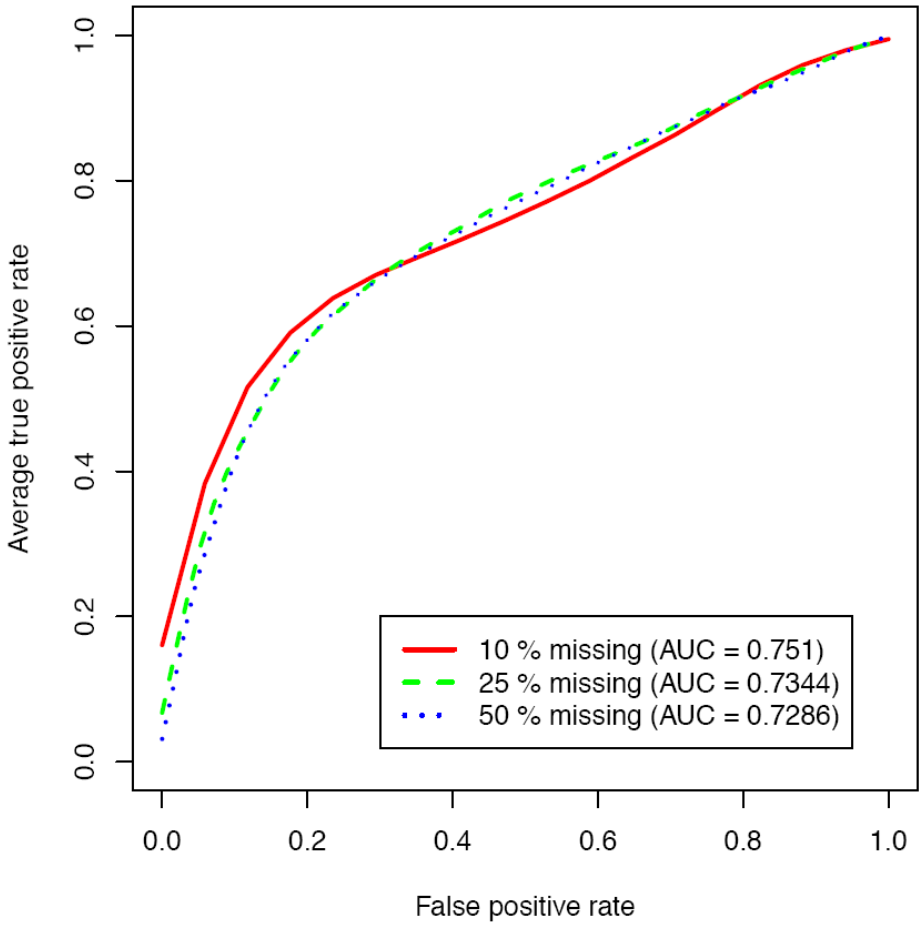

Figure V-5: ROC curve for maximum increase in density missing values inference. Receiver operating characteristic (ROC) curves for inferring missing values of the maximum increase in density statistic using probabilistic principal component analysis. The ROC curves with the corresponding area under the curve (AUC) are given for 10, 25 and 50 % of missing values. Each ROC curve is obtained by averaging 250 experiments. All performances are close to each other, indicating PPCA is relatively robust regarding the number of missing values.

DISCUSSION

Identification of syntrophic associations

In natural environments where methane and oxygen are readily available, methane-oxidizing bacteria can form the basis of a food web (Hutchens et al., 2004; Murase and Frenzel, 2007; Qiu et al., 2009; Dumont et al., 2011). Methane-derived carbon is not only converted to methanotrophic biomass or CO_2 for energy requirements, but can also be leaked as carbon compounds such as methanol or exopolysaccharides, providing food sources for associated heterotrophic organisms (Bowman, 2006). In return, these organisms can stimulate methanotrophic growth by removal of toxic waste products such as methanol or production of complex growth additives such as vitamins, thus creating mutual beneficial interactions. However, only few interactions between methanotrophs and heterotrophs have been resolved and described in detail (Hrsak and Begonja, 2000; Iguchi et al., 2011). In this study, we tried to identify these syntrophic relationships by assuming that co-cultivation results in enhanced growth parameters compared with the methanotrophs in pure culture. Therefore, 225 methanotroph-heterotroph interactions were represented in a miniaturized setup by pairwise co-cultivation in a carbon-free medium under a methane-air atmosphere and screened using three growth parameters. A strong link was observed between the maximum growth density (MD) and the maximum increase in density (MI): when the addition of a heterotroph led to faster growth, the final densities achieved were also higher.

Methylomonas spp. M1 and M5 and *Methylosarcina fibrata* DSM 13736^T (M9) showed significantly increased MD and MI ($p < 0.05$) in co-cultivation, compared to other methanotrophs, suggesting that co-cultures with these organisms benefit from a more general mechanism provided by co-cultivation with heterotrophs. In addition, several clearly beneficial associations promoting growth were observed between *Cupriavidus taiwanensis* LMG 19424 and *Methylomonas* sp. M5 (three-fold increase of MD and four-fold increase of MI; Figure V-2), and *Staphylococcus aureus* R-23700 and *Methylosarcina fibrata* DSM 13736^T (two-fold increase of MD and 2.5-fold increase of MI; Figure V-2). In contrast, *Methylobacterium radiotolerans* LMG 2269 and *Pseudomonas aeruginosa* LMG 12228 significantly inhibited growth of most methanotrophs, while this was not apparent for other heterotrophs, not even for other members of the same genera. Here, we used artificial co-cultures to demonstrate the suitability of our approach and evaluation process for identification of both beneficial and detrimental associations. Via miniaturization,

performed experiments were also very time-efficient (255 co-cultivation experiments were performed and evaluated in a two-week period) and thus ideal for high-throughput screening. Such an approach with more realistic co-cultures combining fresh environmental isolates could be promising to obtain insights in complex *in situ* interactions between different functional guilds, although limitations of laboratory-scale cultivation experiments should always be considered.

Potential mechanisms

Several associations between methanotrophs and heterotrophs were observed but their underlying mechanisms still remain to be resolved. Because of the limited number of methanotrophs included (nine strains from four genera), no obvious link was found with their phylogeny, nitrogen or carbon metabolism, type of methane monooxygenase or other relevant features. However, available physiological data on these strains was limited and their genomes were unavailable. However, using PCA analysis, methanotrophs could be grouped according to their interaction with heterotrophs. For example, a heterotroph that increases growth of *Methylomonas* sp. M1 is likely to inhibit *Methylomonas* sp. M2. Similarly, the heterotrophs could be grouped according to their influence on methanotrophs using *k*-means clustering, which seemed to be governed by *Methylomonas* sp. M3.

In contrast to the methanotrophs, heterotrophic strains were selected based on phylogenetic diversity as well as availability of genome data. This allowed us to search for the underlying genomic mechanisms at the basis of the groups of heterotrophs obtained using *k*-means clustering. To this end, genomic inventories of the carbohydrate and secondary metabolism were extracted based on previous reports on their relevance for methanotroph-heterotroph interactions, and the most determining genes for each cluster were obtained. In selecting potentially relevant genes, we used very strict criteria. The genes of the collective gene sets had to occur in all members of a group while discriminating genes must never appear outside the group of heterotrophs. For larger datasets, it would be more suitable to try to find the optimal trade-off such that relevant genes should occur in as many as possible members of a group, while being rare outside this group.

Typical metagenomic studies sample genetic material from various environmental sites and all identified genes are catalogued (Tringe et al., 2005; Tseng et al., 2009; Jiang et al., 2012), with subsequent linkage to environmental or geographical properties of the sites. Our setting was more controlled, as we could select those organisms with an annotated genome and other desirable properties for the co-culture experiments. The density of these

selected combinations could be measured at multiple time steps, thus resulting in a more detailed growth profile. Only after we obtained patterns in the growth profiles, we tried to relate these obtained groups with properties of selected parts of the gene set of the heterotrophs. As such, we have a more organism-based approach, as the studied microorganisms are part of the experimental setup. This is in contrast with many other studies in which the environment is more central.

Interestingly, several of the found genes were involved in vitamin metabolism, including vitamin B12, which has already been described to stimulate the growth of methanotrophs through co-cultivation with cobalamin-producing rhizobia (Iguchi et al., 2011). Knowing which methanotrophs react positively to organisms that produce certain vitamins can be very useful in biotechnological applications. For example, in order to stabilize a methanotroph-driven bioreactor, a heterotroph that is known to release the relevant vitamins can be added. Here, one uses information about the mechanism of interaction to steer the system, rather than known rules of the thumb about which heterotroph influences which methanotroph.

Predictive modeling

We successfully showed that it is possible to predict interactions of co-cultures that were masked and thus supposedly not measured using data of other co-culture combinations of the same methanotrophs and heterotrophs. This proof-of-principle allows us to only measure a subset of interactions in future experiments, after which the missing values can be inferred in the interaction matrix using a predictive model. Such a model might be a first step in a so-called synthetic ecology framework, in which microbial strains are combined to form a functional tailor-made ecosystem (Curtis et al., 2003; Dunham, 2007). Automatic liquid handlers could be used to make the method even more high-throughput, allowing for larger datasets to be generated. Then, PPCA could substantially reduce both the experimental time and costs by initially testing a quarter or half of the combinations, predicting the remainders *in silico*, and validating the most promising pairs in the lab, if desired. For example, in our analysis the most optimal predictor, which corresponds in the ROC curve to the point closest to the upper left corner, has a true positive rate of 60 % and a corresponding false positive rate of 20 % (Figure V-5). For a hypothetical set of 100 co-cultures with 40 combinations that give a real increase in MI, our model would select 36 combinations as positive pairs, with 24 true positive interactions and 12 false positives. Without the use of modeling, 22 false positives (60% of the cases) would be retrieved for

the same sample size. In reality the ratio of positively versus negatively interacting combinations is unknown. Hence, the model used in this study can also be useful to estimate the size of the experiment that has to be performed to find a certain fraction of positively interacting organisms. Such approaches are common in fields such as drug design (Gönen, 2012), protein network prediction (Vert et al., 2007; Qiu and Noble, 2008) and the prediction of user ratings in media retrieval (Hu et al., 2008).

In general, most methods that are used in practice to infer microbial networks can be thought of as unsupervised (Su and Khoshgoftaar, 2009). Patterns are looked for in an indirect manner, for example by searching for significantly coexisting microorganisms in multiple environments (Chaffron et al., 2010). Though these techniques are also commonly used in inferring gene regulatory networks, these problems are often underdetermined and lack a clear objective function (De Smet and Marchal, 2010). A more direct approach was chosen in this study since the heterotrophs are directly dependent on the carbon flux of the methanotrophs. Since we try to predict masked values in our dataset by learning a model that can reproduce the observed values, our method is denoted as supervised. As we have a clearly defined objective, namely reproducing the observed data, we can thus make an explicit evaluation of our method's performance using the ROC curve.

In contrast to our statistical approach that is data driven, many researchers employ mechanistic models. An elementary way to describe the growth of several interacting species can be done using generalized Lotka-Volterra equations (Mounier et al., 2008). Trying to explain how different species keep each other in balance also resulted in establishing general insights into microbial biodiversity (Reichenbach et al., 2007). More detailed models for bacterial interaction are based on metabolic flux balance analysis, in which interaction is assumed to be determined by potential fluxes of exchanged metabolites. Constraint-based flux balance analysis was used to predict the composition of different nutritional media that induced mutualism, antagonism and neutralism for seven model species (Klitgord and Segrè, 2010). This approach was performed with 6,903 bacterial combinations, indicating that these methods can also be applied in more large-scale settings (Freilich et al., 2011). An important challenge for modeling microbial interactions consists of combining such mechanistic approaches with statistical methods as ours to harness both prior knowledge as well as information based on acquired data.

Although we only provide a proof-of-principle with a rather small data set, these tools could potentially become stronger by expanding the dataset in two ways. Firstly, by using larger data sets containing more methanotrophs and heterotrophs, but which may contain a

larger fraction of unobserved values. Since methods used in collaborative filtering are designed for dealing with very large sparse matrices, one can expect that these methods can cope with such a setting (Su and Khoshgoftaar, 2009). Secondly, the data set could be enriched by providing a suitable feature description of the different microorganisms. Potential features could be the set of metabolic genes in the genome of the organism or some physiological or environmental properties. At this moment our PPCA model is based only on implicit latent features that characterize the structure of the interaction matrix, but an extension to using visible features is possible. By incorporating these features, one could use classification or regression methods to predict missing growth parameters. The success of the approach would of course mainly depend on the biological relevance of the used features.

Conclusions

In conclusion, the growth of 225 pairwise co-cultures of methanotrophs with heterotrophs could be rapidly evaluated in a miniaturized setup by automatic extraction of growth parameters. As such, strain-specific beneficial or harmful interactions by co-cultivation compared with growth of the methanotrophs in pure culture were discovered, the mechanisms of which can be explored in future experiments. Furthermore, it was shown that predicting interactions of co-cultures that were not measured based on data of co-cultures of the same methanotrophs and heterotrophs is possible, thus providing the microbiologist with a tool to reduce the amount of data that needs to be generated when conducting large studies.

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REFERENCES

Bowman, J. (2006) The Methanotrophs - The Families *Methylococcaceae* and *Methylocystaceae*. Chapter 3.1.14. In *The Prokaryotes, third ed.* Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E. (eds), pp. 266-289.

Cavanaugh, C.M., Levering, P.R., Maki, J.S., Mitchell, R.,Lidstrom, M.E., 1987. Symbiosis of methylotrophic bacteria and deep-sea mussels. Nature 325, 346-348.

Chaffron, S., Rehrauer, H., Pernthaler, J.,von Mering, C., 2010. A global network of coexisting microbes from environmental and whole-genome sequence data. Genome research 20, 947-959.

Childress, J.J., Fisher, C.R., Brooks, J.M., Kennicutt, M.C., Bidigare, R.,Anderson, A.E., 1986. A methanotrophic marine molluscan (*Bivalvia, Mytilidae*) symbiosis - Mussels fueled by gas. Science 233, 1306-1308.

Curtis, T.P., Head, I.M.,Graham, D.W., 2003. Theoretical ecology for engineering biology. Environmental Science and Technology 37, 64A-70A.

De Smet, R.,Marchal, K., 2010. Advantages and limitations of current network inference methods. Nature reviews. Microbiology 8, 717-729.

Dumont, M.G., Pommerenke, B., Casper, P.,Conrad, R., 2011. DNA-, rRNA- and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment. Environ Microbiol 13, 1153-1167.

Dunham, M.J., 2007. Synthetic ecology: a model system for cooperation. Proceedings of the National Academy of Science 104, 1741-1742.

Faust, K.,Raes, J., 2012. Microbial interactions: from networks to models. Nat Rev Microbiol 10, 538-550.

Fawcett, T., 2004. ROC graphs: notes and practical considerations for researchers. Machine Learning, 1-38.

Freilich, S., Zarecki, R., Eilam, O., Segal, E.S., Henry, C.S., Kupiec, M. et al., 2011. Competitive and cooperative metabolic interactions in bacterial communities. Nature communications 2, 589.

Fuhrman, J.a., 2009. Microbial community structure and its functional implications. Nature 459, 193-199.

Gönen, M., 2012. Predicting drug-target interactions from chemical and genomic kernels using Bayesian matrix factorization. Bioinformatics 28, 2304-2310.

Hanson, R.S.,Hanson, T.E., 1996. Methanotrophic bacteria. Microbiol. Rev 60, 439-471.

Hoefman, S., van der Ha, D., De Vos, P., Boon, N.,Heylen, K., 2012. Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria. Microb. Biotechnol 5, 368-378.

Hrsak, D.,Begonja, A., 1998. Growth characteristics and metabolic activities of the methanotrophic-heterotrophic groundwater community. J Appl Microbiol 85, 448-456.

Hrsak, D.,Begonja, A., 2000. Possible interactions within a methanotrophic-heterotrophic groundwater community able to transform linear alkylbenzenesulfonates. Appl Environ Microbiol 66, 4433-4439.

Hu, Y.F., Koren, Y.,Volinsky, C. (2008) Collaborative filtering for implicit feedback datasets. In *ICDM 2008: Eighth IEEE International Conference on Data Mining, Proceedings*. Gunopulos, D., Turini, F., Zaniolo, C., Ramakrishnan, N., and Wu, X.D. (eds), pp. 263-272.

Hutchens, E., Radajewski, S., Dumont, M.G., McDonald, I.R.,Murrell, J.C., 2004. Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. Environ Microbiol 6, 111-120.

Iguchi, H., Yurimoto, H.,Sakai, Y., 2011. Stimulation of Methanotrophic Growth in Cocultures by Cobalamin Excreted by Rhizobia. Appl Environ Microbiol 77, 8509-8515.

Jiang, X., Langille, M.G.I., Neches, R.Y., Elliot, M., Levin, S.A., Eisen, J.A. et al., 2012. Functional biogeography of ocean microbes revealed through non-negative matrix factorization. PloS ONE 7, e43866.

King, G.M., 1994. Associations of methanotrophs with the roots and rhizomes of aquatic vegetation. Appl Environ Microbiol 60, 3220-3227.

Kip, N., van Winden, J.F., Pan, Y., Bodrossy, L., Reichart, G.J., Smolders, A.J.P. et al., 2010. Global prevalence of methane oxidation by symbiotic bacteria in peat-moss ecosystems. Nature Geoscience 3, 617-621.

Klitgord, N.,Segrè, D., 2010. Environments that induce synthetic microbial ecosystems. PLoS computational biology 6.

Michener, W.K.,Jones, M.B., 2012. Ecoinformatics: supporting ecology as a data-intensive science. Trends in ecology & evolution 27, 85-93.

Modin, O., Fukushi, K.,Yamamoto, K., 2007. Denitrification with methane as external carbon source. Water Res 41, 2726-2738.

Mounier, J., Monnet, C., Vallaeys, T., Arditi, R., Sarthou, A.-S., Hélias, A.,Irlinger, F., 2008. Microbial interactions within a cheese microbial community. Appl Environ Microbiol 74, 172-181.

Murase, J.,Frenzel, P., 2007. A methane-driven microbial food web in a wetland rice soil. Environmental Microbiology 9, 3025-3034.

Nishiyama, a., Tokihiro, T., Badoual, M.,Grammaticos, B., 2010. Modelling the morphology of migrating bacterial colonies. Physica D: Nonlinear Phenomena 239, 1573-1580.

Overbeek, R., Begley, T., Butler, R.M., Choudhuri, J.V., Chuang, H.-Y., Cohoon, M. et al., 2005. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic acids research 33, 5691-5702.

Park, J.M., Kim, T.Y.,Lee, S.Y., 2009. Constraints-based genome-scale metabolic simulation for systems metabolic engineering. Biotechnol Adv 27, 979-988.

Patil, K.R., Akesson, M.,Nielsen, J., 2004. Use of genome-scale microbial models for metabolic engineering. Curr Opin Biotechnol 15, 64-69.

Petersen, J.M.,Dubilier, N., 2009. Methanotrophic symbioses in marine invertebrates. Environmental Microbiology Reports 1, 319-335.

Picioreanu, C., van Loosdrecht, M.C.,Heijnen, J.J., 1998. A new combined differential-discrete cellular automaton approach for biofilm modeling: application for growth in gel beads. Biotechnol Bioeng 57, 718-731.

Qiu, J.,Noble, W.S., 2008. Predicting co-complexed protein pairs from heterogeneous data. PLoS computational biology 4, e1000054.

Qiu, Q., Conrad, R.,Lu, Y., 2009. Cross-feeding of methane carbon among bacteria on rice roots revealed by DNA-stable isotope probing. Environmental Microbiology Reports 1, 355–361.

Raghoebarsing, A.A., Smolders, A.J.P., Schmid, M.C., Rijpstra, W.I.C., Wolters-Arts, M., Derksen, J. et al., 2005. Methanotrophic symbionts provide carbon for photosynthesis in peat bogs. Nature 436, 1153-1156.

Read, S., Marzorati, M., Guimaraes, B.C.,Boon, N., 2011. Microbial Resource Management revisited: successful parameters and new concepts. Appl Microbiol Biotechnol 90, 861-871.

Reshef, D.N., Reshef, Y.a., Finucane, H.K., Grossman, S.R., McVean, G., Turnbaugh, P.J. et al., 2011. Detecting novel associations in large data sets. Science 334, 1518-1524.

Stacklies, W., Redestig, H., Scholz, M., Walther, D.,Selbig, J., 2007. pcaMethods--a bioconductor package providing PCA methods for incomplete data. Bioinformatics 23, 1164-1167.

Su, X.,Khoshgoftaar, T.M., 2009. A Survey of Collaborative Filtering Techniques. Advances in Artificial Intelligence 2009, 1-19.

Tipping, M.,Bishop, C., 1997. Probabilistic principal component analysis. Journal of the Royal Statistical Society 3, 611-622.

Tringe, S.G., von Mering, C., Kobayashi, A., Salamov, A.A., Chen, K., Chang, H.W. et al., 2005. Comparative metagenomics of microbial communities. Science 308, 554-557.

Tseng, Y.Y., Dundas, J.,Liang, J., 2009. Predicting protein function and binding profile via matching of local evolutionary and geometric surface patterns. J Mol Biol 387, 451-464.

van der Ha, D., Bundervoet, B., Verstraete, W.,Boon, N., 2011. A sustainable, carbon neutral methane oxidation by a partnership of methane oxidizing communities and microalgae. Water Res 45, 2845-2854.

van der Ha, D., Vanwonterghem, I., Hoefman, S., De Vos, P.,Boon, N., 2012. Selection of associated heterotrophs by methane-oxidizing bacteria at different copper concentrations. Antonie Leeuwenhoek 103, 527-537.

Vert, J.-P., Qiu, J.,Noble, W.S., 2007. A new pairwise kernel for biological network inference with support vector machines. BMC Bioinformatics 8.

Chapter VI

Methyloparacoccus murrellii, a novel methanotroph isolated from pond water in South Africa and Japan

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Author Contributions:

SH performed the experiments, analyzed the data and wrote the paper. YS provided strain OS501. PDV, KH, PV, NB, DvdH, YS, IH, YH commented on the manuscript.

Ongoing:

The deposit of the type strain in a second culture collection is ongoing.

SUMMARY

Two novel methanotrophic strains, R-49797^T and OS501, were isolated from pond water in South Africa and in Japan, respectively. Strains R-49797^T and OS501 share a 16S rRNA gene sequence similarity of 99.7%. Cells of both strains are gram-negative, non-motile cocci with a diplococcoid tendency. Colonies and liquid cultures appear creamy to white. Strain R-49797^T displays an intracytoplasmic membrane system typical to gammaproteobacterial methanotrophs. Both strains possess a particulate methane monooxygenase (pMMO). Soluble methane monoooxygenase (sMMO) activity and the *mmoX* gene encoding for sMMO were not detected. Methane and methanol are utilized as sole carbon source. Strains were negative for nitrogen fixation and the *nifH* gene encoding for nitrogenase was not detected. The strains grow optimally at 25-33°C (range 20-37°C) and at pH 6.3-6.8 (range 5.8-9.0). The strains do not support growth in media supplemented with 1% (w/v) NaCl. For both strains, the two major fatty acids are $C_{16:1}$w7c and $C_{16:0}$ and the DNA G+C content is 65.6%. The isolates belong to the family *Methylococcaceae* of the *Gammaproteobacteria* and cluster most closely among the genera *Methylocaldum*, *Methylococcus* and *Methylogaea*, with a 16S rRNA gene similarity of 94.2% between R-49797^T and its closest related type strain (*Methylocaldum gracile* VKM 14L^T).

Based on the low 16S rRNA sequence similarities towards its nearest phylogenetic neighbor genera, the formation of a separate lineage based on 16S rRNA and *pmoA* phylogenetic analysis, and the unique combination of phenotypic characteristics of the two isolated strains compared with the genera *Methylocaldum*, *Methylococcus* and *Methylogaea*, we propose to classify these strains as a novel genus and species, *Methyloparacoccus murrellii*, within the *Methylococcaceae* family with strain R-49797^T (= LMG 27482^T) as type strain.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *pmoA* and *mxaF* gene sequences of strain R-49797^T are HF558990, HF954363 and HF954364, respectively; and of strain OS501 are AB636299, AB636304 and HF954365, respectively.

INTRODUCTION

Aerobic methanotrophic bacteria are defined by their ability to oxidize methane and utilize it as sole carbon and energy source using a methane monooxygenase (MMO) of which a soluble (sMMO) and a particulate (pMMO) form have been described (Semrau et al., 2010). These methanotrophs are ubiquitous in nature and have been found in different habitats where oxygen and methane are readily available, such as in soils, sediments, wetlands, fresh water and marine environments (Hanson and Hanson, 1996; Bowman, 2006). Although non-proteobacterial methanotrophs have been described (Op den Camp et al., 2009; Ettwig et al., 2010), the methanotrophs that belong to the *Gammaproteobacteria* or the *Alphaproteobacteria* have been studied much more intensively (Bowman et al., 1993). Twelve methanotrophic genera have been formally named and described within the family *Methylococcaceae* of the *Gammaproteobacteria*: the genera *Methylococcus, Methylocaldum, Methylogaea, Methylothermus, Methylohalobius, Methylosoma, Methylovulum, Methylosphaera, Methylobacter, Methylomicrobium, Methylosarcina* and *Methylomonas* (Geymonat et al., 2011). Within the *Alphaproteobacteria*, the genera *Methylosinus* and *Methylocystis* in the family *Methylocystaceae* and the genera *Methylocella, Methyloferula* and *Methylocapsa* in the family *Beijerinckiaceae* have been named and described (Dedysh, 2009; Vorobev et al., 2010).

Recently, the genus *Methylogaea* was named through the formal characterization of a non-thermotolerant strain within the *Methylococcus-Methylocaldum* clade which typically harbors thermotolerant members (Geymonat et al., 2011). Here, we report the characterization of two novel isolates within this *Methylococcus-Methylocaldum-Methylogaea* clade that (i) share similar cell-shape characteristics with *Methylococcus* strains, (ii) are genotypically most closely related to *Methylocaldum* strains and (iii) are non-thermotolerant like the type strain of *Methylogaea*. Furthermore, the *nifH* gene which is commonly present in members of this clade could not be detected for the two novel isolates.

EXPERIMENTAL PROCEDURES

Strain R-49797^T (= LMG 27482^T) was isolated from a facultative waste stabilization pond in South Africa (Chapter II) by miniaturized extinction culturing (Hoefman et al., 2012a) in diluted Ammonium Mineral Salts (dAMS) medium, which was a modification of diluted Nitrate Mineral Salts medium [dNMS, (Hoefman et al., 2012a)] amended with 2 mM NH_4Cl as sole nitrogen source, 4 mM phosphate buffer and 10 µM $CuSO_4$. Strain OS501 (= LMG 27483) was isolated from pond water from Inukai Pond in Suita City, Osaka, Japan (34º49'9"N 135º31'42"E) by plating of enrichment cultures (NMS medium, 20% CH_4 in air, 28°C, shaking) on NMS agar (ATCC 1306 medium) and incubation in gastight jars under a CH_4:air atmosphere.

In the present study, both strains were routinely cultured at 28°C in dNMS (liquid or solidified with agar) with a modified copper concentration (10 µM Cu^{2+}) under a CH_4:air atmosphere in gastight flasks (20% methane in air) or jars (CH_4:air, 1:1).

Cell morphology and motility were assessed by phase-contrast microscopy. Gram staining was performed, and cytochrome c oxidase and catalase activities were assessed according to Cleenwerck et al., (2002). Flagella staining was performed according to Heimbrook et al., (1989). The presence of *Azotobacter*-type cysts was evaluated by the staining procedure of Vela & Wyss (1964) on cultures grown to stationary phase. Heat resistance and desiccation resistance were assessed on turbid suspensions (OD_{600nm} 1) of cultures grown to stationary phase. Suspensions were incubated for 15 minutes at 85°C, rapidly cooled on ice and plated onto dNMS agar and incubated at 28°C for heat resistance assessment. Drops of suspensions were air dried on sterile glass slides and kept at 28°C for three weeks after which desiccation resistance of the cultures was assessed by rehydration and incubation in dNMS (both on agar plates and in liquid cultures). Transmission electron microscopy was performed according to Spieck and Lipski (2011).

The ability of the strains to oxidize methane at 1%, 0.1% and 0.01% CH_4 concentrations was assessed in liquid cultures by monitoring the CH_4, O_2 and CO_2 levels with a Compact GC (Global Analyzer Solutions, Belgium), equipped with one channel connected to a flame ionization detector and two channels connected to a thermal conductivity detector. Utilization of the following carbon sources (0.1%) was evaluated by monitoring of growth (OD_{600nm} levels) in liquid dNMS without methane added to the headspace: methanol, formate, urea, methylamine, dimethylamine, dimethyl carbonate, formamide, acetate, pyruvate, succinate, malate, ethanol, citrate, glycine, D-xylose, maltose and glucose.

Liquid dNMS without addition of carbon was included as a negative control. Utilization of the following nitrogen sources at a concentration of 2 mM was evaluated in liquid dMS (dNMS without nitrogen source) with methane added to the headspace by monitoring of growth (OD_{600nm} levels): nitrate, nitrite, ammonium (NH_4Cl and $(NH_4)_2SO_4$), formamide, methylamine, glycine, hydroxylamine, urea, L-serine, L-proline, L-aspartate, L-leucine, L-cysteine, L-arginine and yeast extract (0.1% w/v yeast extract, instead of 2 mM). Liquid dMS without addition of nitrogen was included as a negative control. The ability to fix nitrogen was assessed by evaluating growth (OD_{600nm} levels over time) in nitrogen-free medium (liquid dMS) with 20% CH_4 added to air at normal (21%) and ten-fold reduced (2.1%) oxygen levels. Growth of strains R-49797^T and OS501 in nitrogen-free medium was compared with (i) growth of a positive control, *Methylomonas methanica* NCIMB 11130^T, in nitrogen-free medium and (ii) growth of strains R-49797^T and OS501 in the same medium amended with 10 mM KNO_3 or 10 mM NH_4Cl. Salt tolerance [tested at 0.23% (40 mM), 0.58% (100 mM), 1%, 5% and 10% NaCl spiked in dNMS], the pH optimum and range (tested at pH 4, 5, 5.8, 6.3, 6.8, 7.3, 7.8, 9, 10) and the temperature optimum and range (tested at 5°C, 15°C, 20°C, 25°C, 28°C, 33°C, 37°C, 45°C and 52°C) were assessed by monitoring of growth (OD_{600nm}) in liquid dNMS.

Nitrate reduction was evaluated by growth in liquid dNMS amended with 10 mM KNO_3 and monitoring of nitrite production by colorimetry (Griess, 1879) and N_2O production by gas chromatography (Compact GC, see above). Soluble methane monooxygenase activity was tested using a modified version of the naphthalene oxidation assay (Hoefman et al., 2012a). Cryopreservation of strains R-49797^T and OS501 with 5% DMSO as cryoprotectant at -80°C and in liquid nitrogen was performed as described by Hoefman et al., (2012b).

DNA was extracted and 16S rRNA gene sequences were generated as described in Hoefman et al., (2012a). Sequences were assembled with BioNumerics 5.1 (Applied Maths, Belgium). Preliminary genus identification was obtained by query in the "Classifier" program of Ribosomal Database Project II (Cole et al., 2005). Sequences of strains R-49797^T and OS501 were aligned among a selection of methanotrophic type strains using the integrated aligner of ARB (Ludwig et al., 2004). Based on the 16S rRNA gene alignment, a Maximum Likelihood (ML) phylogenetic tree was constructed using RAxML v7.3.5 based on the General Time Reversible substitution model with gamma-distributed rate variation (GTR+Gamma). Bootstrap analysis based on 1000 replicates was performed. The best scoring ML tree was exported in newick format and analyzed using

the MEGA 5 software (Tamura et al., 2011).

Amplification of the *pmoA* gene encoding the 27 kDa peptide of particulate methane monooxygenase (pMMO) was carried out with the A189f/mb661r primer set according to Costello and Lidstrom (1999). Amplification of the *mmoX* gene encoding the α-subunit of the hydroxylase component of soluble methane monooxygenase (sMMO) was tested with primer sets 534f /1393r (Horz et al., 2001), mmoXA/mmoXB (Auman et al., 2000) and mmoX206f/mmoX886r (Hutchens et al., 2004). *pxmA* [encoding a sequence-divergent particulate monooxygenase (pXMO)], *nifH* (encoding the highly conserved Fe protein of nitrogenase) and *mxaF* (encoding the large subunit of methanol dehydrogenase) gene amplification was performed using the primer sets pmoA189F/pxmA634R (Tavormina et al., 2008), F1/nifH439R (De Meyer et al., 2011) and f1003/r1561 (McDonald et al., 1995), respectively. For detection of each of the above mentioned genes, the PCR mix and temperature program of the original studies were applied; the outcome of each test was verified using a set of methanotrophic type strains as positive and negative controls.

When functional gene amplification was positive, sequences were generated as explained for 16S rRNA gene analysis and subsequently placed in the correct reading frame as determined by Transeq (www.ebi.ac.uk/tools/emboss/transeq) and protein translation analysis using pBLAST (Altschul et al., 1990). Sequences were aligned using the MEGA 5 software based on translated protein sequences and DNA-based ML phylogenetic trees were constructed using RAxML as explained above.

DNA was extracted and purified as described by Logan et al., (2000) to determine the G+C content by HPLC (Mesbah et al., 1989). Phospholipid fatty acid analysis was carried out as described by Vancanneyt et al., (1996). Fatty acids were identified using the Microbial Identification System (MIDI) software (Sherlock Microbial Identification Systems, version 3.0; Library TSBA 50, version 5.0).

RESULTS & DISCUSSION

One-week old colonies of strains R-49797^T (= LMG 27482^T) and OS501 (= LMG 27483) are white to creamy colored and round. Two-week old colonies of strain OS501, but not of strain R-49797^T, were still mostly white but displayed a distinct green shine. Cells of both strains were gram-negative, non-motile cocci (diameter 0.8 µm to 1.5 µm) which displayed a diplococcoid tendency (Figure VI-1 a), similar to what was observed by Foster and Davis (1966) in *Methylococcus capsulatus* cultures. Cells exhibited catalase and cytochrome c oxidase activities. *Azotobacter*-type cysts were not observed. Cells were neither heat resistant nor desiccation resistant. Transmission electron micrographs showed cells with intracytoplasmic membrane systems appearing as bundles of vesicular disks (Figure VI-1 b,c), a typical feature of the gammaproteobacterial methanotrophs (Hanson and Hanson, 1996). Similarly as was observed for the type strain of *Methylogaea oryzae* (Geymonat et al., 2011), cells store two types of compounds displayed as light and dark entities (Figure VI-1 b). The inclusions of low electron density probably represent poly-β-hydroxybutyrate granules, known to be produced by several methanotrophs (Whittenbury et al., 1970; Heyer et al., 2005; Helm et al., 2006). Strains R-49797^T and OS501 can be cryopreserved successfully at -80°C and in liquid nitrogen using 5% DMSO as cryoprotectant.

Strains R-49797^T and OS501 only utilized methane or methanol as sole carbon and energy source. Medium amended with 0.1% methanol did not support growth, however, strains grew on methanol fumes as sole carbon source when incubated on dNMS plates with a few drops of methanol placed on the inside lid of the petri dish. Although growth of OS501 could be improved by the addition of cobalamin during cultivation (Iguchi et al., 2011), this characteristic appeared to be strain-specific since such improved growth was not observed for R-49797^T with 0.1 µg L^{-1} vitamin B12 amended to the medium.

Strains R-49797^T and OS501 showed nearly complete methane oxidation activity after one week incubation with 1% and 0.1% methane amended to the headspace. At 0.01%, methane oxidation activity was reduced to 81% (± 0.2%, n=2) for R-49797^T and 56% (± 7.7%, n=2) for OS501 after three weeks of incubation. In contrast, Knief and Dunfield (2005) found that the strains *Methylococcus capsulatus* Bath and *Methylocaldum* sp. E10a, members of the two most closely related taxa of strains R-49797^T and OS501 based on 16S rRNA gene sequence analysis (see below), lost their methane oxidation activity when incubated with 0.1% CH_4 in the headspace.

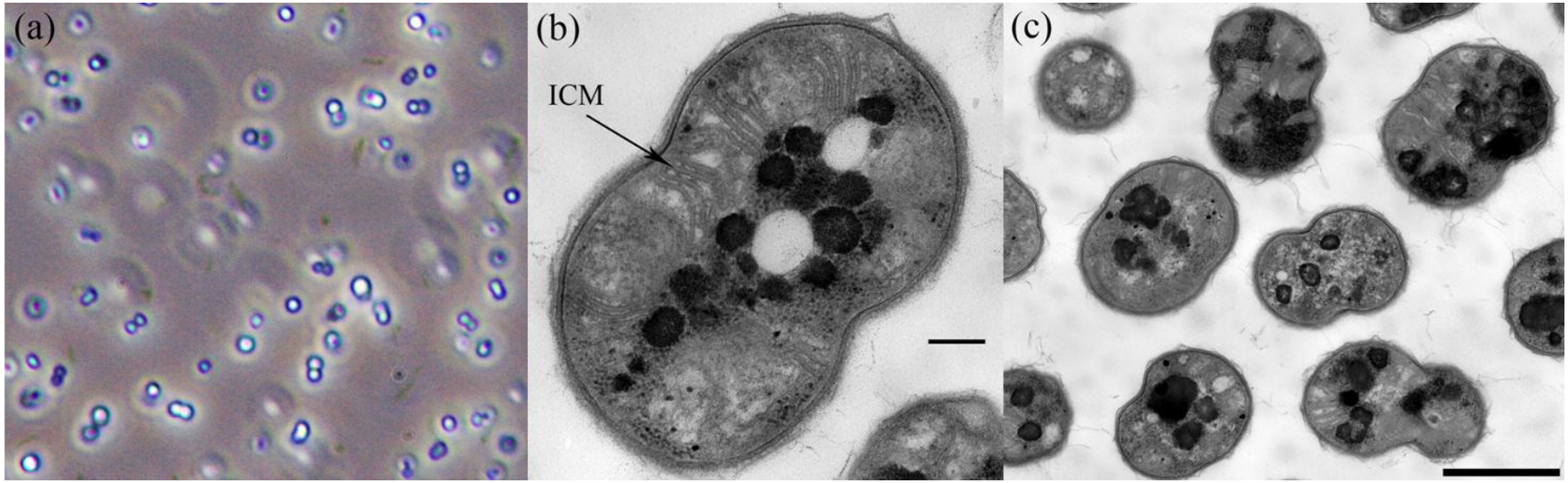

Figure VI-1: (a) Phase-contrast micrograph of actively growing cells of strain R-49797^T showing cocci with a diplococcoid tendency, similar as reported for *Methylococcus capsulatus* (Foster and Davis, 1966). (b), (c) Electron micrographs of ultrathin sections of actively growing cells of strain R-49797^T showing a cell shape resembling *Methylococcus* cultures and displaying typical gammaproteobacterial methanotrophic intracytoplasmic membrane arrangements [ICM (b)]. Two types of unidentified storage compounds are observed [dark and light circles (b)]. Bars: 0.2 µm (b) 1 µm (c).

The isolates grew with ammonium [both NH_4Cl and $(NH_4)_2SO_4$], nitrate, nitrite, urea, proline, aspartate, arginine and yeast extract as sole nitrogen source. The isolates could not grow in nitrogen-free medium at high (21%) or low (2.1%) oxygen tension and the *nifH* gene could not be amplified. The strains grew at temperatures between 20 and 37°C (optimum: 25-33°C), media with pH between 5.8 to 9 (optimum 6.3-6.8) and salt addition up to 100 mM NaCl. Nitrate reduction of both strains was confirmed by the formation of nitrite and N_2O following incubation with 10 mM KNO_3 as sole nitrogen source. The strains were negative for sMMO activity and the *mmoX* gene could not be amplified. The *pmoA* gene was present (Figure VI-2), while the *pxmA* gene could not be detected by PCR.

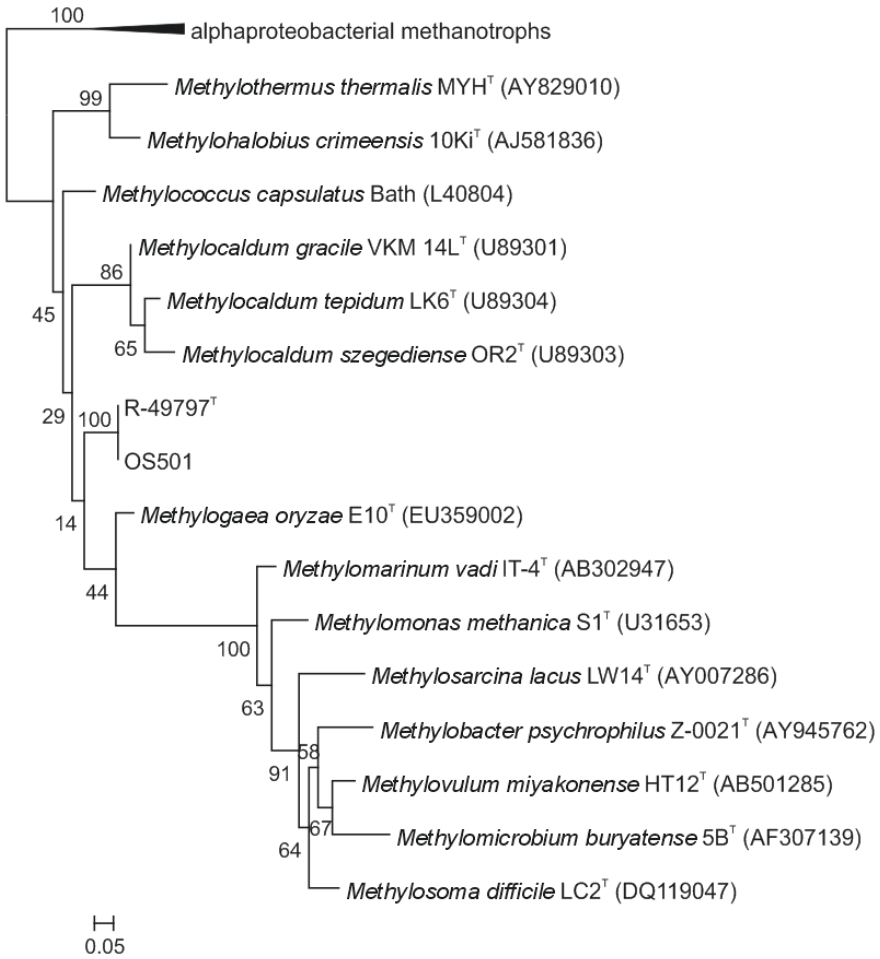

Figure VI-2: *pmoA* gene phylogenetic maximum-likelihood tree (438 nucleotide alignment; GTR+Gamma substitution model; 1000-replicate bootstrapping) of strains R-49797^T and OS501 with the available *pmoA* sequences of the type strains within *Methylococcus*, *Methylocaldum* and *Methylogaea* and one *pmoA* sequence per remaining genus within the *Methylococcaceae*. The alphaproteobacterial methanotrophic type strains *Methylocystis parvus* OBBP^T (U31651), *Methylosinus trichosporium* IMET 10561^T (AJ459001) and *Methylocapsa acidiphila* B2^T (AJ278727) were used as an outgroup. Bar: 0.05 substitutions per nucleotide position.

16S rRNA gene sequences of R-49797^T (1451 bp) and OS501 (1497 bp) were obtained. Phylogenetic analysis showed that the two strains formed a new cluster within the *Methylococcus-Methylocaldum-Methylogaea* clade (Figure VI-3). Pairwise 16S rRNA sequence comparison showed that R-49797^T had a sequence similarity of 93.8-94.2% with the type strains of the three *Methylocaldum* species (strains VKM 14L^T, OR2^T and LK6^T), 93.8% with *Methylococcus capsulatus* texas^T, 90.0% with *Methylogaea oryzae* E10^T and 99.7% with OS501. The 16S rRNA gene sequence results are supported by phylogenetic analysis of the *pmoA* gene, which confirms that strains R-49797^T and OS501 form a line of descent different from those of the other members of the *Methylococcus-Methylocaldum-Methylogaea* clade (Figure VI-2).

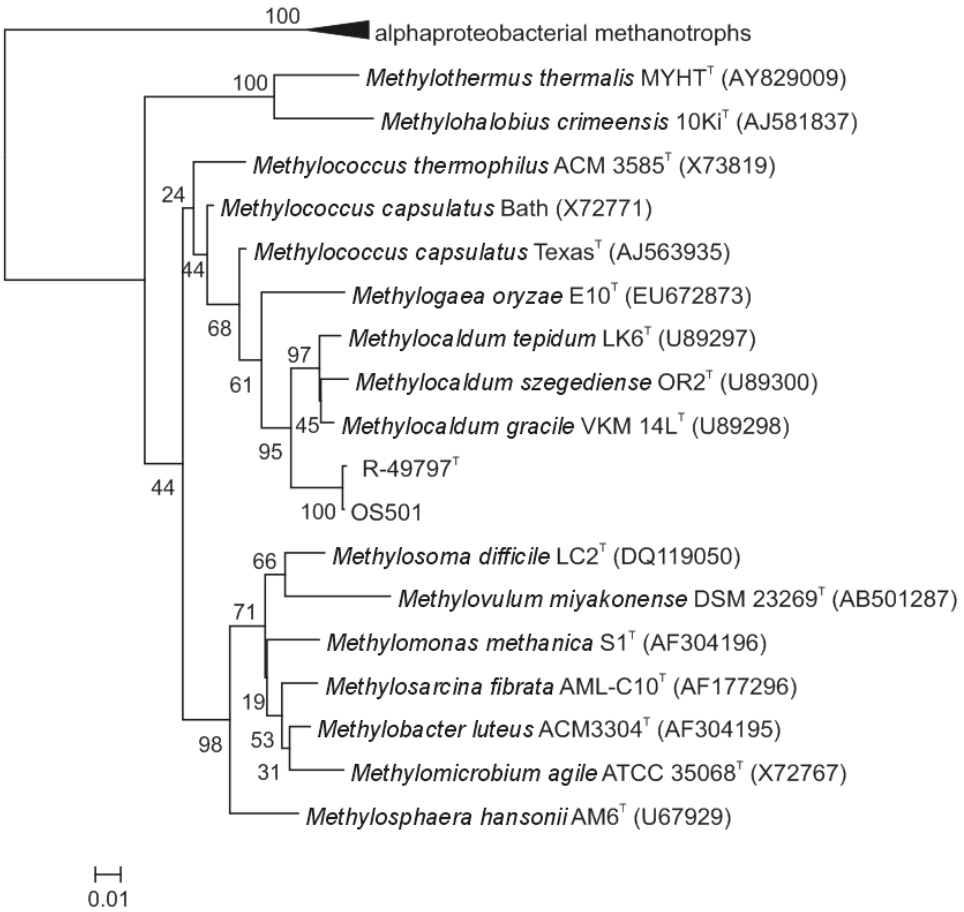

Figure VI-3: 16S rRNA gene phylogenetic maximum-likelihood tree (1223 nucleotide alignment; GTR+Gamma substitution model; 1000-replicate bootstrapping) of strains R-49797^T and OS501 with the type strains of the recognized species within *Methylococcus*, *Methylocaldum* and *Methylogaea* and the type strains of the remaining genera within the *Methylococcaceae*. The alphaproteobacterial methanotrophic type strains *Methylocystis parvus* NCIMB 11129^T (Y18945), *Methylosinus trichosporium* OB3b^T (Y18947), *Methyloferula stellata* AR4^T (FR686343), *Methylocella palustris* K^T (Y17144) and *Methylocapsa acidiphila* B2^T (AJ278726) were used as an outgroup. Bar: 0.01 substitutions per nucleotide position.

The G+C content as determined by HPLC was 65.6% for R-49797^T and OS501. Similar values are observed for other members of the *Methylococcus-Methylocaldum-Methylogaea* clade (Table VI-2). Phospholipid fatty acid analysis identified $C_{16:1}$w7c and $C_{16:0}$ as the two major fatty acids. This finding is consistent with the other members of the *Methylococcus-Methylocaldum-Methylogaea* clade, although the most dominant fatty acid of strains R-49797^T and OS501 is $C_{16:1}$w7c, while for the other members of the clade $C_{16:0}$ is more dominant than $C_{16:1}$w7c (Table VI-1).

The major morphological and physiological characteristics distinguishing strains R-49797^T and OS501 from other genera within the *Methylococcus-Methylocaldum-Methylogaea* clade are presented in Table VI-2. The cells of strains R-49797^T and OS501 are clearly different in appearance when compared to *Methylogaea* and *Methylocaldum* cells, since rod-shapes, pleomorphism and motility were absent for strains R-49797^T and OS501. The non-motile cocci most closely resemble the appearance of cells of *Methylococcus* strains. Although *Methylococcus* and *Methylocaldum* strains are thermotolerant, strains R-49797^T, OS501 and *Methylogaea oryzae* E10^T are not. Further distinctions between strains R-49797^T and OS501 and *Methylococcus* strains include the sensitivity to 1% NaCl and sensitivity to 0.1% methanol and absence of the *nifH* and *mmoX* genes resulting in the absence of activity of nitrogenase and sMMO, respectively, for strains R-49797^T and OS501.

Given the above mentioned genotypic and phenotypic differences between strains R-49797^T and OS501 and other members of the *Methylococcus-Methylocaldum-Methylogaea* clade, we propose to assign a novel genus and species to this clade, *Methyloparacoccus murrellii* gen. nov., sp. nov., with R-49797^T as type strain.

Table VI-1: Composition of cellular fatty acids distinguishing strains R-49797^T and OS501 from other genera within the *Methylococcus-Methylocaldum-Methylogaea* clade. Values are percentages of the total fatty acids. Fatty acid data of the three *Methylocaldum* type strains is not available (Bodrossy et al., 1997), instead data from Eshinimaev et al., (2004) is used to represent *Methylocaldum*.

Fatty acid	**R-49797^T & OS501**	***Methylococcus* spp.[a]**	***Methylogaea* oryzae[b]**	***Methylocaldum* sp.[c]**
$C_{12:0}$	-	NR	2.11	0-0.10
$C_{14:0}$	3.77-4.71	0.8-6.2	5.84	1.97-2.40
$C_{15:1}$w8c	0.29-0.34	NR	NR	NR
$C_{15:0}$	3.19-3.34	0-12.7	1.03	2.50-3.51
$C_{16:1}$w9c	5.14-6.45	-	7.36	NR
$C_{16:1}$w7c[d]	52.4-54.2	10.6-45.9	10.33	NR
$C_{16:1}$w5c	4.17-5.70	0-9.0	NR	-
$C_{16:1}$	-	-	-	11.9-13.3
$C_{16:0}$	23.7-24.8	33.5-56	62.05	63.7-65
$C_{16:0}$ 3-OH	2.61-2.72	NR	2.93	-
iso-$C_{16:0}$ 3-OH	-	NR	3.69	NR
$C_{17:0}$ cyclo	-	0-15.1	-	6.07-8.99

-, not detected; NR, not reported

Data was extracted from [a] Bowman et al., (1993), [b] Geymonat et al., (2011) and [c] Eshinimaev et al., (2004)

[d] This peak in the chromatogram represents $C_{16:1}$w7c and/or iso-$C_{15:0}$ 2-OH according to the MIDI system, however in this study the peak is assigned to $C_{16:1}$w7c since this fatty acid is common among the studied methanotrophs

Table VI-2: Major characteristics distinguishing strains R-49797^T and OS501 from other genera within the *Methylococcus-Methylocaldum-Methylogaea* clade.

Characteristic	**R-49797^T & OS501**	***Methylococcus* spp.**[a]	***Methylogaea oryzae***[b]	***Methylocaldum* spp.**[c]
Cell shape	Cocci	Cocci-rods	Curved rods	Rods-pleomorphic
Cell size (µm)	0.8-1.5	0.8-1.5 x 1.0-1.5	0.5-0.7 x 2.0-2.2	0.4-1.2 x 1.0-2.0
Pigmentation	White	White to brown	White	Cream to Brown
Motility	-	Variable	-	+
Chain formation	-	-	NR	Variable
Cyst formation	-	+	-	+
Temperature range (°C)	20-37	28-55	20-37	20-62
Temperature optimum (°C)	25-33	37-50	30-35	42-55
pH range	5.8-9	5.5-9.0	5-8	6-8.5
pH optimum	6.3-6.8	NR	6.5-6.8	7.1-7.2
Tolerance to 1% NaCl	-	+	-	-
nifH presence	-	+	+	+[d]
N_2 Fixation	-	+	-	NR
sMMO	-	+	-	-
Methanol 0.1%	-	+	+	-
G+C content (mol%)	65.6	59-66	63.1	57-59

+, positive result; -, negative result; NR, not reported

Data was extracted from [a] Bowman et al., (1993), [b] Geymonat et al., (2011) and [c] Bodrossy et al., (1997) and Echinimaev et al., (2004)

[d] Data on *nifH* presence of type strains of *Methylocaldum* has not been reported, however *nifH* sequences of strains reported in Echinimaev et al., (2004) are available

Description of *Methyloparacoccus* gen. nov.

Methyloparacoccus (Me.thy.lo.pa.ra.coc'cus. N.L. n. methylum (from French méthyle), the methyl group; N.L. pref. methylo-, pertaining to the methyl radical; Gr. prep. para, beside, alongside of, near, like; N.L. masc. n. coccus (from Gr. n. kokkos), a grain or berry; N.L. masc. n. *Methyloparacoccus* referring to a methyl-using organism resembling but clearly different from other methyl-using cocci). Cells are aerobic, gram-negative, non-motile, coccoid and show a diplococcoid tendency. Cells possess the typical intracytoplasmic membrane system for gammaproteobacterial methanotrophs forming bundles of membrane vesicles. Resting stages are not observed. Cells only utilize methane or methanol as sole carbon source. Methane is oxidized by pMMO; sMMO and pXMO are not present. N_2 is not fixed, and the *nifH* gene is absent. Cells are neutrophilic, mesophilic and non-thermotolerant. The most dominant cellular fatty acids are $C_{16:1}$w7c (52-54%) and $C_{16:0}$ (24-25%). The most closely related genera are *Methylocaldum*, *Methylococcus* and *Methylogaea* within the family *Methylococcocaceae* in the class *Gammaproteobacteria*. The type species is *Methyloparacoccus murrellii*.

Description of *Methyloparacoccus murrellii* sp. nov.

Methyloparacoccus murrellii (mur.rel'li.i. *murrellii* of Murrell, named in honor of the British microbiologist Colin Murrell, for his numerous contributions to expanding the knowledge on methanotroph physiology, biochemistry, diversity and molecular ecology). Displays all properties described for the genus *Methyloparacoccus*. In addition, cocci have a diameter of 0.8 to 1.5 µm. Cells still display methane oxidation activity with 0.01 % CH_4 amended to the headspace. Cells grow optimally between 25°C to 33°C at a pH between 6.3 and 6.8 and can utilize ammonium [both NH_4Cl and $(NH_4)_2SO_4$], nitrate, nitrite, urea, proline, aspartate, arginine and yeast extract as sole nitrogen source. Cells grow with 0.58% NaCl amended to the medium, but are sensitive to 1% NaCl additions. The G+C content is 65.6%. The type strain R-49797^T (= LMG 27482^T) was isolated from pond water in South Africa. Strain OS501 (=LMG 27483) was isolated from pond water in Japan.

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REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W.,Lipman, D.J., 1990. BASIC LOCAL ALIGNMENT SEARCH TOOL. J Mol Biol 215, 403-410.

Auman, A.J., Stolyar, S., Costello, A.M.,Lidstrom, M.E., 2000. Molecular characterization of methanotrophic isolates from freshwater lake sediment. Appl Environ Microbiol 66, 5259-5266.

Bodrossy, L., Holmes, E.M., Holmes, A.J., Kovacs, K.L.,Murrell, J.C., 1997. Analysis of 16S rRNA and methane monooxygenase gene sequences reveals a novel group of thermotolerant and thermophilic methanotrophs, Methylocaldum gen. nov. Arch Microbiol 168, 493-503.

Bowman, J. (2006) The Methanotrophs - The Families *Methylococcaceae* and *Methylocystaceae*. Chapter 3.1.14. *Prokaryotes* **5**: 266-289, DOI: 10.1007/0-387-30745-1_15.

Bowman, J.P., Sly, L.I., Nichols, P.D.,Hayward, A.C., 1993. Revised taxonomy of the methanotrophs – Description of *Methylobacter* Gen Nov, emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. International Journal of Systematic Bacteriology 43, 735-753.

Cleenwerck, I., Vandemeulebroecke, K., Janssens, D.,Swings, J., 2002. Re-examination of the genus Acetobacter, with descriptions of Acetobacter cerevisiae sp. nov. and Acetobacter malorum sp. nov. Int J Syst Evol Microbiol 52, 1551-1558.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M. et al., 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res 33, D294-D296.

Costello, A.M.,Lidstrom, M.E., 1999. Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. Appl Environ Microbiol 65, 5066-5074.

De Meyer, S.E., van Hoorde, K., Vekeman, B., Braeckman, T.,Willems, A., 2011. Genetic diversity of rhizobia associated with indigenous legumes in different regions of Flanders (Belgium). Soil Biol Biochem 43, 2384-2396.

Dedysh, S.N., 2009. Exploring methanotroph diversity in acidic northern wetlands: Molecular and cultivation-based studies. Microbiology 78, 655-669.

Eshinimaev, B.T., Medvedkova, K.A., Khmelenina, V.N., Suzina, N.E., Osipov, G.A., Lysenko, A.M.,Trotsenko, Y.A., 2004. [New thermophilic methanotrophs of the genus Methylocaldum] Translated from Russian. Mikrobiologiia 73, 530-539.

Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M.M. et al.,

2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature 464, 543-+.

Foster, J.W.,Davis, R.H., 1966. A methane-dependent coccus, with notes on classification and nomenclature of obligate, methane-utilizing bacteria. J Bacteriol 91, 1924-1931.

Geymonat, E., Ferrando, L.,Tarlera, S.E., 2011. Methylogaea oryzae gen. nov., sp nov., a mesophilic methanotroph isolated from a rice paddy field. Int J Syst Evol Microbiol 61, 2568-2572.

Griess, P., 1879. Bemerkungen zu der abhandlung der H.H. Weselsky und Benedikt "Ueber einige azoverbindungen." Chem. Ber 12, 426-428.

Hanson, R.S.,Hanson, T.E., 1996. Methanotrophic bacteria. Microbiol. Rev 60, 439-471.

Heimbrook, M.E., Wang, W.L.L.,Campbell, G., 1989. STAINING BACTERIAL FLAGELLA EASILY. Journal of Clinical Microbiology 27, 2612-2615.

Helm, J., Wendlandt, K.D., Rogge, G.,Kappelmeyer, U., 2006. Characterizing a stable methane-utilizing mixed culture used in the synthesis of a high-quality biopolymer in an open system. J Appl Microbiol 101, 387-395.

Heyer, J., Berger, U., Hardt, M.,Dunfield, P.F., 2005. *Methylohalobius crimeensis* gen. nov., sp. nov., a moderately halophilic, methanotrophic bacterium isolated from hypersaline lakes of Crimea. Int J Syst Evol Microbiol 55, 1817-1826.

Hoefman, S., van der Ha, D., De Vos, P., Boon, N.,Heylen, K., 2012a. Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria. Microb. Biotechnol 5, 368-378.

Hoefman, S., Van Hoorde, K., Boon, N., Vandamme, P., De Vos, P.,Heylen, K., 2012b. Survival or revival: long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria. PLoS One 7, e34196.

Horz, H.P., Yimga, M.T.,Liesack, W., 2001. Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of pmoA, mmoX, mxaF, and 16S rRNA and ribosomal DNA, including pmoA-based terminal restriction fragment length polymorphism profiling. Appl Environ Microbiol 67, 4177-4185.

Hutchens, E., Radajewski, S., Dumont, M.G., McDonald, I.R.,Murrell, J.C., 2004. Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. Environ Microbiol 6, 111-120.

Iguchi, H., Yurimoto, H.,Sakai, Y., 2011. Stimulation of Methanotrophic Growth in Cocultures by Cobalamin Excreted by Rhizobia. Appl Environ Microbiol 77, 8509-8515.

Knief, C.,Dunfield, P.F., 2005. Response and adaptation of different methanotrophic bacteria to low methane mixing ratios. Environ Microbiol 7, 1307-1317.

Logan, N.A., Lebbe, L., Hoste, B., Goris, J., Forsyth, G., Heyndrickx, M. et al., 2000. Aerobic endospore-forming bacteria from geothermal environments in northern Victoria Land, Antarctica, and Candlemas Island, South Sandwich archipelago, with the proposal of Bacillus fumarioli sp nov. Int J Syst Evol Microbiol 50, 1741-1753.

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

McDonald, I.R., Kenna, E.M.,Murrell, J.C., 1995. Detection of methanotrophic bacteria in environmental samples with the PCR. Appl Environ Microbiol 61, 116-121.

Mesbah, M., Premachandran, U.,Whitman, W.B., 1989. Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. International Journal of Systematic Bacteriology 39, 159-167.

Op den Camp, H.J.M., Islam, T., Stott, M.B., Harhangi, H.R., Hynes, A., Schouten, S. et al., 2009. Environmental, genomic and taxonomic perspectives on methanotrophic Verrucomicrobia. Environmental Microbiology Reports 1, 293-306.

Semrau, J.D., DiSpirito, A.A.,Yoon, S., 2010. Methanotrophs and copper. FEMS Microbiol Rev 34, 496-531.

Spieck, E.,Lipski, A. (2011) Cultivation, growth physiology, and chemotaxonomy of nitrite-oxidizing bacteria. In *Methods in Enzymology: Research on Nitrification and Related Processes, Vol 486, Part A*. Klotz, M.G. (ed), pp. 109-130.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M.,Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28, 2731-2739.

Tavormina, P.L., Ussler, W.,Orphan, V.J., 2008. Planktonic and sediment-associated aerobic methanotrophs in two seep systems along the North American margin. Appl Environ Microbiol 74, 3985-3995.

Vancanneyt, M., Witt, S., Abraham, W.R., Kersters, K.,Fredrickson, H.L., 1996. Fatty acid content in whole-cell hydrolysates and phospholipid fractions of pseudomonads: A taxonomic evaluation. Syst Appl Microbiol 19, 528-540.

Vela, G.R.,Wyss, O., 1964. Improved stain for visualization of *Azotobacter* encystment. J Bacteriol 87, 476-&.

Vorobev, A.V., Baani, M., Doronina, N.V., Brady, A.L., Liesack, W., Dunfield, P.F.,Dedysh, S.N., 2010. Methyloferula stellata gen. nov., sp. nov., an acidophilic, obligately methanotrophic bacterium possessing only a soluble methane monooxygenase. Int J Syst Evol Microbiol.

Whittenbury, R., Phillips, K.C.,Wilkinson, J.F., 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61, 205-217.

Chapter VII

Methylomonas lenta, a novel methanotroph isolated from manure and a denitrification tank in Belgium

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Redrafted from:

Hoefman S, Heylen K and De Vos P. *Methylomonas lenta* sp. nov., a novel methanotroph isolated from manure and a denitrification tank in Belgium. Submission pending.

Author Contributions:

SH performed the experiments, analyzed the data and wrote the paper. PDV and KH commented on the manuscript.

Ongoing:

The deposit of the type strain in a second culture collection is ongoing.

SUMMARY

Two methanotrophic strains, R-45377^T and R-45370, were isolated from a covered but aerobic slurry pit of a cow stable (Melle, Belgium) and from the top layer of a denitrification tank of a wastewater treatment plant (Ossemeersen, Gent, Belgium), respectively. The strains show 99.9% 16S rRNA gene sequence similarity. Cells are gram-negative motile rods containing typical type I methanotroph intracytoplasmic membranes. Colonies and liquid cultures appear white to pale-pink for R-45377^T and pale-pink for R-45370. The *pmoA* gene encoding for particulate methane monooxygenase (pMMO) is present. Soluble methane monoooxygenase (sMMO) activity and the presence of the *mmoX* gene encoding for sMMO could not demonstrated. The *pxmA* gene encoding a sequence-divergent particulate monooxygenase (pXMO) was not detected. Methane and methanol are utilized as sole carbon source. The *nifH* gene encoding for nitrogenase is present, but strains did not grow in nitrogen-free medium under a normal and reduced oxygen atmosphere. The strains grow optimally at 20°C (range 15-28°C) and at pH 6.8-7.3 (range 6.3-7.8). They grow in media supplemented with up to 200 mM NaCl. The major cellular fatty acids are $C_{16:1}$w8c, $C_{16:1}$w5c, $C_{16:1}$w7c, $C_{14:0}$, $C_{15:0}$ and $C_{16:0}$. The DNA G+C content varied between 46.9-47.2%. 16S rRNA gene and *pmoA* based phylogenetic analysis showed that the isolates cluster among members of *Methylomonas* within the *Gammaproteobacteria*, with pairwise 16S rRNA gene sequence similarities of 97.5% and 97.2% between R-45377^T and its closest related type strains *M. scandinavica* SR5^T and *M. paludis* MG30^T, respectively.

Based on low 16S rRNA sequence similarities towards the nearest neighbor species, the formation of a separate lineage based on 16S rRNA and *pmoA* phylogenetic analysis, and the unique phenotypic characteristics compared to the existing *Methylomonas* species, we propose to classify these strains in a novel species, *Methylomonas lenta*, with R-45377^T (= LMG 26260^T) as type strain.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *pmoA* and *nifH* gene sequences of strain R-45377^T are FR798962, HF954359 and HF954360, respectively; and of strain R-45370 are FR798956, HF954361 and HF954362, respectively.

INTRODUCTION

Aerobic methanotrophic bacteria are grouped by their ability to oxidize the greenhouse gas methane and utilize it as sole carbon and energy source by a particulate or soluble methane monooxygenase (pMMO or sMMO) which converts methane to methanol in the first step of methane oxidation (Semrau et al., 2010). The first isolation of a methanotroph in pure culture, *Bacillus methanicus,* later renamed to *Methylomonas methanica*, was reported by Söhngen (1906) after which only three more methanotrophs were described until 1970 (Anthony, 1982). At that time, Whittenbury and colleagues isolated and characterized more than 100 methanotrophic cultures, establishing the basic phylogenetic framework of the intensively-studied proteobacterial methanotrophs, which are positioned within the *Gammaproteobacteria* and *Alphaproteobacteria* (Whittenbury et al., 1970), although a few non-proteobacterial MOB have also been described (Op den Camp et al., 2009; Ettwig et al., 2010). Five methanotrophic genera have been described within the *Alphaproteobacteria*: *Methylocella, Methyloferula* and *Methylocapsa* in the family *Beijerinckiaceae* and *Methylosinus* and *Methylocystis* in the family *Methylocystaceae* (Dedysh, 2009; Vorobev et al., 2010). Currently, twelve methanotrophic genera have been formally described within the family *Methylococcaceae* of the *Gammaproteobacteria*: *Methylomonas*, *Methylobacter*, *Methylococcus*, *Methylomicrobium*, *Methylosarcina*, *Methylocaldum*, *Methylogaea*, *Methylothermus*, *Methylohalobius*, *Methylosoma*, *Methylovulum* and *Methylosphaera* (Geymonat et al., 2011). Six species have been described within the genus *Methylomonas* (Danilova et al., 2012) and three of them no longer have extant type strains, namely *M. scandinavica, M. fodinarum* and *M. aurantiaca*. Two *Methylomonas* species were described very recently, *M. koyamae* and *M. paludis* (Danilova et al., 2012; Ogiso et al., 2012). The type strain of *M. methanica,* the type species of the genus, represents the first methanotroph ever isolated (Anthony, 1982). *Methylomonas* colonies are typically pink, orange or red. Most cells are rod-shaped, motile by single polar flagellum, neutrophilic and mesophilic and have been isolated from a wide variety of environments, such as rice paddies, wetlands, waste water, coal mine drainage water (Bowman et al., 1993; Hanson and Hanson, 1996; Bowman, 2006; Danilova et al., 2012; Ogiso et al., 2012). Here, we report the characterization of two new *Methylomonas* isolates from manure and a denitrification tank in Belgium that we propose to assign to a novel species, *Methylomonas lenta* with R-45377^T (LMG 26260^T) as type strain.

EXPERIMENTAL PROCEDURES

Strain R-45377^T (= LMG 26260^T) was isolated from a covered but aerobic slurry pit of a cow stable (Melle, Belgium). Strain R-45370 (= LMG 26613) was isolated from the top layer of a denitrification tank of a wastewater treatment plant (WWTP, Ossemeersen, Gent, Belgium). Both isolates were obtained by miniaturized extinction culturing (Hoefman et al., 2012a). The strains were routinely cultured at 20°C in diluted Nitrate Mineral Salts [dNMS, (Dunfield et al., 2003)] medium (liquid or solidified with agar) with a modified copper concentration (10 µM Cu^{2+}) and a 2 mM phosphate buffer (pH 6.8) under a CH_4:air (1:1) atmosphere when cultivated in jars, or with 20% CH_4 added to the headspace when cultivated in gastight flasks.

Cell morphology and motility were assessed by phase-contrast microscopy. Gram staining was performed and cytochrome c oxidase and catalase activities were according to Cleenwerck et al., (2002). Flagella staining was performed according to Heimbrook et al., (1989). The presence of *Azotobacter*-type cysts was evaluated by the staining procedure of Vela & Wyss (1964) on cultures grown to stationary phase. Transmission electron microscopy was performed on strain R-45377^T according to Spieck and Lipski (2011).

Heat resistance and desiccation resistance were assessed on turbid suspensions (OD_{600nm} 1) of cultures grown to stationary phase. To measure the heat resistance, suspensions were incubated for 15 minutes at 85°C, rapidly cooled on ice and plated onto dNMS agar and incubated. For evaluation of desiccation resistance, drops of suspensions were air dried on sterile glass slides and kept at 28°C for three weeks. Desiccation resistance of the cultures was assessed by rehydration and incubation in dNMS (both on agar plates and in liquid cultures).

Utilization of the following carbon sources (0.1%) was evaluated by monitoring of growth (OD_{600nm} levels) in liquid dNMS without methane added to the headspace: methanol, formate, urea, methylamine, dimethylamine, dimethyl carbonate, formamide, acetate, pyruvate, succinate, malate, ethanol, citrate, glycine, D-xylose, maltose and glucose. Utilization of the following nitrogen sources at a concentration of 2 mM by replacing nitrate in dNMS was evaluated with methane added to the headspace and monitoring of growth (OD_{600nm} levels): nitrite, ammonium (NH_4Cl and $(NH_4)_2SO_4$), formamide, methylamine, glycine, hydroxylamine, urea, L-serine, L-proline, L-aspartate, L-leucine, L-cysteine, L-arginine and yeast extract (0.1% w/v yeast extract, instead of 2 mM).

Growth by dinitrogen gas fixation was evaluated by incubation of the strains in duplicate in

liquid nitrogen-free dNMS with 20% CH_4 added to air at normal (21%) and ten-fold reduced (2.1%) oxygen levels. Growth was determined through OD_{600nm} measurements and verified by visual inspection after 4, 7, 11, 14 and 21 days of incubation. In addition, the methane oxidation activity was assessed by measuring the CH_4, O_2 and CO_2 levels via gas chromatography (Compact Gas Chromatograph equipped with one channel connected to a flame ionization detector and two channels connected to a thermal conductivity detector Global Analyzer Solutions, Belgium). *M. methanica* NCIMB 11130^T was included as a positive control, as this strain is known to fix dinitrogen gas (Auman et al., 2001; Boulygina et al., 2002).

Salt tolerance [tested at 0.23% (40 mM), 0.58% (100 mM), 1%, 5% and 10% NaCl spiked in dNMS], the pH optimum and range (tested at pH 4, 5, 5.8, 6.3, 6.8, 7.3, 7.8, 9, 10) and the temperature optimum and range (tested at 5°C, 15°C, 20°C, 25°C, 28°C, 33°C, 37°C, 45°C and 52°C) were assessed by monitoring of growth (OD_{600nm}) in liquid dNMS.

Soluble methane monooxygenase activity was tested using a modified version of the naphthalene oxidation assay (Hoefman et al., 2012a). Cryopreservation of strains R-45377^T and R-45370 was performed as described by Hoefman et al., (2012b).

DNA was extracted and 16S rRNA gene sequences were generated as described in Hoefman et al., (2012a). Sequences were assembled with BioNumerics 5.1 (Applied Maths, Belgium). Preliminary genus identification was obtained by query in the "Classifier" program of Ribosomal Database Project II (Cole et al., 2005). Sequences of strains R-45377^T and R-45370 were aligned among a selection of methanotrophic type strains using the integrated aligner of ARB (Ludwig et al., 2004). Based on the 16S rRNA gene alignment, a Maximum Likelihood (ML) phylogenetic tree was constructed using RAxML v7.3.5 based on the General Time Reversible substitution model with gamma-distributed rate variation (GTR+Gamma). Bootstrap analysis based on 1000 replicates was performed. The best scoring ML tree was exported in newick format and analyzed using the MEGA 5 software (Tamura et al., 2011).

Functional genes were amplified and sequenced when applicable. Amplification of the *pmoA* gene encoding the 27 kDa peptide of particulate methane monooxygenase (pMMO) was carried out with the A189f/mb661r primer set according to Costello and Lidstrom (1999). Amplification of the *mmoX* gene encoding the α-subunit of the hydroxylase component of soluble methane monooxygenase (sMMO) was tested with primer sets 534f /1393r (Horz et al., 2001), mmoXA/mmoXB (Auman et al., 2000) and mmoX206f/mmoX886r (Hutchens et al., 2004). *pxmA* [encoding a sequence-divergent

particulate monooxygenase (pXMO)] and *nifH* (encoding the highly conserved Fe protein of nitrogenase) gene amplification was performed using the primer sets pmoA189F/pxmA634R (Tavormina et al., 2008), F1/nifH439R (De Meyer et al., 2011) and f1003/r1561 (McDonald et al., 1995), respectively. For each of the above primer sets, the PCR mix and temperature program recommended in the original literature were applied and success of each assay was verified using a set of methanotrophic type strains as positive and negative controls.

When functional gene amplification was positive, sequences were generated as explained for 16S rRNA gene analysis. Protein translation analysis using Transeq (www.ebi.ac.uk/tools/emboss/transeq) and pBLAST (Altschul et al., 1990) confirmed that the sequences encoded for part of the evaluated enzymes. Sequences were aligned using the MEGA 5 software based on translated protein sequences and DNA-based ML phylogenetic trees were subsequently constructed using RAxML as explained above.

DNA was extracted and purified as described by Logan et al., (2000) to determine the G+C content by HPLC (Mesbah et al., 1989). Phospholipid fatty acid analysis was carried out as described by Vancanneyt et al., (1996). Fatty acids were identified using the Microbial Identification System (MIDI) software (Sherlock Microbial Identification Systems, version 3.0; Library TSBA 50, version 5.0). For identification of the methanotroph-specific fatty acid $C_{16:1}$w8c, not present in the database of the MIDI system, in-house generated data of the type strains *Methylomonas methanica* NCIMB 11130^T and *Methylomonas koyamae* NCIMB 14606^T, known to form this fatty acid, were used as references.

RESULTS & DISCUSSION

Colonies of strain R-45377^T and R-45370 start to develop after one week of incubation under optimal conditions. After one to two weeks of incubation, colonies of strain R-45377^T are small (<1 mm), round and white with a light pink shine, whereas colonies of strain R-45370 are similar in appearance although they are clearly pink and not white. The colonies develop a lot slower (4 to 5 days difference) and appear less slimy than typical *Methylomonas methanica* and *Methylomonas koyamae* cultures, cultivated in parallel in identical conditions. Cells of R-45377^T and R-45370 are gram-negative rods (1.3 to 2.0 µm long and 0.6 to 0.9 µm wide), motile by single polar flagellum exhibiting intracytoplasmic membrane systems appearing as stacks of vesicular disks (Figure VII-1). Cells are sensitive to heat and desiccation and *Azotobacter*-type cysts are not observed. Cells show positive cytochrome c oxidase, but negative catalase reactions. Both strains can be preserved successfully for longer periods at -80°C and in liquid nitrogen using 5% DMSO as cryoprotectant.

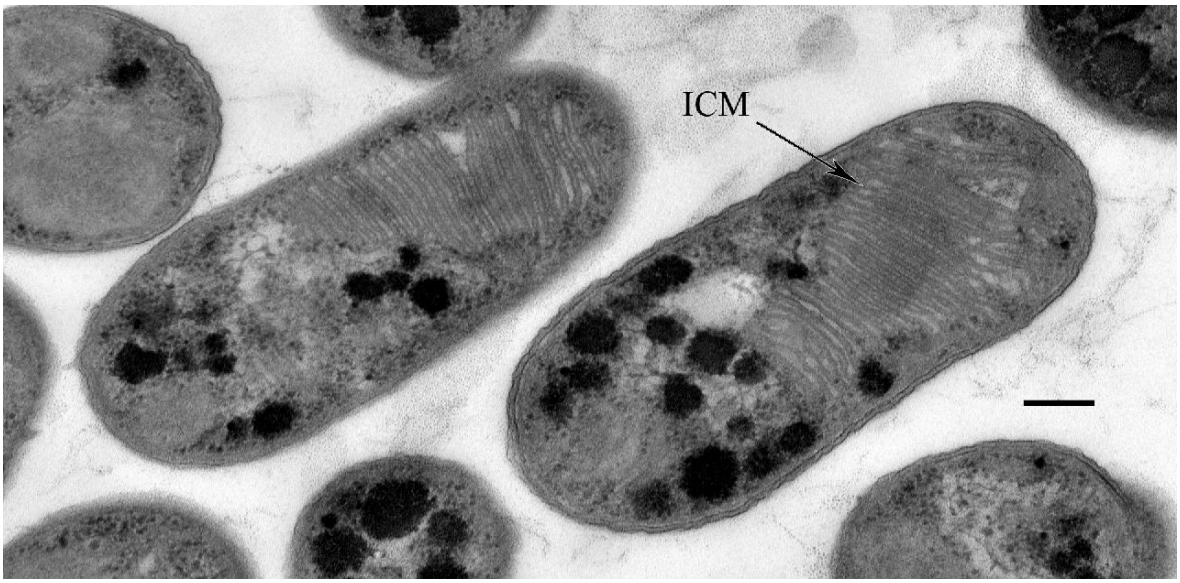

Figure VII-1: Phase-contrast micrograph of actively growing cells of strain R-45377^T showing rods with typical intracytoplasmic membrane arrangements (ICM) of gammaproteobacterial methanotrophs. Bar: 0.2 µm.

Only methane or methanol are used as sole carbon and energy source. Cells are sensitive to 0.1% and 0.5% methanol amendments, but grow with 0.04% methanol added to the medium. Both strains grow with nitrate, nitrite, ammonium [both NH_4Cl and $(NH_4)_2SO_4$], urea, serine, leucine and yeast extract as sole nitrogen source. Although the *nifH* gene

could be amplified, the strains did not grow after three weeks of incubation in nitrogen-free medium under tested conditions, neither at high nor low oxygen tension. This was concluded by a lack of visual turbidity confirmed with OD_{600nm} analysis, the absence of a drop in CH_4 and O_2 levels and the absence of an increase in CO_2 levels measured by gas chromatography. In parallel, growth and methane oxidation was observed for the strains with 10 mM KNO_3 and 10 mM NH_4Cl added to the medium. Using the same setup, the positive control strain, *M. methanica* NCIMB 11130^T, was positive for N_2 fixation at low oxygen tension. Strains R-45377^T and R-45370 grow optimally at 20°C (range 15 to 28°C). The optimal pH for growth is 6.8 to 7.3 (range 6.3 to 7.8). The strains tolerate salt additions up to 200 mM but are sensitive to 5% NaCl amendments. Both strains were negative for sMMO activity and the *mmoX* gene could not be amplified. The *pmoA* gene was present, while the presence of *pxmA* gene could not be demonstrated.

16S rRNA gene sequences (1486 bp) of R-45377^T and R-45370 used in a phylogenetic analysis showed that the two strains formed a separate cluster within *Methylomonas* (Figure VII-2) adjacent to *M. scandinavica* SR5^T.

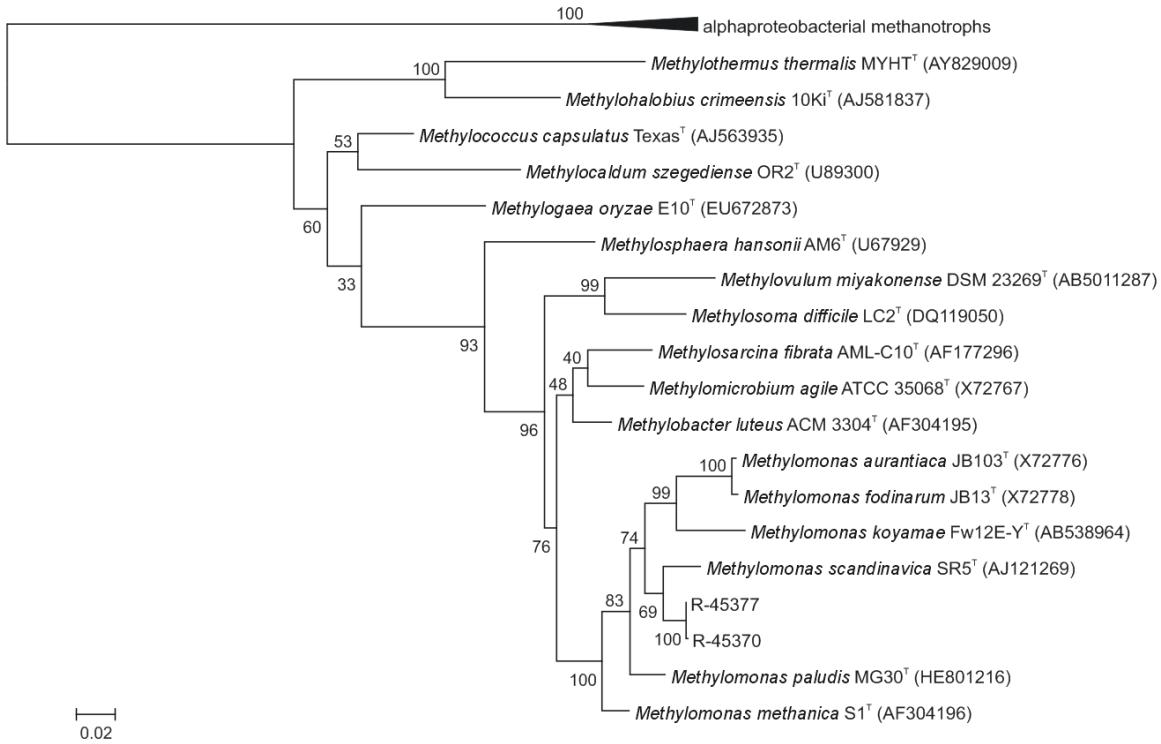

Figure VII-2: 16S rRNA gene phylogenetic maximum-likelihood tree (1403 nucleotide alignment; GTR+Gamma substitution model; 1000-replicate bootstrapping) of strains R-45377^T and R-45370 with the type strains of the recognized species within *Methylomonas* and the type strains of the remaining genera within the *Methylococcaceae*. The alphaproteobacterial methanotrophic type strains *Methylocystis parvus* NCIMB 11129^T (Y18945), *Methylosinus trichosporium* OB3b^T (Y18947), *Methyloferula stellata* AR4^T (FR686343), *Methylocella palustris* K^T (Y17144) and *Methylocapsa acidiphila* B2^T (AJ278726) were used as an outgroup. Bar: 0.02 substitutions per nucleotide position.

Pairwise 16S rRNA sequence comparison revealed sequence similarities for R-45377^T of 97.5 % with *M. scandinavica* SR5^T, 97.2% with *M. paludis* MG30^T, 96% with *M. methanica* S1^T, 95.9% with *M. koyamae* Fw12E-Y^T, 95% with *M. aurantiaca* JB103^T, 94.9% with *M. fodinarum* JB13^T, and 99.9% with strain R-45370. The 16S rRNA gene sequence results are supported by phylogenetic analysis of the *pmoA* gene (Figure VII-3) and the *nifH* gene (Figure VII-4), which confirms a distinct line of descent for strains R-45377^T and R-45370 different from the other members of *Methylomonas*, although several *Methylomonas* species could not be included in the analysis since their *pmoA* and *nifH* genes were never reported, and since the type strains of *M. scandinavica*, *M. aurantiaca* and *M. fodinarum* are no longer extant (personal communication VKM and ACM collections).

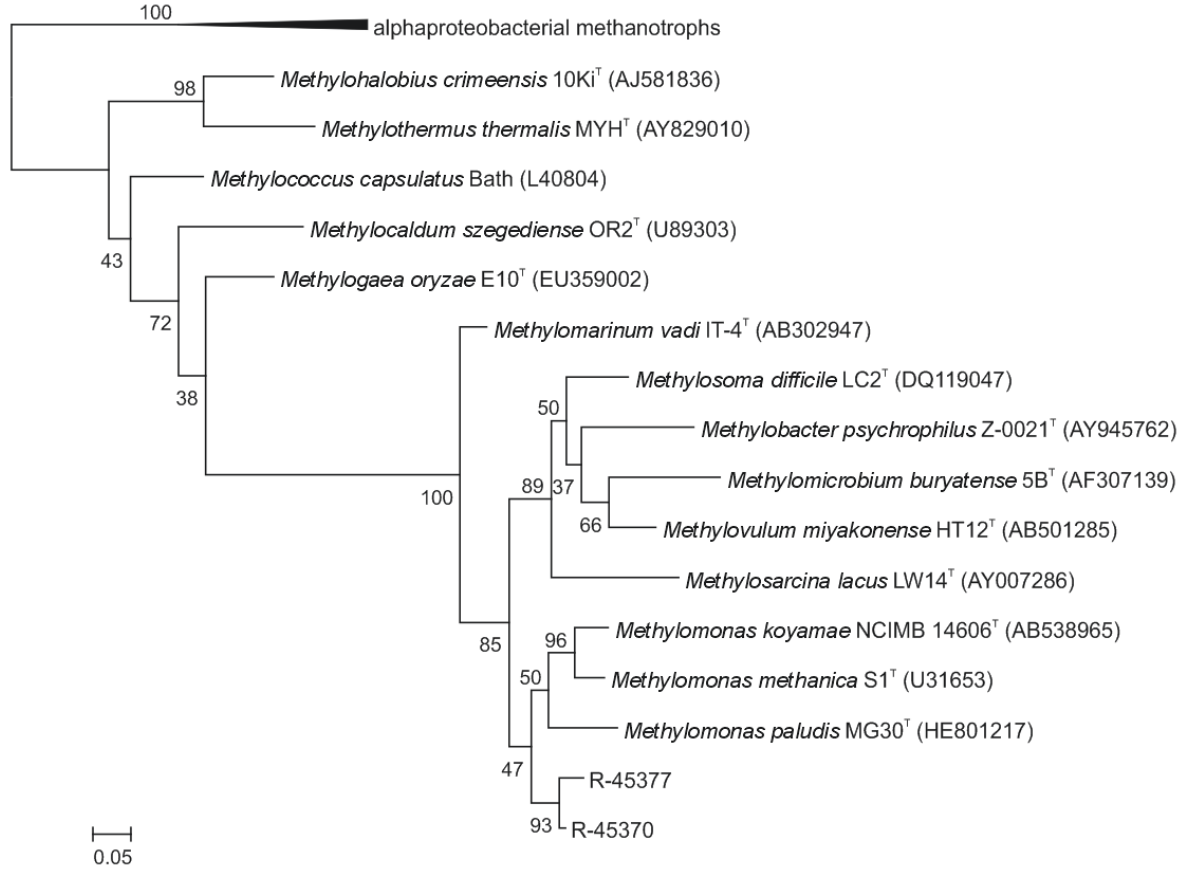

Figure VII-3: *pmoA* gene phylogenetic maximum-likelihood tree (429 nucleotide alignment; GTR+Gamma substitution model; 1000-replicate bootstrapping) of strains R-45377^T and R-45370 with the available *pmoA* sequences of the type strains within *Methylomonas* and one *pmoA* sequence per remaining genus within the *Methylococcaceae*. The alphaproteobacterial methanotrophic type strains *Methylocystis parvus* OBBP^T (U31651), *Methylosinus trichosporium* IMET 10561^T (AJ459001) and *Methylocapsa acidiphila* B2^T (AJ278727) were used as an outgroup. Bar: 0.05 substitutions per nucleotide position.

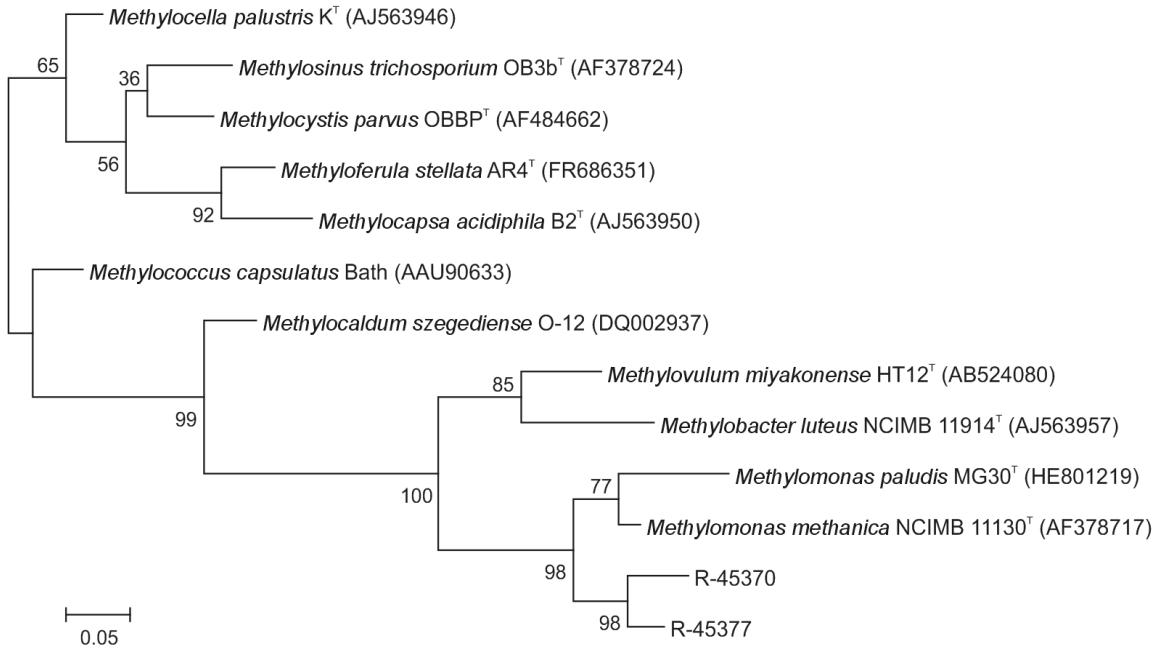

Figure VII-4: *nifH* gene phylogenetic maximum-likelihood tree (285 nucleotide alignment; GTR+Gamma substitution model; 1000-replicate bootstrapping) of strains R-45377^T and R-45370 with the available *nifH* sequences of the type strains within *Methylomonas* and available *nifH* sequences within the *Methylococcaceae*. The alphaproteobacterial methanotrophic type strains of *Methylocystis parvus*, *Methylosinus trichosporium*, *Methyloferula stellata*, *Methylocella palustris* and *Methylocapsa acidiphila* were used as an outgroup. Bar: 0.05 substitutions per nucleotide position.

Both strains display typical *Methylomonas* PLFA profiles, with $C_{16:1}$w8c, $C_{16:1}$w5c, $C_{16:1}$w7c, $C_{14:0}$ and $C_{16:0}$ as major cellular fatty acids (Table VII-1). However, the strains possess (i) an unusually high amount of the fatty acid $C_{15:0}$ (5.3% to 5.8%) compared with other *Methylomonas* strains (0% to 1.2%), and (ii) a high amount of $C_{16:1}$w5c (11.7% to 18.3%) similar as observed for the type strain of *Methylomonas koyamae*, compared with other *Methylomonas* strains (1.8% to 6.3%). Since the production of cellular fatty acids might be dependent on the applied growth conditions, the type strains of *M. koyamae* and *M. methanica* as well as several recently isolated strains belonging to both species were included for PLFA analysis in parallel with R-45377^T and R-45370. PLFA analysis appeared to be highly robust for *Methylomonas* strains, since the PLFA profiles generated in house for *M. koyamae* and *M. methanica* were highly similar with the data reported in literature for strains of these species, supporting the above mentioned differences in PLFA profiles between R-45377^T and R-45370 with other *Methylomonas* members.

Table VII-1: Composition of cellular fatty acids distinguishing strains R-45377^T and R-45370 from other species within the genus *Methylomonas*. Values are percentages of the total fatty acids.

Fatty acid	**R-45377^T & R-45370**	***M. paludis*[a]**	***M. koyamae*[b]**	***Methylomonas* spp.[c]**
$C_{12:0}$	0.60-2.51	NR	NR	NR
$C_{13:0}$	0.57-0.86	NR	NR	NR
$C_{14:0}$	6.38-9.78	11.80	22.90	18.9-24.6
$C_{15:1}$w8c	0.98-2.30	NR	NR	NR
$C_{15:1}$w6c	0.51-0.65	NR	NR	0.00
$C_{15:1}$w5c	0.97-1.06	NR	NR	NR
$C_{15:0}$	5.34-5.83	0.50	1.16	0-1.2
$C_{16:1}$w8c[d]	40.85-42.42	22.10	39.40	18.7-41.3
$C_{16:1}$w7c[e]	9.10-10.47	13.90	4.35	7.7-15.3
$C_{16:1}$w6c	-	5.00	-	4.5-13.3
$C_{16:1}$w5c	11.67-18.26	1.80	16.70	1.9-6.3
$C_{16:1}$w5t	-	34.8	-	7.9-16.6
$C_{16:0}$	5.00	5.60	7.70	4.3-8.7
$C_{17:1}$w8c	0.00-0.82	NR	NR	0.00
$C_{16:0}$ 3-OH	4.11-4.16	NR	3.79	NR
$C_{18:1}$w7c	-	0.30	NR	0.2-2.5

Data was extracted from [a] Danilova et al., (2012) [b] Ogiso et al., (2012) and [c] Bowman et al., (1993) representing *M. methanica*, *M. fodinarium* and *M. aurantiaca*

-, not detected; NR, not reported; No fatty acid data was reported for *Methylomonas scandinavica* (Kalyuzhnaya et al., 1999)

[d]Fatty acid $C_{16:1}$w8c was identified using in-house generated data of *Methylomonas methanica* NCIMB 11130^T and *Methylomonas koyamae* NCIMB 14606^T as references.

[e] This peak in the chromatogram represents $C_{16:1}$w7c and/or iso-$C_{15:0}$ 2-OH according to the MIDI system, however in this study the peak is assigned to $C_{16:1}$w7c since this fatty acid is common among the studied methanotrophs.

Table VII-2: Major characteristics distinguishing strains R-45377^T and R-45370 from other species within *Methylomonas*.

Characteristic	**R-45377^T & R-45370**	***M. paludis*[a]**	***M. koyamae*[b]**	***M. scandinavica*[c]**	***M. methanica*[d]**	***M. fodinarum*[e]**	***M. aurantiaca*[e]**
Cell shape	rods	rods	rods	rod-like ovoids	rods	rods	rods
Cell size (µm)	0.6-0.9 x 1.3-2.0	1-1.5 x 1.0-4.0	0.8-1.1 x 1.2-2.5	0.6-0.8 x 1.5-1.7	0.5-1.0 x 0.5-2.0	0.7-1.0 x 0.8-1.2	0.5-0.8 x 0.8-1.5
Pigmentation	white to pink	pale-pink	pink-orange	pink	pink	orange	orange
Motility	+	-	+	+	+	+	+
Chain formation	-	+	-	+	-	+	+
Temperature range (°C)	15-28	8-30	10-40	5-30	10-37	10-35	15-40
Temperature optimum (°C)	20	20-25	30	17	25-30	25	35
Growth at 5°C	-	-	-	+	-	-	-
Growth at 37°C	-	-	+	-	+	-	+
pH range	6.3-7.8	3.8-7.3	5.5-7.0	5.0-9.0	5.5-9.0	5.5-9.0	5.0-9.0
pH optimum	6.8-7.3	5.8-6.4	6.5	6.8-7.6	7.0	7.0	6.5
Methanol 0.1%	-	+	+	+	+	+	+
Tolerance to 1% NaCl	+	-	-	+	+	+	+
Oxidase	+	NR	+	NR	+	-	-
Catalase	-	NR	+	NR	+	+	+
nifH presence	+	+	+[f]	NR	+[f]	NR	NR
N2 Fixation	-	+	+[f]	NR	Variable	+	+
sMMO	-	-	-	NR	Variable	NR	NR
pxmA presence	-	NR	+[f]	NR	+	NR	NR
G+C content (mol%)	47	48.5	57.1	53.8	52	58	56.5

Data was extracted from [a] Danilova et al., (2012), [b] Ogiso et al., (2012), [c] Kalyuzhnaya et al., (1999), [d] Bowman et al., (1993), [e] Bowman et al. (1990).

+, positive; -, negative; NR, not reported; [f] not reported in original description but positive based on in house data

The major morphological and physiological characteristics distinguishing strains R-45377^T and R-45370 from other species within *Methylomonas* are presented in Table VII-2. Both strains differ from other *Methylomonas* species by their sensitivity to 0.1% methanol. Their DNA G+C content of 47% is the lowest reported for *Methylomonas* members so far. Similar as the type strains of *M. paludis* and *M. scandinavica*, R-45377^T and R-45370 have a lower temperature optimum than most *Methylomonas* strains. Strains R-45377^T and R-45370 differ from *M. paludis* MG30^T by (i) their motility by single polar flagellum, (ii) their inability to form chains, (iii) their tolerance to 1% NaCl and (iv) their higher sensitivity to acidic environments. The strains are distinct from *M. scandinavica* SR5^T by their inability to grow at 5°C. Based on the above mentioned genotypic and phenotypic differences between strains R-45377^T and R-45370 and other members of *Methylomonas*, we propose the addition of a novel *Methylomonas* species, *Methylomonas lenta* sp. nov., with R-45377^T as type strain.

Description of *Methylomonas lenta* sp. nov.

Methylomonas lenta (len'ta. L. fem. adj. *lenta* slow, delayed, referring to the slow growth of the microorganism compared with typical *Methylomonas* cultures). Cells are aerobic, gram-negative, rods (1.3-2.0 µm long; 0.6-0.0 µm wide) motile by single polar flagellum and possess intracytoplasmic membrane systems typical to gammaproteobacterial methanotrophs. Colonies appear white to pink. Resting stages are not observed. Cultures grow optimally at 20°C in a range of 15°C to 28°C and at optimal pH of 6.3 to 7.3 in a range 6.3 to 7.8. Cells utilize methane and methanol as sole carbon and energy source but are sensitive to ≥0.5% methanol concentrations. Strains grow with nitrate, nitrite, ammonium [both NH_4Cl and $(NH_4)_2SO_4$], urea, serine, leucine and yeast extract as sole nitrogen source and tolerate NaCl amendments up to 200 mM. Cultures are positive for cytochrome c oxidase but negative for catalase activities. The *pmoA* gene is present. The *pxmA* gene could not be detected. The *nifH* is present but strains did not grow in nitrogen-free medium under the tested conditions. Presence of *mmoX* gene and sMMO activity could not be demonstrated. The DNA G+C content is 47% for the type strain and the major fatty acids are $C_{16:1}$w8c, $C_{16:1}$w5c, $C_{16:1}$w7c, $C_{14:0}$, $C_{15:0}$ and $C_{16:0}$. The type strain R-45377^T (= LMG 26260^T) was isolated from a covered but aerobic slurry pit of a cow stable (Melle, Belgium). An additional member, strain R-45370 (= LMG 26613) showing 99.9% 16S rRNA gene sequence similarity to the type strain, was isolated from the top layer of a denitrification tank of a wastewater treatment plant (Ossemeersen, Gent, Belgium).

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REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W.,Lipman, D.J., 1990. BASIC LOCAL ALIGNMENT SEARCH TOOL. J Mol Biol 215, 403-410.

Anthony, C. 1982 Biochemistry of methylotrophs. London: The Academic Press.

Auman, A.J., Stolyar, S., Costello, A.M.,Lidstrom, M.E., 2000. Molecular characterization of methanotrophic isolates from freshwater lake sediment. Appl Environ Microbiol 66, 5259-5266.

Auman, A.J., Speake, C.C.,Lidstrom, M.E., 2001. nifH sequences and nitrogen fixation in type I and type II methanotrophs. Appl Environ Microbiol 67, 4009-4016.

Boulygina, E.S., Kuznetsov, B.B., Marusina, A.I., Turova, T.P., Kravchenko, I.K., Bykova, S.A. et al., 2002. [Study of nucleotide sequences of nifH genes in methanotrophic bacteria]. Mikrobiologiia 71, 500-508.

Bowman, J. (2006) The Methanotrophs - The Families *Methylococcaceae* and *Methylocystaceae*. Chapter 3.1.14. *Prokaryotes* **5**: 266-289, DOI: 10.1007/0-387-30745-1_15.

Bowman, J.P., Sly, L.I., Nichols, P.D.,Hayward, A.C., 1993. Revised taxonomy of the methanotrophs – Description of *Methylobacter* Gen Nov, emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. International Journal of Systematic Bacteriology 43, 735-753.

Cleenwerck, I., Vandemeulebroecke, K., Janssens, D.,Swings, J., 2002. Re-examination of the genus Acetobacter, with descriptions of Acetobacter cerevisiae sp. nov. and Acetobacter malorum sp. nov. Int J Syst Evol Microbiol 52, 1551-1558.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M. et al., 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res 33, D294-D296.

Costello, A.M.,Lidstrom, M.E., 1999. Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. Appl Environ Microbiol 65, 5066-5074.

Danilova, O.V., Kulichevskaya, I.S., Rozova, O.N., Detkova, E.N., Bodelier, P.L., Trotsenko, Y.A.,Dedysh, S.N., 2012. Methylomonas paludis sp. nov., the first acid-tolerant member of the genus Methylomonas, from an acidic wetland. Int J Syst Evol Microbiol.

De Meyer, S.E., van Hoorde, K., Vekeman, B., Braeckman, T.,Willems, A., 2011. Genetic diversity of rhizobia associated with indigenous legumes in different regions of Flanders (Belgium). Soil Biol Biochem 43, 2384-2396.

Dedysh, S.N., 2009. Exploring methanotroph diversity in acidic northern wetlands: Molecular and cultivation-based studies. Microbiology 78, 655-669.

Dunfield, P.F., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A.,Dedysh, S.N., 2003. *Methylocella silvestris* sp nov., a novel methanotroph isolated from an acidic forest cambisol. Int J Syst Evol

Microbiol 53, 1231-1239.

Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M.M. et al., 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature 464, 543-+.

Geymonat, E., Ferrando, L.,Tarlera, S.E., 2011. Methylogaea oryzae gen. nov., sp nov., a mesophilic methanotroph isolated from a rice paddy field. Int J Syst Evol Microbiol 61, 2568-2572.

Hanson, R.S.,Hanson, T.E., 1996. Methanotrophic bacteria. Microbiol. Rev 60, 439-471.

Heimbrook, M.E., Wang, W.L.L.,Campbell, G., 1989. STAINING BACTERIAL FLAGELLA EASILY. Journal of Clinical Microbiology 27, 2612-2615.

Hoefman, S., van der Ha, D., De Vos, P., Boon, N.,Heylen, K., 2012a. Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria. Microb. Biotechnol 5, 368-378.

Hoefman, S., Van Hoorde, K., Boon, N., Vandamme, P., De Vos, P.,Heylen, K., 2012b. Survival or revival: long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria. PLoS One 7, e34196.

Horz, H.P., Yimga, M.T.,Liesack, W., 2001. Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of pmoA, mmoX, mxaF, and 16S rRNA and ribosomal DNA, including pmoA-based terminal restriction fragment length polymorphism profiling. Appl Environ Microbiol 67, 4177-4185.

Hutchens, E., Radajewski, S., Dumont, M.G., McDonald, I.R.,Murrell, J.C., 2004. Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. Environ Microbiol 6, 111-120.

Kalyuzhnaya, M.G., Khmelenina, V.N., Kotelnikova, S., Holmquist, L., Pedersen, K.,Trotsenko, Y.A., 1999. Methylomonas scandinavica sp. nov., a new methanotrophic psychrotrophic bacterium isolated from deep igneous rock ground water of Sweden. Syst Appl Microbiol 22, 565-572.

Logan, N.A., Lebbe, L., Hoste, B., Goris, J., Forsyth, G., Heyndrickx, M. et al., 2000. Aerobic endospore-forming bacteria from geothermal environments in northern Victoria Land, Antarctica, and Candlemas Island, South Sandwich archipelago, with the proposal of Bacillus fumarioli sp nov. Int J Syst Evol Microbiol 50, 1741-1753.

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

McDonald, I.R., Kenna, E.M.,Murrell, J.C., 1995. Detection of methanotrophic bacteria in environmental samples with the PCR. Appl Environ Microbiol 61, 116-121.

Mesbah, M., Premachandran, U.,Whitman, W.B., 1989. Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. International Journal of Systematic Bacteriology 39, 159-167.

Ogiso, T., Ueno, C., Dianou, D., Huy, T.V., Katayama, A., Kimura, M.,Asakawa, S., 2012. Methylomonas koyamae sp nov., a type I methane-oxidizing bacterium from floodwater of a rice paddy field. Int J Syst Evol Microbiol 62, 1832-1837.

Op den Camp, H.J.M., Islam, T., Stott, M.B., Harhangi, H.R., Hynes, A., Schouten, S. et al., 2009. Environmental, genomic and taxonomic perspectives on methanotrophic Verrucomicrobia. Environmental Microbiology Reports 1, 293-306.

Semrau, J.D., DiSpirito, A.A.,Yoon, S., 2010. Methanotrophs and copper. FEMS Microbiol Rev 34, 496-531.

Söhngen, N.L., 1906. Uber Bakterien, welche methan ab kohlenstoffnahrung und energiequelle gerbrauchen. Parasitenkd. Infectionskr. Abt. 2. 15, 513-517.

Spieck, E.,Lipski, A. (2011) Cultivation, growth physiology, and chemotaxonomy of nitrite-oxidizing bacteria. In *Methods in Enzymology: Research on Nitrification and Related Processes,*

Vol 486, Part A. Klotz, M.G. (ed), pp. 109-130.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M.,Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28, 2731-2739.

Tavormina, P.L., Ussler, W.,Orphan, V.J., 2008. Planktonic and sediment-associated aerobic methanotrophs in two seep systems along the North American margin. Appl Environ Microbiol 74, 3985-3995.

Vancanneyt, M., Witt, S., Abraham, W.R., Kersters, K.,Fredrickson, H.L., 1996. Fatty acid content in whole-cell hydrolysates and phospholipid fractions of pseudomonads: A taxonomic evaluation. Syst Appl Microbiol 19, 528-540.

Vela, G.R.,Wyss, O., 1964. Improved stain for visualization of *Azotobacter* encystment. J Bacteriol 87, 476-&.

Vorobev, A.V., Baani, M., Doronina, N.V., Brady, A.L., Liesack, W., Dunfield, P.F.,Dedysh, S.N., 2010. Methyloferula stellata gen. nov., sp. nov., an acidophilic, obligately methanotrophic bacterium possessing only a soluble methane monooxygenase. Int J Syst Evol Microbiol.

Whittenbury, R., Phillips, K.C.,Wilkinson, J.F., 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61, 205-217.

Reflection & Discussion

In Chapters I, II and IV, MOB strains were isolated that were used in the different experiments described in Chapters III to VII. In Chapter VI, strain OS501 (obtained from Yasuyoshi Sakai, Kyoto University, Japan) was included with strain R-49797 for the description of *Methyloparacoccus murrellii*. Both strains share a 16S rRNA gene sequence similarity of 99.7%.

An overview of the strains used throughout Chapters III to VII and their 16S rRNA, *pmoA*, *mmoX*, *nifH* and *pxmA* gene sequence results are presented in Table C-1. Furthermore, the 16S rRNA gene sequence phylogeny of the strains compared with selected reference strains is displayed in Figure C-1.

Crucial to the generation of a large part of the data presented in Chapters III to VII, was the use of 96-well microtiter plates for cultivation of the methanotrophs in different conditions. In Chapter III, it was confirmed with batch experiments that the data generated in such a miniaturized setup was relevant. Miniaturized culturing proved useful in many aspects. Firstly, the growth rate or cell yield of several MOB could be rapidly enhanced by using customized media based on data obtained by miniaturized cultivation (Chapter III). This is interesting from a biotechnological point of view because current applications of MOB remain limited in part due to the relative slow growth rate and low cell density of the available strains. For poorly growing strains, this could ensure that these strains do not become lost upon periodic sub-cultivation. Secondly, miniaturized extinction culturing for isolation purposes allows many cultivation conditions to be tested in parallel, and as such can lead to the isolation of MOB with desired properties. For example, an isolate retrieved from a high (70 mM) ammonium enrichment showed growth at ammonium levels up to 100 mM (Chapter IV). Thirdly, miniaturized culturing can be used to rapidly extract growth parameters of MOB in co-cultivation with heterotrophs (Chapter V). Such exploratory studies can rapidly reveal preference of certain MOB to grow specifically with certain heterotrophs, after which the most interesting combinations can be studied further

by batch or continuous cultivation experiments. Furthermore, with this data set, a proof of principle was provided for the prediction of unknown results of co-cultures. This may proof useful for reducing the amount of data that needs to be generated when conducting large co-cultivation studies.

Nitrogen metabolism

Interestingly, the data generated by miniaturized cultivation revealed a large variability between closely related strains within the genus *Methylomonas* in many aspects, such as their growth preference (Chapter III), their nitrogen metabolism (Chapter IV) and their co-cultivation interaction pattern (Chapter V). As discussed in Chapter IV, these findings have important implications when interpreting ecological studies that utilize tools with a low taxonomic resolution, for example in the case of studying the effect of nitrogenous fertilization on methane oxidation activity. The organisms responsible for this activity can be tracked by stable isotope probing (^{13}C-labelled methane) analyzing the incorporation of labeled methane into the DNA, rRNA, mRNA or fatty acids of specific groups of methanotrophs (Mohanty et al., 2006; Dumont et al., 2011). However, these techniques only allow to distinguish between different methanotrophic genera, at best. Therefore, intra genus and intra species strain diversity is not evaluated with currently applied tools. Since we show that strains within a genus differ physiologically in many aspects, these findings might explain contradictory reports on the effect of nitrogen addition. Consider the case of two undisturbed wetland soils A and B, each containing a similar population of Type I and Type II MOB, but soil A also contains a *Methylomonas* strain (Type I) tolerant to ammonium amendments while all other MOB are sensitive to such a disturbance. Biodiversity measured by 16S rRNA gene sequence analysis will be considered equal for both soils. Upon ammonium amendment, methane oxidation activity will decline in soil B, while in soil A activity either remains indifferent (functional redundancy) or can even be stimulated (in the case of a relief of nitrogen limitation) due to presence of the ammonium tolerant *Methylomonas* strain. Furthermore, SIP analyses will show that in soil A, *Methylomonas* or Type I MOB in general became dominant, while this will not be apparent in soil B, even though both soils contained an equal fraction of Type I MOB prior to the disturbance. In future work, we would like to thoroughly evaluate if indeed niche partitioning among individuals within taxa (genus or species) can be important to ensure functional stability in a disturbed ecosystem, and not higher level community structure per se. To validate this hypothesis, we plan to conduct artificial lab-scale community

experiments. Different communities will have a fixed species richness, evenness and genotypic diversity (on a higher taxonomic level), but will differ in individual strains with individual phenotypic traits. The differential effect of nitrogen amendment will then be evaluated between communities. From a biotechnological point of view, strains with specific characteristics might be desired. For example, pig manure is typically highly loaded with ammonium and accounts for almost 50% of the methane emissions from livestock in Flanders. It would be interesting to evaluate the methane mitigation potential in pig manure storage facilities of MOB strains that could thrive in such high ammonium conditions.

In addition to the examples provided in Chapters III, IV and V, several other observations of strain dependency between closely related strains were witnessed. For example, the three isolated *Methylocystis* strains R-49792, R-49794 and R-49796 cannot grow with nitrate as nitrogen source, while most methanotrophs can, including their most closely related type strain *Methylocystis parvus* NCIMB 11129^T (99.4-99.5% 16S rRNA gene sequence similarity). Furthermore, we could not obtain a *nifH* sequence for the three *Methylocystis* strains, and consequently, these strains were also negative in our experiments testing growth by nitrogen fixation. This finding is very unusual for Type II MOB, as members of this group are commonly known to fix nitrogen. Moreover, we could obtain a *nifH* sequence for *Methylocystis parvus* NCIMB 11129^T. Additionally, the three *Methylocystis* strains possessed polar tufts of flagella, which were previously reported for *Methylosinus* cultures but not for *Methylocystis* cultures. Moreover, *Methylocystis* R-49796 shaped into a pear-form upon entering the stationary phase, a characteristic previously reported for *Methylosinus trichosporium* cultures, but not for *Methylocystis* cultures (Whittenbury et al., 1970).

Pigmentation

Closely related *Methylomonas* strains also showed additional strain dependent features. *Methylomonas* strains typically produce pink, red and orange carotenoid pigments, which are thought to promote survival of cells under oxidative stress by scavenging of free radicals (Bowman et al., 1993; Sharpe et al., 2007; Kocher et al., 2009). As such, the type of pigmentation is sometimes used to distinguish *Methylomonas* strains (Bowman et al., 1990; Bowman et al., 1993; Ogiso et al., 2012). For example, cultures of the type strain of *M. koyamae* were described as orange to pink (Ogiso et al., 2012). Interestingly, strain R-45378 which was most closely related to this type strain (99.5% 16S rRNA sequence

similarity) showed a bright orange to yellow pigmentation in ammonium medium (dAMS), while this was much less apparent in nitrate medium, i.e. dNMS (Figure C-1).

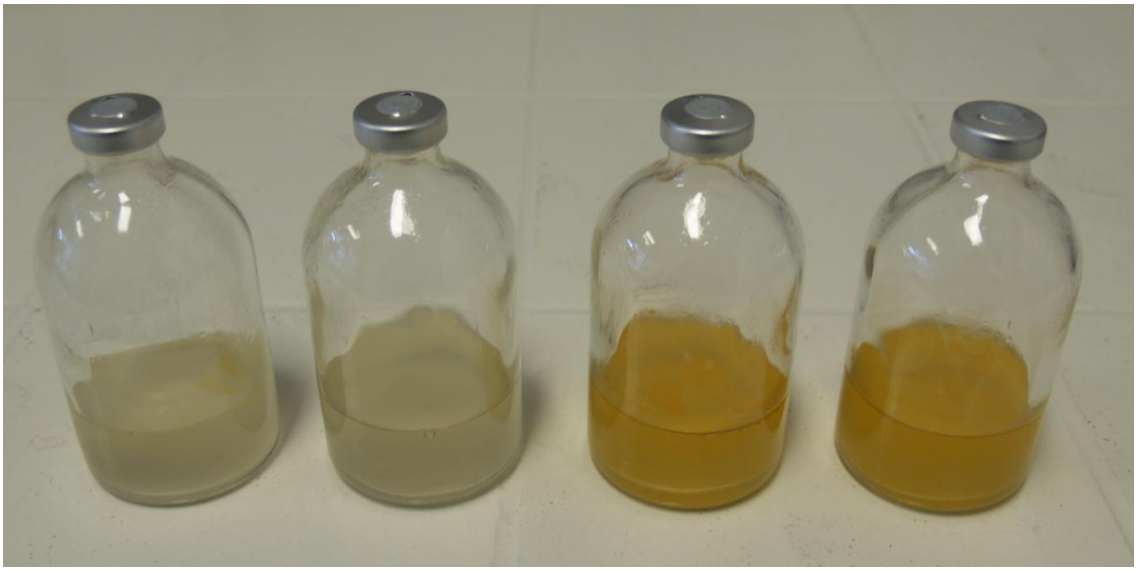

Figure C-1: Batch cultivation of *Methylomonas* sp. R-45378 in dNMS medium with 10 mM KNO_3 (two bottles on the left) and dAMS medium with 10 mM NH_4Cl (two bottles on the right). Increased pigment production was observed for the strain when cultivated with ammonium instead of nitrate. The initial biomass inoculated in the four cultures was the same while the OD_{600nm} values at the time of taking the picture were similar 0.498 ± 0.043 in dNMS compared with 0.420 ± 0.030 in dAMS medium.

This discrepancy in pigmentation between cultivation in ammonium and nitrate medium was observed in several experiments with different nitrogen concentrations. Upon centrifugation of the cultures, cells were clearly pink, thus resembling typical *Methylomonas* cells, while the supernatant was yellow. In addition, colonies of *Methylomonas* sp. R-45378 appeared red on dAMS plates, while they were orange on dNMS plates. Yellow spent media have been observed before for other methanotrophs and this was attributed to the production of small peptides named methanobactins, copper-siderophores or chalkophores, which appeared yellow-red and were secreted in solution to capture copper ions (Kim et al., 2004). However, these methanobactins are optimally produced under copper-limiting conditions (Semrau et al., 2010), while in our experiments rather high copper levels (10 µM) are amended to both dNMS and dAMS, although the copper-to-biomass levels were not measured. Perhaps, more so in ammonium than nitrate medium, this *Methylomonas* strain produces such a type of compound to capture copper ions and thus enhance growth, since enhanced cell yields of this strain were observed in semi-continuous cultures in an optimized growth medium where one of the factors was the

replacement of nitrate by ammonium as sole nitrogen source (Chapter III). Alternatively, since the strain cannot express sMMO, perhaps the expression of a type of methanobactin could provide an advantage for this strain once copper-to-biomass ratios become lower upon cultivation.

All other *Methylomonas* strains never showed a clear difference in pigmentation when cultivated in different media. However, *Methylomonas* sp. R-45378 was isolated along with several other *Methylomonas* isolates which displayed an identical GTG_5 rep-profile (Chapter I). *Methylomonas* sp. R-45378 was chosen as representative strain of this cluster. Interestingly, another isolate from this cluster appeared pink in liquid and on solid media and did not appear to produce a yellow compound. Pigmentation is thus a strain dependent trait and care should be taken when using such features to distinguish between *Methylomonas* species. For example, *M. koyamae* NCIMB 14606^T, R-49807 and R-49799 share a 100% 16S rRNA gene relatedness. While the former two appeared orange in liquid cultures and on solid media, R-49799 always appeared to be pink. Another example of a strain dependent trait between these three strains is the formation of flocs regardless of the amended nitrogen source for R-49807, while this was never apparent for the other two closely related strains.

Link with taxonomy

As shown by the above case, strain dependent features impact the description of novel taxa. This is especially true for MOB, since many methanotrophic taxa, both species and genera, have been described based on the isolation of a single strain. It is therefore unknown whether phenotypic characteristics described for a type strain are common for that species or strain dependent. In fact, in such a case the description of a novel methanotroph almost becomes solely governed by 16S rRNA gene sequencing. Consider the case where one methanotroph is isolated, and this isolate has a 16S rRNA gene sequence similarity below 97% (or above 97%, but with DNA-DNA hybridization values below 70%) with the most closely related type strain of a species based on the description of a single strain. Then it is sufficient to find one different phenotypic characteristic between both strains to propose a novel taxon for the isolate. However, in this case the relevance of this phenotypic characteristic remains unknown, as it is possible, as shown from our results, that even two strains sharing 100% 16S rRNA sequence similarities differ in phenotypic traits that are used to describe novel methanotrophic taxa. To illustrate this

further, we currently have the perfect example within our strain set. *Methylomonas* sp. R-45383 shares a 97.9% 16S rRNA sequence similarity with its most closely related type strain *Methylomonas koyamae* NCIMB 14606^T. According to Stackebrandt and Ebers (2006), this already provides genotypic evidence that the two strains belong to different species. To be sure, we conducted DNA-DNA hybridizations between the two strains, and found only a 19% (± 9%) relatedness which provides enough genotypic evidence that the strains belong to two different species. However, we isolated only one strain, and *Methylomonas koyamae* was described based on a single isolate (Ogiso et al., 2012). Therefore, we cannot know if the different phenotypic characteristics observed between both strains truly reflect differences between species: among other differences, *M. koyamae* NCIMB 14606^T colonies are orange and the strain produced nitrous oxide in our experiments, while *Methylomonas* sp. R-45383 colonies are pink and never showed nitrous oxide production. According to the rules, we have a case to propose the description of a novel *Methylomonas* species with strain R-45383 as type strain without the need to perform any additional experiments. However, the question can be raised whether this is useful without conducting additional phenotypic tests and/or isolating additional strains, preferably from different habitats and/or different geographic locations.

Such problems caused by a lack of cultured methanotrophic diversity are a direct result of the laborious nature and difficulty to isolate MOB, which we have tried to address by introducing miniaturized extinction culturing. This technique proved useful in isolating a set of closely related strains. In all fairness, in this thesis we propose the description of two novel taxa in Chapters VI and VII, based on only 2 strains per taxon, which is still not a lot of strains, although the isolates were obtained from different origins that were geographically separated and had many features in common that differed from the closest related taxa.

Table C-1: Overview of the strains used in this study, showing origin, identification and 16S rRNA and functional gene sequencing results

Origin	Strain Nr.	Identification	16S rRNA gene similarity[a]		*pmoA*	*mmoX*	*pxmA*	*nifH*
Denitrification tank,	R-45362	*Methylomonas*	*M. methanica*	98.6%	+	+	+	+
Belgium	R-45363	*Methylomonas*	*M. methanica*	98.6%	+	+	+	+
	R-45364	*Methylomonas*	*M. methanica*	98.3%	+	+	+	+
	R-45371	*Methylomonas*	*M. methanica*	98.5%	+	+	+	+
	R-45372	*Methylomonas*	*M. methanica*	98.4%	+	+	+	+
	R-45374	*Methylomonas*	*M. methanica*	98.6%	+	+	+	+
	R-45370	*Methylomonas*	*M. scandinavica*	97.5%	+	-	-	+
Manure, Belgium	R-45377	*Methylomonas*	*M. scandinavica*	97.5%	+	-	-	+
Wetland, Belgium	R-45378	*Methylomonas*	*M. koyamae*	99.5%	+	-	+	+
	R-45383	*Methylomonas*	*M. koyamae*	97.9%	+	-	+	+
	R-45379	*Methylosinus*	*M. sporium*	98.9%	+	+	-	+
70mM NH_4Cl Enrichment	R-49799	*Methylomonas*	*M. koyamae*	100%	+	-	+	+
Facultative pond,	R-49807	*Methylomonas*	*M. koyamae*	100%	+	-	+	+
South Africa	R-49792	*Methylocystis*	*M. parvus*	99.4%	+	-	-	-
	R-49794	*Methylocystis*	*M. parvus*	99.4%	+	-	-	-
	R-49796	*Methylocystis*	*M. parvus*	99.5%	+	-	-	-
	R-49797	*Methyloccocaceae*	*Methylocaldum gracile*	94.2%	+	-	-	-
Pond water, Japan	OS501	*Methyloccocaceae*	*Methylocaldum gracile*	94.4%	+	-	-	-

[a]16S rRNA gene sequence similarity with closest related type strain

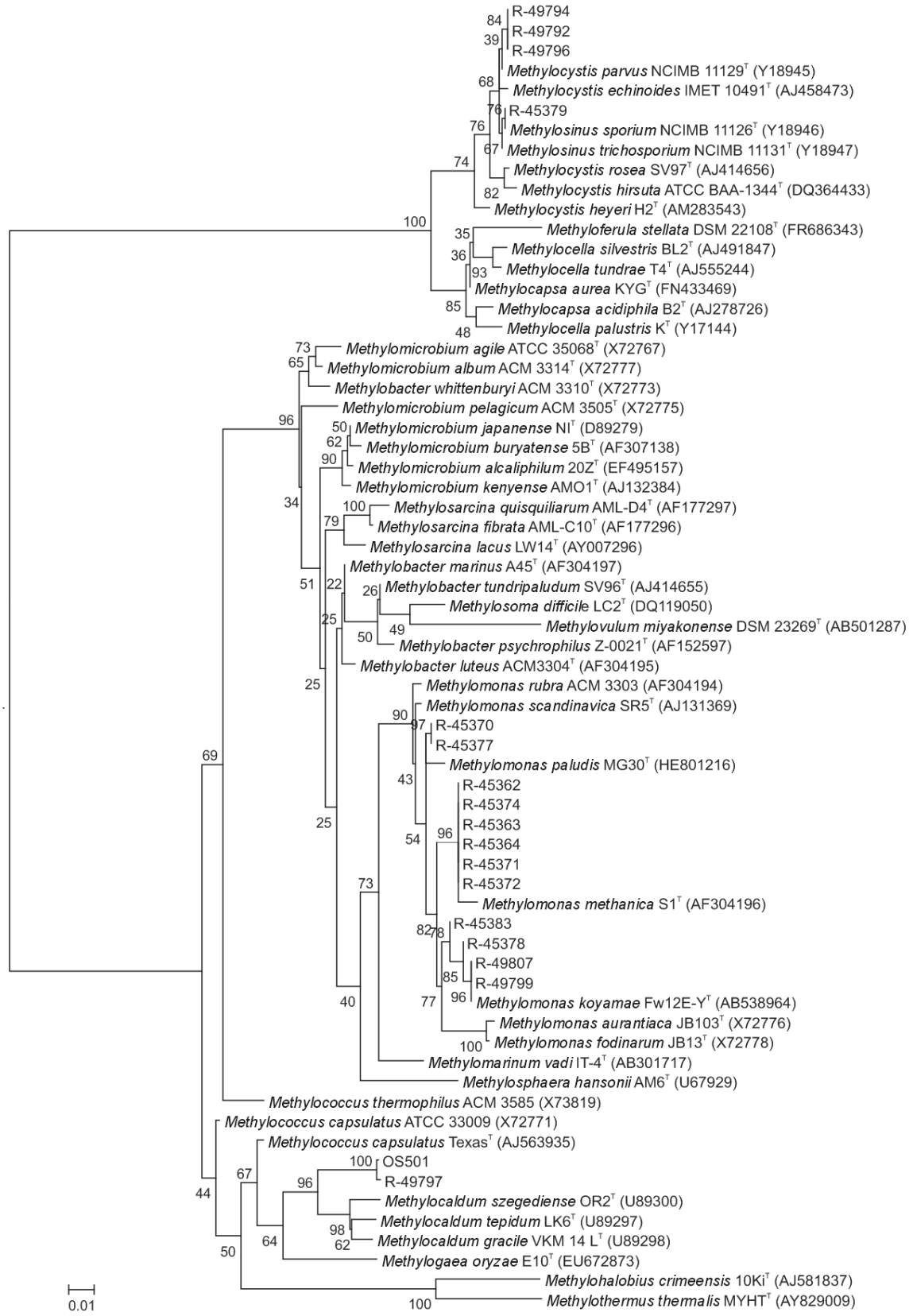

Figure C-1: 16S rRNA gene phylogenetic maximum-likelihood tree (1220 nucleotide alignment; GTR+Gamma substitution model; 1000-replicate bootstrapping; rooted between alphaproteobacterial and gamma-proteobacterial MOB) of strains used throughout Chapters III to VII with selected reference strains Bar: 0.01 substitutions per nucleotide position.

REFERENCES

Bowman, J.P., Sly, L.I., Cox, J.M.,Hayward, A.C., 1990. *Methylomonas fodinarum* sp nov and *Methylomonas aurantiaca* sp nov – 2 closely related Type I obligate methanotrophs. Syst Appl Microbiol 13, 279-287.

Bowman, J.P., Sly, L.I., Nichols, P.D.,Hayward, A.C., 1993. Revised taxonomy of the methanotrophs – Description of *Methylobacter* Gen Nov, emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. International Journal of Systematic Bacteriology 43, 735-753.

Dumont, M.G., Pommerenke, B., Casper, P.,Conrad, R., 2011. DNA-, rRNA- and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment. Environ Microbiol 13, 1153-1167.

Kim, H.J., Graham, D.W., DiSpirito, A.A., Alterman, M.A., Galeva, N., Larive, C.K. et al., 2004. Methanobactin, a copper-acquisition compound from methane-oxidizing bacteria. Science 305, 1612-1615.

Kocher, S., Breitenbach, J., Muller, V.,Sandmann, G., 2009. Structure, function and biosynthesis of carotenoids in the moderately halophilic bacterium Halobacillus halophilus. Arch Microbiol 191, 95-104.

Mohanty, S.R., Bodelier, P.L.E., Floris, V.,Conrad, R., 2006. Differential effects of nitrogenous fertilizers on methane-consuming microbes in rice field and forest soils. Appl Environ Microbiol 72, 1346-1354.

Ogiso, T., Ueno, C., Dianou, D., Huy, T.V., Katayama, A., Kimura, M.,Asakawa, S., 2012. Methylomonas koyamae sp nov., a type I methane-oxidizing bacterium from floodwater of a rice paddy field. Int J Syst Evol Microbiol 62, 1832-1837.

Semrau, J.D., DiSpirito, A.A.,Yoon, S., 2010. Methanotrophs and copper. FEMS Microbiol Rev 34, 496-531.

Sharpe, P.L., Dicosimo, D., Bosak, M.D., Knoke, K., Tao, L., Cheng, Q.,Ye, R.W., 2007. Use of transposon promoter-probe vectors in the metabolic engineering of the obligate methanotroph Methylomonas sp. strain 16a for enhanced C40 carotenoid synthesis. Appl Environ Microbiol 73, 1721-1728.

Stackebrandt, E.,Ebers, J., 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 33, 152-155.

Whittenbury, R., Davies, S.L.,Davey, J.F., 1970. Exospores and cysts formed by methane-utilizing bacteria. J Gen Microbiol 61, 219-226.

Part D

Preservation of methane-, ammonia- and nitrite-oxidizing bacteria

Chapter VIII

Survival or Revival:

Long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria

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Redrafted from:

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Author Contributions:

SH, KH and PDV designed the experiments. SH performed the experiments and wrote the paper. KVH performed the statistical analysis. KH, PDV, NB, PV and KVH commented on the manuscript.

SUMMARY

Knowledge on long-term preservation of micro-organisms is limited and research in the field is scarce despite its importance for microbial biodiversity and biotechnological innovation. Preservation of fastidious organisms such as methane-oxidizing bacteria (MOB) has proven difficult. Most MOB do not survive lyophilization and only some can be cryopreserved successfully for short periods. A large-scale study was designed for a diverse set of MOB applying fifteen cryopreservation or lyophilization conditions. After three, six and twelve months of preservation, the viability (via live-dead flow cytometry) and culturability (via most-probable number analysis and plating) of the cells were assessed. All strains could be cryopreserved without a significant loss in culturability using 1% trehalose in 10-fold diluted TSB (TT) as preservation medium and 5% DMSO as cryoprotectant. Several other cryopreservation and lyophilization conditions, all of which involved the use of TT medium, also allowed successful preservation but showed a considerable loss in culturability. We demonstrate here that most of these non-culturables survived preservation according to viability assessment indicating that preservation induces a viable but non-culturable (VBNC) state in a significant fraction of cells. Since this state is reversible, these findings have major implications shifting the emphasis from survival to revival of cells in a preservation protocol. We showed that MOB cells could be significantly resuscitated from the VBNC state using the TT preservation medium.

INTRODUCTION

Long-term preservation of micro-organisms is an often neglected but very important aspect of both applied and environmental microbiology (Stahl and Wagner, 2006; Emerson and Wilson, 2009; Joint et al., 2010). Preservation of microbial resources allows (i) validation of previously obtained results, (ii) ensures that strains do not get lost during research, (ii) catalogs biodiversity for future research and (iv) enables scientists to apply well documented cultures for biotechnological or commercial use. Novel isolates can either be preserved in the research lab or deposited in public culture collections, safeguarding thousands of strains available for the scientific community upon request (Smith, 2003; Janssens et al., 2010). Long-term storage is most often achieved by lyophilization or cryopreservation in liquid nitrogen or at -80°C (De Paoli, 2005). The former requires specialized equipment and thus is mainly used by culture collections, and offers advantages over cryopreservation such as ease of storage and transport (Morgan et al., 2006). Besides the choice and concentration of cryo- or lyoprotectant, many other parameters may influence the success of preservation, such as the growth, preservation and resuscitation medium, growth rate, culture density, the rate of freezing and thawing or the instrument settings during lyophilization (Morgan et al., 2006). Therefore, long-term preservation is considered an empirical research field, where it is very difficult to compare or apply data from literature. Nonetheless, a wide variety of micro-organisms have already been successfully maintained for decades using these procedures (Hubalek, 2003). However, standard preservation protocols appear to be less successful for many fastidious organisms, such as methane-oxidizing bacteria (MOB). Lyophilization of MOB is generally unsuccessful, while cryopreservation is possible but usually only for short periods (Green and Woodford, 1992; Bowman, 2006). Therefore, many of these cultures can only be maintained by periodic sub-cultivation or are kept at low metabolic rates (4°C, under appropriate atmosphere). As a result, several MOB are no longer extant (Bowman et al., 1993), despite being a well-studied and functionally important group of bacteria (Hanson and Hanson, 1996).

Upon resuscitation (after cryopreservation) or rehydration (after lyophilization), a preservation method is considered successful when strains are culturable (qualitatively) after preservation, preferably in high numbers (quantitatively), as evaluated by counting either most probable numbers (MPN) or colony forming units (CFU) (Cleland et al., 2004). Besides culturability, viability of cells can also be assessed based on certain cellular

functions, such as membrane integrity (Joux and Lebaron, 2000). For example, fluorescent dyes such as SYBR®Green can penetrate cells with intact cytoplasmic membranes, while propidium iodide only penetrates damaged membranes, resulting in green or red fluorescence respectively (Wang et al., 2010). Cells in these different physiological states can then be counted rapidly by flow cytometry (Porter et al., 1995). By applying such methods, it was discovered that cells can enter a state of dormancy (Xu et al., 1982), termed the viable but non-culturable (VBNC) state, at which cells are still alive but fail to grow using conditions that would normally allow their growth (Oliver, 2005, 2010). The VBNC state is induced by certain stress factors, such as starvation, decreased temperatures or elevated osmotic concentrations (Oliver, 2005), which are relevant for preserved cells, although the VBNC state has thus far not been linked to preservation. Considering that the VBNC state has been found to be reversible (Whitesides and Oliver, 1997), it would be most informative to know whether a preservation method failed due to cell death or induction of the VBNC state, in order to plan resuscitation efforts accordingly.

The aim of the present study was to evaluate the success of long-term preservation of a diverse set of MOB by applying 15 preservation conditions using both lyophilization and cryopreservation. Viability was assessed through live/dead flow cytometry while culturability was evaluated through MPN analysis and plating. Since both viability and culturability were assessed quantitatively, it was explored whether non-culturable cells were either dead or still viable and whether the VBNC fraction could be reduced by resuscitation efforts. The obtained results showed that all MOB could be successfully preserved by cryopreservation and lyophilization, and demonstrated that a VBNC state induced by preservation could be significantly reduced using a specifically adapted preservation medium.

EXPERIMENTAL PROCEDURES

Strains, growth conditions and preservation cultures

Most of the aerobic methane-oxidizing diversity obtained so far is phylogenetically positioned within the *Alphaproteobacteria* (Type II MOB) and the *Gammaproteobacteria* (Type Ia/b MOB) (Dedysh, 2009). Ten methane-oxidizing type strains were obtained from the DSMZ and NCIMB culture collections (www.dsmz.de and www.ncimb.com) and their main characteristics are listed in Table VIII-1. Nine strains were cultivated in a diluted Nitrate Mineral Salts (dNMS) medium, as described previously (Hoefman et al., 2011b), while DSM 19304^T was cultivated in DSM1180 medium (pH 9.0) (www.dsmz.de). Cultures were grown either in broth with 20% (v/v) CH_4 added to the headspace or on solid medium in gas-tight jars (Oxoid, UK) under a CH_4:air (1:1) atmosphere.

Prior to preservation, the MOB strains were grown in broth at optimal temperature (Table VIII-1) to early stationary phase in standard growth medium (dNMS or DSM1180) as well as in a carbon-rich preservation medium, i.e. a ten-fold diluted trypticase soy broth (BD, France) medium supplemented with 1% trehalose (TT medium). DSM 19304^T could only be cultivated in DSM1180 medium. To allow harvesting of cells at early stationary phase, growth curves for all cultures and in each medium were established in preliminary experiments (data not shown).

MPN and plate enumeration

To enumerate the culturable MOB at early stationary phase, either most probable number (MPN) or plate counts were performed. For MPN counts, a dilution series (10^{-2} up to 10^{-9}) was prepared in triplicate in 96-well microtiter plates (200 µL/well) and incubated in dNMS or DSM1180 medium at optimal temperature (Table VIII-1) in gas-tight jars (Oxoid, UK) under a CH_4:air (1:1) atmosphere. After one and two weeks of incubation, growth was monitored by measuring the optical density at 600 nm (Spectramax+384, Molecular Devices, USA) and most probable numbers were calculated using MPN-Tables (USDA-FSIS, 2008). Plate counts were estimated by plating dilutions 10^{-1}, 10^{-3} and 10^{-5} in duplicate (50 µL/plate) on dNMS or DSM1180 medium and incubated as mentioned higher. The amount of colony forming units per volume (CFU/mL) was estimated after one and two weeks of incubation. For logistic reasons, culturability was quantified by MPN for the eight strains grown at 28°C, and by plate counting for the remaining two strains.

Table VIII-1: Preservation conditions with corresponding abbreviations (left) and MOB type strains with corresponding species name, type of MOB and standard cultivation conditions (right). Use of TT preservation medium for growth, preservation and resuscitation of cells is mentioned in brackets. Standard medium was used for the other preservation conditions. DSM 19304^T could not be cultivated in TT medium.

Preservation Condition[a]	Abbreviation	Strain	Species	Type	Standard medium	Temp. (°C)
Lyophilization		NCIMB 11130^T	*Methylomonas methanica*	Ia	dNMS	28
20% Sucrose / 10% BSA	S/BSA	DSM 13736^T	*Methylosarcina fibrata*	Ia	dNMS	28
20% Sucrose / 10% BSA (TT)	S/BSA/TT	DSM 19304^T	*Methylomicrobium alcaliphilum*	Ia	DSM1180	28
7.5 % Trehalose in horse serum	T/HS	NCIMB 11912^T	*Methylocaldum gracile*	Ib	dNMS	28
7.5% Trehalose in horse serum (TT)	T/HS/TT	NCIMB 11853^T	*Methylococcus capsulatus*	Ib	dNMS	37
10% Skimmed Milk	S.Milk	DSM 17706^T	*Methylosinus sporium*	II	dNMS	28
12% Glycine Betaine	LPA-GB	NCIMB 11131^T	*Methylosinus trichosporium*	II	dNMS	28
Cryopreservation (liquid nitrogen)		DSM 18500^T	*Methylocystis hirsuta*	II	dNMS	28
15% Glycerol	Glyc	NCIMB 11129^T	*Methylocystis parvus*	II	dNMS	28
15% Glycerol (TT)	Glyc/TT	DSM 15673^T	*Methylocella tundrae*	II	dNMS	20
5% DMSO	DMSO					
5% DMSO (TT)	DMSO/TT					
5% DMSO[b]	DMSO/-80					
10% Methanol	Methanol					
20% Sucrose	Sucrose					
12% Glycine Betaine	CPA-GB					
Microbank Beads[b]	Beads					

[a]Final concentrations are listed.

[b]Conditions preserved at -80°C and not in liquid nitrogen.

Viability counts by flow cytometry

MOB were quantified by live/dead flow cytometry (FCM). The cultures were diluted to optimal cell density (10^4 – 10^6 cells/mL) for FCM measurements and 10 µL mL^{-1} live/dead stain, 5 mM Na_2EDTA and 10 µL mL^{-1} Cytocount™ beads at 1,064 particles $µL^{-1}$ (Dako, Denmark) were added. The live/dead stain was composed of 30 mM propidium iodide (Invitrogen Switzerland) mixed with SYBR®Green I (10^{-2} dilution in dimethyl sulfoxide (DMSO)) at a ratio of 1:50. Following staining, samples were mixed thoroughly and incubated in the dark for 15 min at room temperature. Samples were analyzed using a CyAn™ High Performance Flow Cytometer (Dakocytomation, Belgium) equipped with a 50-mW sapphire solid-state diode laser, emitting at a fixed wavelength of 488 nm. The optimal settings for each strain were determined as described by (Wang et al., 2010). Briefly, the instrument voltage values for side angle light scatter (SSC), green fluorescence and red fluorescence were optimized for log-phase MOB cells. No compensation was applied to sample analysis. For each sample run, log-phase cells of stained MOB strains and non-stained MOB strains were used as positive and negative controls, respectively. Heat-killed (90°C for 3 min) cells were used as inactivation control for the stains applied in the flow cytometric analysis. Samples were run until a bead count of approximately 300 was reached at an EPS (Events Per Second) rate below 10,000/s. Green fluorescence was collected at 510-550 nm, red fluorescence at 603-623 nm. Data were analyzed using Summit™ 4.3 software. Following the principle that only SYBR®Green I penetrates cells with intact membranes resulting in green fluorescence, and both SYBR®Green I and propidium iodide penetrate cells with destroyed membranes resulting in red fluorescence, the amount of viable cells mL^{-1} (based on bead counts) of the MOB cultures was calculated.

Preservation

(i) Lyophilization

Ten MOB type strains were lyophilized. Samples were centrifuged for 15 min at 6,000 g and resuspended in a lyoprotectant (LPA). The six LPA combinations are listed in Table VIII-1. For conditions in TT medium, besides the preservation of cultures in this medium in combination with an LPA, cultures were also grown prior to preservation and rehydrated after preservation in TT medium. For clarity, TT medium will be further termed as a ‘preservation medium’, although this is a simplification for ‘pre-preservation growth, preservation and rehydration medium’. The combination of cultures with protectants

resulted in 58 suspensions. Ampoules (AR-glass, 7 mm diameter, 0.9 mm thickness) were filled with 100 µL of each suspension in triplicate (one for each evaluation time-point), a cotton plug was implemented and the suspensions were lyophilized: after an initial freezing step at -50°C for 60 min in a pre-cooled plate (-50°C), the ampoules were subjected to a primary (-18 °C, 0.5 mbar, 410 min) and secondary drying phase (20 °C, 0.012 mbar, 746 min). Ampoules were subsequently heat-sealed under vacuum (<0.13 mbar) and stored at 4°C in the dark.

(ii) Cryopreservation

The ten MOB type strains cultivated as explained above were subjected to long-term preservation by cryopreservation in liquid nitrogen. Cryotube™ vials (Nunc, Denmark) were used to add 500 µL sample to 500 µL cryoprotectant (CPA). The eight CPA combinations are listed in Table VIII-1. As mentioned above, for conditions in TT medium, this medium was used as a pre-preservation growth, preservation and resuscitation medium, but is further termed 'preservation medium' for clarity. Vials were stored in liquid nitrogen, in the gas phase just above the liquid phase. DMSO as a CPA was also tested at -80°C. The combination of cultures with protectants resulted in 78 suspensions, prepared in triplicate (one for each evaluation time-point). When glycerol, sucrose and betaine were used as a CPA, contact between cells and CPA was at least 60 min to allow cellular uptake of CPA prior to preservation. Manipulations with DMSO and methanol in contact with cells were executed at 4°C and care was taken to preserve the cells immediately after addition of these CPA, to avoid toxic effects. The MOB were also cryopreserved using Microbank™ beads (Pro-Lab Diagnostics, USA). After centrifuging (15 min, 6,000 g) 1 mL of each culture grown in carbon-deficient media, the cell pellet was harvested using sterile cotton swabs and beads were inoculated with cell material according to the manufacturer's instructions. The vials were preserved at -80°C.

Rehydration and resuscitation

The lyophilized suspensions were rehydrated after 3, 6 and 12 months. Ampoules were opened and 1 mL fresh medium (either dNMS, DSM1180 or TT medium) was added to rehydrate the cells and the suspensions were transferred to eppendorf tubes. After a rehydration step at room temperature for approximately one hour, the culturable and viable recovery of the cells was evaluated as above.

The cryopreserved suspensions were resuscitated after 3, 6 and 12 months. Vials were thawed at 37°C, and upon thawing, immediately transferred to eppendorf tubes and centrifuged (6,000 g, 15 min). After supernatant removal, an equal volume of fresh medium (either dNMS, DSM1180 or TT medium) was added to avoid toxic effects of DMSO and methanol at room temperature. After a resuscitation step at room temperature for approximately one hour, the culturable and viable recovery of the cells was evaluated as above. The survival of the cultures on Microbank^{TM} beads was evaluated on plates according to the manufacturer's instructions. Since quantification is not possible using the beads, culturability was only assessed qualitatively for this preservation condition.

Quality control

Prior to preservation, the ten MOB type strains were verified for absence of heterotrophic satellites on trypticase soy agar after 2 weeks of incubation without any additional CH_4 in the atmosphere. In addition, 16S rRNA gene sequence analysis was performed on the cultures, as described previously (Hoefman et al., 2011a). Both quality control tests were also performed on the cryopreserved cultures after a 12-month preservation period with DMSO as CPA in standard medium as well as in TT medium.

Statistical analysis

Prior to statistical analysis, recorded measurements were log transformed. Imputation of missing values was performed by implementing the expectation-maximization (EM) maximum likelihood estimation (MLE) algorithm, with the maximum number of iterations set at 1000. Nested analysis of variance (ANOVA) designs with repeated measures were used for evaluating the possible effect of the duration of preservation and the effect of LPA or CPA conditions used on culturability and viability measured by MPN and FCM, respectively. Depending on whether or not the assumption of sphericity was respected, no correction (Mauchly's test of sphericity, $p > 0,05$) or a Greenhouse-Geisser or Huynh-Feldt correction (Mauchly's test of sphericity, $p < 0,05$ with estimated epsilon value $\varepsilon < 0,75$ or $\varepsilon < 0,75$ respectively) was taken into account. ANOVA implementing Scheffé's post-hoc test was used to assess which preservation condition scored significantly better than others. The effect of adding TT was assessed using a paired samples T-test. All statistical data analyses were performed using IBM SPSS Statistics 19 (IBM, Brussels, Belgium).

RESULTS

Dataset generation for long-term preservation of MOB

Currently, there is no universal protocol for the long-term preservation of methane-oxidizing bacteria. Therefore, a diverse set of ten type strains of MOB, representing eight genera and the three major MOB types (Type Ia, Ib & II), was tested using 15 preservation conditions (Table VIII-1). The conditions were evaluated after 3, 6 and 12 months of preservation, either by cryopreservation or lyophilization. The success rate of different preservation conditions can be evaluated by simply checking if resuscitated cultures can still grow, or more thoroughly, by counting the fraction of cells that are viable (based on FCM) and the fraction that can grow (based on MPN and plating). As a result of cell death due to the harsh conditions during preservation, it is expected that both these fractions will drop. Indeed, Figure VIII-1 displays the average log transformed outcome of all combinations of cultures and preservation conditions and shows a significant drop (p-value <0.05) in both viability and culturability after preservation.

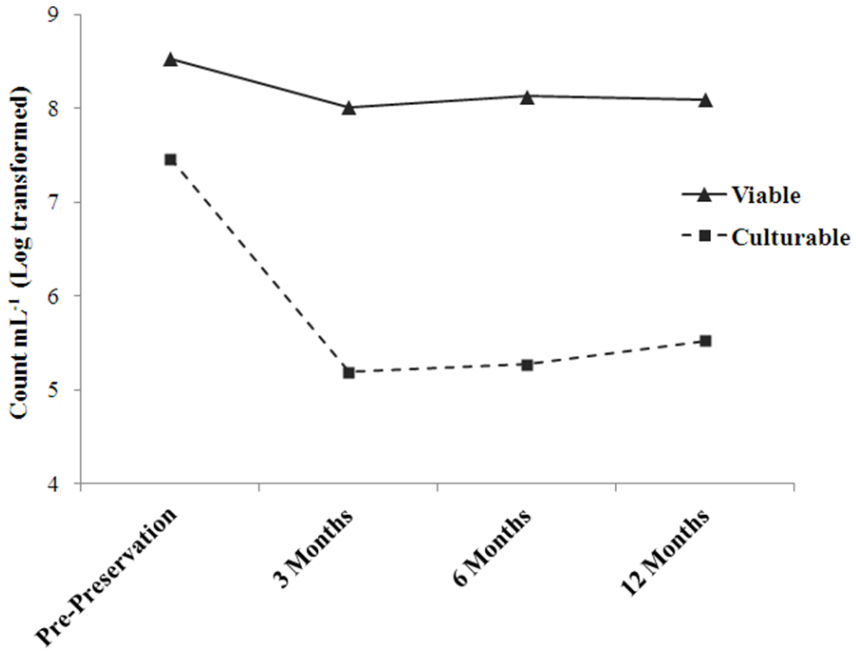

Figure VIII-1: Effect of long-term preservation on MOB viability and culturability after 3, 6 and 12 months. The average viable (black triangle) and culturable (black square) log transformed cells per mL of all tested strains in all preservation conditions was plotted over time: before preservation & 3, 6 and 12 months post-preservation. For both the viable as well as the culturable counts, counts were significantly lower after preservation (p-value < 0.05), while the 3 tested time points post-preservation were similar compared to one another (p-value > 0.05).

Interestingly, the three tested time points post-preservation were found to be highly similar, indicating that there was no decline (p-value <0.05) in viability or culturability over the tested time frame. Therefore, the data obtained after 12-month preservation were selected to investigate differences between preservation conditions and counts more deeply.

Qualitative evaluation of MOB culturability

For each condition, the percentage of cultures that could still grow after 12 months preservation was calculated (Table VIII-2). Three conditions were found to preserve all strains successfully: DMSO/TT (cryopreservation), Glyc/TT (cryopreservation) and S/BSA/TT (lyophilization). Interestingly, in these three preservation conditions TT medium was used instead of standard growth medium as pre-preservation growth, preservation and resuscitation medium and performed better than their corresponding condition in standard medium, even when excluding results of DSM 19304^T, which was not tested in TT medium (Table VIII-2). The lowest culturability rate (10%) was observed with glycine betaine as a lyoprotectant, while most of the other preservation conditions resulted in culturability rates around 60-90%. NCIMB 11912^T appeared to be the most difficult strain to grow after preservation with overall culturability of 47%, while this ranged between 60-93% for the other strains. Strain authenticity was confirmed by 16S rRNA gene sequence analysis and absence of growth on TSA plates.

Table VIII-2: The percentage of strains that were culturable or viable per preservation condition (left) and the percentage of preservation conditions for which culturability or viability was achieved per strain (right) after 12 months of preservation. Values between brackets exclude DSM 19304^T, the strain not tested in TT medium, to allow a more accurate comparison of the four conditions tested in TT medium with their corresponding conditions in standard medium.

Condition	Culturable (%)	Viable (%)	Type Strain	Culturable (%)	Viable (%)
Lyophilization			DSM 18500^T	87	93
S/BSA	80 (78)	80 (90)	DSM 13736^T	87	64
S/BSA/TT	100	100	DSM 17706^T	93	93
T/HS	60 (67)	100 (100)	DSM 15673^T	87	100
T/HS/TT	89	100	NCIMB 11912^T	47	92
S.Milk	70	83	NCIMB 11130^T	73	100
LPA-GB	10	44	NCIMB 11129^T	93	100
Cryopreservation			NCIMB 11131^T	93	100
Glyc	80 (78)	100 (100)	NCIMB 11853^T	60	93
Glyc/TT	100	100	DSM 19304^T	64	89
DMSO	90 (89)	100 (100)			
DMSO/TT	100	100			
DMSO/-80	90	100			
Methanol	80	90			
Sucrose	80	90			
CPA-GB	90	100			
Beads	60	NT			

Quantitative evaluation of MOB culturability

To investigate the success of each condition in more detail, the culturability was examined quantitatively: a condition is more successful when the drop in culturability after preservation is lower. Based on the average culturability log transformed drops, a ranking of the preservation conditions was calculated (Table VIII-3).

Table VIII-3: Ranking and separation into homogenous subsets using Scheffé's test of tested preservation conditions based on average log transformed culturability data.

Ranking	Condition	Method	Group (Scheffé's test[a,b])		
			A	B	C
1	DMSO/TT	Cryopreservation	0.0[c]		
2	DMSO	Cryopreservation	0.7		
3	Glyc/TT	Cryopreservation	0.8		
4	CPA-GB	Cryopreservation	1.0		
5	DMSO/-80	Cryopreservation	1.0		
6	Methanol	Cryopreservation	1.2		
7	T/HS/TT	Lyophilization	1.3		
8	Sucrose	Cryopreservation	1.5		
9	S/BSA/TT	Lyophilization	1.7		
10	Glyc	Cryopreservation		2.0	
11	S/BSA	Lyophilization		2.1	
12	T/HS	Lyophilization		3.2	
13	S.Milk	Lyophilization		3.4	
14	LPA-GB	Lyophilization			5.3

[a]Groups A & B and B & C are homogenous subsets (no significant difference). Members of group A are significantly different from members of group C (p-value <0.05).
[b]Average mean culturable log drop (compensated for missing values).
[c]Increasing culturable log drops from top to bottom.

Interestingly, the condition DMSO/TT, which allowed preservation of all strains successfully based on qualitative data (Table VIII-2), also resulted in the lowest culturability drops quantitatively. This suggests that MOB can be stored most successfully through cryopreservation in liquid nitrogen with 5% DMSO using TT preservation medium. Four out of six lyophilization conditions were less successful than most cryopreservation conditions (group B&C, Table VIII-3). However, the other two lyophilization conditions, S/BSA/TT and T/HS/TT, notably the only ones using the TT medium, were grouped among the best conditions (group A, Table VIII-3). Considering that S/BSA/TT was one of the three conditions suited for successful preservation of all strains, lyophilization of MOB combining sucrose and BSA as LPA in TT as preservation medium can be considered as a formidable alternative to cryopreservation. The preservation condition using glycerol, the most common CPA, was found among the worst preservation conditions of this study. However, glycerol scored markedly better in combination with TT medium, similar as was observed for the other preservation conditions using TT medium. No significant difference was observed when comparing the

preservation of DMSO in liquid nitrogen or at -80°C. Lyophilization of MOB using glycine betaine was the least successful condition in the present study, both qualitatively (Table VIII-2) and quantitatively (Table VIII-3).

VBNC state induced by long-term preservation of MOB

Besides data on culturability, Table VIII-2 also lists the percentage of strains that were still viable after 12 months preservation, according to flow cytometric live-dead counts. Qualitatively, nine preservation conditions resulted in 100% viability of the tested strains, while this was only true for three preservation conditions based on culturability (Table VIII-2). Quantitatively, the drop in culturability was found to be significantly larger than the drop in viability (p-value <0.05), as visualized for the total dataset (Figure VIII-1) as well as for each preservation condition (Figure VIII-2) and each strain per condition (Table VIII-4). Indeed, Figure VIII-2 shows that the average culturable drop is more extensive (larger bars) than the average viable drop. These results indicate that in addition to a fraction of cells that die off during preservation, a viable but non-culturable (VBNC) state is also induced. The VBNC state was defined here as the increased difference between viable FCM counts and culturable counts induced by preservation (arrow Figure VIII-2), while resuscitation from the VBNC state was assumed when this difference could be reduced. To evaluate the effect of TT medium on the VBNC fraction, a separate dataset was created excluding DSM 19304^{T} and including only the four preservation conditions with TT medium and their corresponding conditions in standard medium. Statistical analysis showed that the fraction of viable cells was similar for both media. However, for the TT medium a significantly higher culturable fraction was observed, or in other words, the fraction of VBNC cells was significantly reduced by use of TT medium.

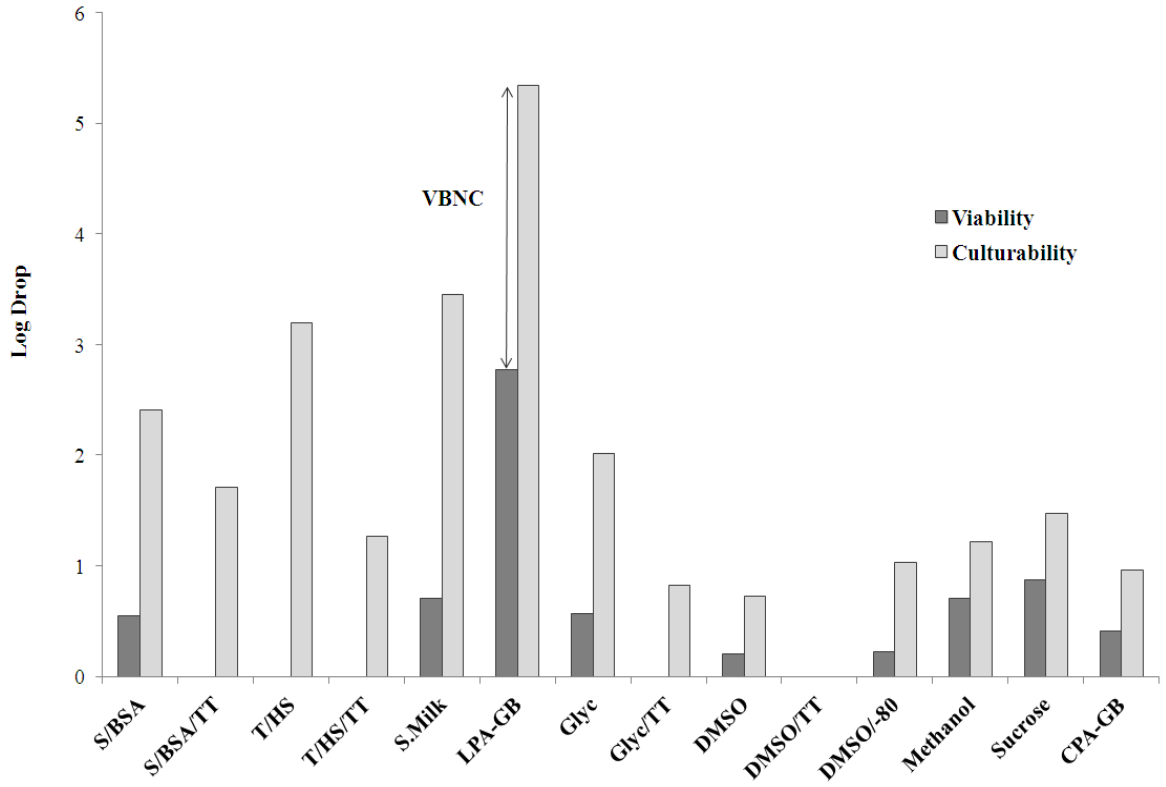

Figure VIII-2: Average viable (dark grey bars) and culturable (light grey bars) log drops of all tested strains plotted for each preservation condition. Log drops were obtained by subtraction of 12 month post-preservation counts by pre-preservation counts using log transformed data. Following preservation, the drop in culturability is mostly more extensive (larger bars) than the drop in viability indicating that a vast number of cells became viable-but-non-culturable (VBNC) after the preservation process (arrow indicating VBNC as difference between viable and culturable log drop for condition LPA-GB). Bars that are not visible indicate that no drop was observed after preservation (e.g. DMSO/TT for both viable and culturable log drop counts). The use of TT medium before, during and after preservation reduced the amount of VBNC cells significantly (p-value < 0.05; e.g. T/HS/TT compared with T/HS) in a comparison between the four conditions using TT with the four corresponding conditions in standard medium. When the viability drops, the culturability also drops but to a higher extent, it may indicate that FCM can be used to partially predict the success of subsequent cultivation attempts following long-term preservation.

Table VIII-4: The log drop in viability and culturability and the VBNC fraction for each strain tested with each preservation condition. The VBNC fraction was calculated by subtraction of the culturable drop by the viable drop. Detection limit values were used to calculate drops with counts that were below this limit, such calculations are marked with ≥, indicating that the true drop is potentially even higher. Calculations of drops that resulted in negative values were indicated as 0, since these would implicate an impossible increase after preservation, attributed to variations in the measurements.

		Preservation Condition[a,b]													
		Lyophilization						Cryopreservation							
Data Processed	Type Strain	S/BSA	S/BSA/TT	T/HS	T/HS/TT	S.Milk	LPA-GB	Glyc	Glyc/TT	DMSO	DMSO/TT	DMSO/-80	Methanol	Sucrose	CPA-GB
Viable Drop	DSM 18500^T	0.0	0.0	ND	0.5	0.0	≥ 4.6	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0
	DSM 13736^T	≥ 3.8	0.0	0.0	0.0	≥ 2.6	≥ 4.0	1.2	0.0	0.8	0.0	0.0	≥ 4.0	≥ 3.9	1.6
	DSM 17706^T	0.0	0.0	0.8	0.0	0.0	≥ 4.2	0.1	0.0	0.0	0.0	1.4	0.0	0.0	0.0
	DSM 15673^T	0.0	0.0	0.0	1.1	ND	0.8	0.1	0.0	0.0	0.0	0.5	0.2	0.1	0.1
	NCIMB 11912^T	0.0	0.0	ND	0.0	ND	≥ 4.2	0.8	0.0	0.7	0.0	0.8	0.8	0.5	0.1
	NCIMB 11130^T	0.0	0.0	0.2	0.0	ND	ND	0.9	0.0	0.2	0.0	0.4	1.1	0.0	1.0
	NCIMB 11129^T	0.0	0.0	0.0	0.0	0.1	1.2	0.0	0.0	0.1	0.0	0.1	0.4	0.0	0.0
	NCIMB 11131^T	0.0	0.3	0.0	0.1	0.6	0.0	0.0	0.7	0.0	0.0	0.0	0.1	0.0	0.0
	NCIMB 11853^T	0.0	0.9	0.1	0.0	0.6	≥ 4.0	1.3	0.0	0.0	0.1	0.0	0.4	1.5	0.9
	DSM 19304^T	≥ 3.7	NT	0.0	NT	ND	0.0	1.7	NT	1.1	NT	0.1	0.0	1.5	0.9
Culturable Drop	DSM 18500^T	2.0	2.6	≥ 6.5	4.6	2.3	≥ 6.5	3.6	3.6	1.0	0.6	0.6	0.6	0.6	1.3
	DSM 13736^T	1.6	3.0	0.0	1.3	2.6	≥ 4.5	0.3	1.0	1.0	0.5	0.8	≥ 4.5	2.6	0.8
	DSM 17706^T	0.6	1.0	1.3	1.0	2.6	≥ 6.5	0.6	0.0	0.6	0.0	0.3	1.0	1.3	0.3
	DSM 15673^T	0.0	0.0	≥ 5.3	0.1	0.5	≥ 5.3	0.7	0.0	0.0	0.0	0.0	0.0	0.8	0.3
	NCIMB 11912^T	≥ 1.8	0.0	1.8	0.0	1.8	≥ 1.8	≥ 1.8	0.0	≥ 1.8	0.0	≥ 1.8	≥ 1.8	≥ 1.8	≥ 1.8
	NCIMB 11130^T	5.7	6.4	≥ 6.2	0.6	≥ 6.2	≥ 6.5	1.7	2.0	1.0	1.3	0.7	0.7	3.3	1.7
	NCIMB 11129^T	1.0	1.0	4.6	0.7	3.8	≥ 6.8	2.6	1.5	0.3	0.7	1.0	1.6	0.3	0.6
	NCIMB 11131^T	2.0	1.4	0.0	2.4	4.0	2.7	2.7	1.7	1.2	0.0	1.0	0.7	1.0	0.4
	NCIMB 11853^T	≥ 3.7	0.2	0.0	≥ 2.0	≥ 3.7	≥ 3.7	≥ 3.7	4.3	0.0	0.0	0.0	0.0	1.7	1.1
	DSM 19304^T	2.7	NT	≥ 6.2	NT	≥ 6.2	≥ 6.5	1.7	NT	1.3	NT	5.0	1.7	1.0	1.0
VBNC	DSM 18500^T	2.0	2.6	ND	4.1	2.3	NV	3.6	3.6	1.0	0.6	0.6	0.6	0.0	1.3
	DSM 13736^T	0.0	3.0	0.0	1.3	0.0	NV	0.0	1.0	0.2	0.5	0.8	0.5	0.0	0.0
	DSM 17706^T	0.6	1.0	0.5	1.0	2.6	NV	0.6	0.0	0.6	0.0	0.0	1.0	1.3	0.3
	DSM 15673^T	0.0	0.0	≥ 5.3	0.0	ND	≥ 4.4	0.6	0.0	0.0	0.0	0.0	0.0	0.6	0.1
	NCIMB 11912^T	≥ 1.8	0.0	ND	0.0	ND	NV	≥ 1.0	0.0	≥ 1.1	0.0	≥ 1.1	≥ 1.1	≥ 1.3	≥ 1.7
	NCIMB 11130^T	5.7	6.4	≥ 5.9	0.6	ND	ND	0.7	2.0	0.8	1.3	0.3	0.0	3.2	0.7
	NCIMB 11129^T	1.0	1.0	4.6	0.7	3.7	≥ 5.6	2.6	1.5	0.3	0.7	0.9	1.2	0.3	0.6
	NCIMB 11131^T	2.0	1.2	0.0	2.3	3.4	2.7	2.7	1.1	1.2	0.0	1.0	0.7	1.0	0.4
	NCIMB 11853^T	≥ 3.7	0.0	0.0	≥ 2.0	≥ 3.1	NV	≥ 2.4	4.3	0.0	0.0	0.0	0.0	0.2	0.2
	DSM 19304^T	0.0	NT	≥ 6.2	NT	ND	≥ 6.4	0.0	NT	0.2	NT	4.9	1.6	0.0	0.1

[a]NT: Could not be tested; [b]ND: Could not be determined

As a detailed example, the effect of long-term preservation on the viability of strain NCIMB 11129^{T} is shown in Figure VIII-3. Prior to preservation most of the cells of the culture were considered viable by visual inspection, due to a larger emission of green fluorescence (SYBR®Green dye) than red fluorescence (PI dye). The majority of the cells was no longer viable after preserving this culture with LPA-GB (B, Figure VIII-3), which corresponded to an expected large drop in culturability (below detection limit, Table VIII-4). Since the FCM profiles and counts after preservation with either Glyc or DMSO/TT were highly similar to the profile and counts prior to preservation, similar cultivation counts were expected. Interestingly, the drop in culturability was a lot higher for Glyc (2.6 logs) than for DMSO/TT (0.7 logs), suggesting that VBNC was induced due to preservation but could be reduced depending on the protectant or preservation medium used. Such FCM profiles were obtained and processed for all combinations of strains and preservation conditions for each time-point (data not shown). FCM profiles of preserved samples showed a large increase in background, which was probably caused by detritus of cells destroyed during the preservation process. Therefore, obtained non-viable counts were probably underestimated and were not used in further calculations.

The drops in viability and culturability as well as the VBNC cell fraction caused by preservation (calculated as the drop in culturability subtracted by the drop in viability) per preservation condition for each of the strains are shown in Table VIII-4. When a clear drop in viability was observed (e.g. for conditions S/BSA, S.Milk and LPA-GB), a corresponding more extensive drop in culturability was observed (Figure VIII-3). The opposite was not necessarily so, since for S/BSA/TT the large drop in culturability did not correspond with a clear drop in viability.

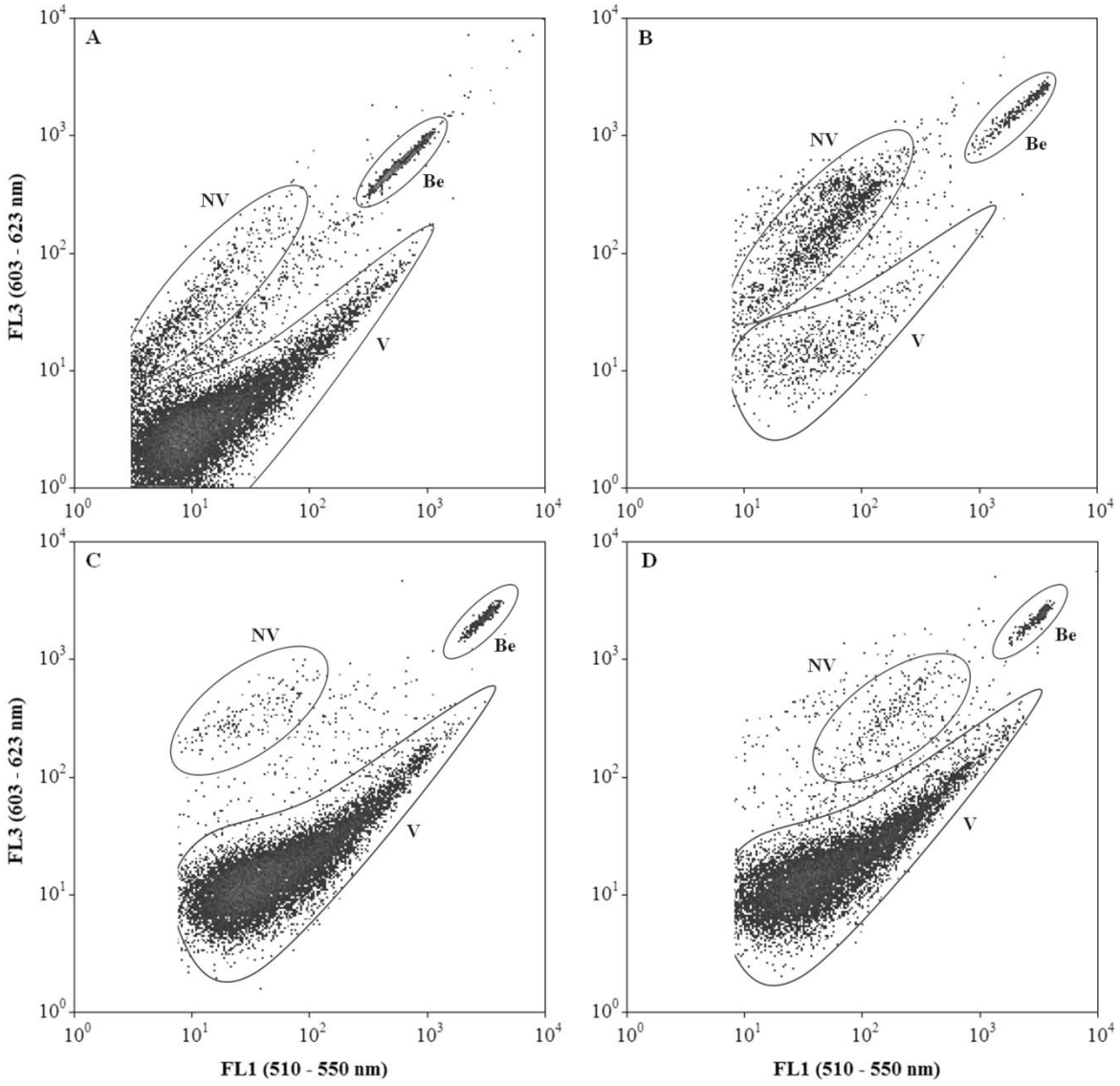

Figure VIII-3: Live-dead staining of *Methylocystis parvus* NCIMB 11129^T prior to preservation (A) and preserved for 12 months using LPA-GB (B), glycerol (C) and DMSO/TT (D) measured by flow cytometry. Green fluorescence (FL1 detector) was plotted against red fluorescence (FL3 detector). Cells emitting more green than red were considered viable (V), cells emitting more red than green were considered non-viable (NV). Cytocount beads (Be) were added to calculate the amount of viable cells per mL. These types of counts were encircled for indicative purposes only. Background counts found at the bottom left are not shown for clarity. Since obtained non-viable counts were expected to be underestimated (detritus of cells causing increase in background), these were not used in actual calculations. Before preservation (A) the majority of cells were viable. However, lyophilization of NCIMB 11129^T with LPA-GB (B) killed the majority of cells (shift from viable to non-viable), which corresponded with an MPN count below detection limit (Table VIII-4). In contrast, a drop in viability could not be observed between pre-preservation of NCIMB 11129^T and post-preservation using glycerol (C) and DMSO/TT (D). Interestingly, despite their similar flow cytometric counts and profiles, the culturable fraction was a lot higher after preserving with DMSO/TT (MPN log drop 0.7; Table VIII-4) than with glycerol (MPN log drop 2.6; Table VIII-4) indicating that the VBNC fraction was limited in the former and extensive in the latter condition.

DISCUSSION

The essence of preserving biomass is the possibility to again grow cells when needed which display identical properties as prior to preservation. Therefore, cultivation is the routine evaluation tool of preservation success. Unfortunately, this approach does not allow differentiation between cell death and survival of cells unable to replicate in given cultivation conditions (i.e. VBNC cells). The VBNC state is known to be induced by stress but has thus far never been linked to preservation. Here, we unequivocally demonstrated that recovery of culturability and not viability of biomass after preservation is most problematic: a significant discrepancy between viability and culturability after preservation was observed for ten MOB type strains (representing the three major types of aerobic MOB; Type Ia, Ib & II) in fifteen different preservation conditions analyzed in parallel by live/dead flow cytometry and cultivation, respectively. To overcome possible subjective and difficult interpretation of FCM data (as described by (Davey, 2011; Davey and Hexley, 2011)), the general trend of high viability was confirmed by highly reproducible results between time points and the observation of an extensive viability drop for the worst preservation conditions. In addition, viable counts with FCM and MPN were highly similar for easily preserved strains *Bacillus cereus* LMG 6923^T and *Pseudomonas aeruginosa* LMG 1242^T that do not demonstrate VBNC due to their high recovery of culturability (data not shown). Injured cells mimicking the VBNC state, and thus not truly dormant cells (Barer, 1997; Lennon and Jones, 2011), were not expected in our dataset since shifts towards increased red fluorescence were not apparent (Figure VIII-3), although this cannot be completely excluded without conclusive tests (Oliver, 2005; Davey, 2011). However, regardless of whether cells are damaged or dormant after preservation, viability assessment proved a valuable asset to preservation methodology for partial prediction of cultivation success (as biomass with high viability drops also showed low culturability). This is of particular interest for slow-growing fastidious micro-organisms, since the rapidly obtained FCM results could be used to focus subsequent laborious cultivation attempts only on biomass with a high viable fraction. Alternatively, culture collections could use FCM as a quick tool as part of their quality control.

Preservation induced a viable but non-culturable state in a significant amount of cells. Since this state can be reversible (Whitesides and Oliver, 1997), additional efforts to resuscitate preserved organisms become crucial aspects of the preservation methodology. Interestingly, the innate cryoprotective effects of carbon-rich growth media is often

overseen (Hubalek, 2003; Siaterlis et al., 2009), because to date most preservation studies focused on heterotrophic bacteria, but it could possibly explain the ease of preserving this kind of microorganisms. Indeed, use of TT medium containing ten-fold diluted TSB and trehalose (which protects cells against desiccation effects during lyophilization and rehydration (Streeter, 2003; Jules et al., 2004)) as preservation medium in the present study did significantly reduce the fraction of VBNC cells of MOB strains, which are normally cultivated in carbon-deficient media (Bowman, 2006). Current data does not allow deduction of the specific cause of this effect, which could be the use of a carbon-rich medium during growth (cellular uptake of protective compounds), preservation or resuscitation, the specific use of TSB and trehalose for this medium, its combination with an extra cryo- and lyoprotectant, or a combination of the above. One can argue that, as preservation is an empirical research field, it is not vital to know the exact mechanisms of the protective role as long as resuscitation improves. Nevertheless extrapolation of our findings to other bacterial groups requires extra tests to pinpoint the resuscitation effect to a specific cause.

In addition to a suitable medium choice, there are alternative ways to resuscitate VBNC cells, such as extended incubation (Song et al., 2009), a temperature shift, addition of peptidoglycan hydrolases (Oliver, 2005, 2010) or signal molecules such as N-acyl homoserine lactones or short peptides (Bruns et al., 2002; Nichols et al., 2008) to the cultivation medium. Filter-sterilized supernatants of fresh cultures are hypothesized to produce resuscitation promoting factors (Aydin et al., 2011) which can increase culturability through quorum sensing (Kaprelyants and Kell, 1993). Unfortunately, tests with filter-sterilized spent medium were unsuccessful here (data not shown). On the other hand, a five-week prolonged MPN analysis did indeed increase culturability for 50% of the tested cases (combination of strains with protectants; data not shown). These observations were unexpected since culturability results after one and two weeks were similar to prior preservation, but could be explained by the scout model proposed by Epstein (Epstein, 2009a, b). This model describes that a small fraction of VBNC cells can ‘wake up’ stochastically and start a new population. In this way, the culturable fraction has a growth rate similar to the culture prior to preservation, while scouts from the VBNC fraction create additional positive wells upon extended incubation.

Preservation of methane-oxidizing bacteria is often reported as problematic and limited in time (Bowman, 2006), but little comparable information is available. By combining fifteen cryopreservation or lyophilization conditions, ten MOB type strains were successfully

preserved for twelve months. Only when using 5% DMSO in combination with a 1% trehalose in ten-fold diluted TSB (TT) preservation medium, all strains could be resuscitated after cryopreservation in liquid nitrogen without a significant quantitative drop in culturability. However, two other conditions could also preserve all strains, namely cryopreservation with glycerol in TT medium and lyophilization with sucrose and BSA in TT medium. Lyophilization was previously deemed unsuccessful for MOB and advised against (Green and Woodford, 1992) but has several advantages over cryopreservation, such as ease of storage and distribution of the material (Morgan et al., 2006). In absence of facilities for liquid nitrogen storage or lyophilization, laboratories can still successfully cryopreserve MOB at -80°C, as was shown in a comparison with DMSO as CPA.

Even without use of TT medium and contradictory to previous reports, cryopreservation with standard CPA's such as DMSO, glycerol and methanol allowed preservation of most strains, without decline over time (no differences in culturability and viability were observed between the datasets after three, six and twelve months of preservation). The presented results indicate that by applying appropriate preservation strategies as also discussed thoroughly by Tindall (Tindall, 2007) and Adams (Adams, 2007), a higher success rate could be achieved using similar conditions as previously reported. Since cell damage is mainly inflicted during freezing and thawing events and not during storage (Pegg, 2007; Seki and Mazur, 2009), a decline in viability or culturability is highly unlikely within testable time frames (Mazur, 1984), if cells are stored at stable low temperatures such as in liquid nitrogen containers. Furthermore, as performed in the present study, protectant exposure time should be adapted based on each type of protectant to improve preservation and resuscitation efficiency or to limit toxicity (Fuller, 2004).

In conclusion, the present study demonstrated that recovery of culturability and not viability is the bottleneck for reviving preserved MOB. Therefore, improved resuscitation procedures should become a key aspect of preservation protocols. It was shown here that the use of a combination of TSB and trehalose in the growth, preservation and resuscitation medium with 5% DMSO as cryoprotective agent significantly reduced the induced viable but non-culturable state. Finally, this was further confirmed by successfully preserving 22 recently isolated MOB (Hoefman et al., 2011a), twenty of which were type I MOB reported to be the most difficult to preserve (Bowman, 2006), as well as eight ammonia-oxidizing bacteria (functionally highly related to MOB) from all four AOB genera (Aakra et al., 2001) (data not shown). Therefore, the here applied methodology is most probably more widely applicable for fastidious organisms.

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REFERENCES

Aakra, A., Utaker, J.B., Pommerening-Roser, A., Koops, H.P.,Nes, I.F., 2001. Detailed phylogeny of ammonia-oxidizing bacteria determined by rDNA sequences and DNA homology values. Int J Syst Evol Microbiol 51, 2021-2030.

Adams, G. (2007) The Principles of Freeze-Drying. In *Cryopreservation and Freeze-Drying Protocols*. Day, J., and Stacey, G. (eds). Totowa, New Jersey: Humana Press, pp. 15-38.

Aydin, I., Saijo-Hamano, Y., Namba, K., Thomas, C.,Roujeinikova, A., 2011. Structural Analysis of the Essential Resuscitation Promoting Factor YeaZ Suggests a Mechanism of Nucleotide Regulation through Dimer Reorganization. Plos One 6.

Barer, M.R., 1997. Viable but non-culturable and dormant bacteria: Time to resolve an oxymoron and a misnomer? J Med Microbiol 46, 629-631.

Bowman, J. (2006) The Methanotrophs - The Families Methylococcaceae and Methylocystaceae. Chapter 3.1.14. *Prokaryotes* **5**: 266-289, DOI: 10.1007/0-387-30745-1_15.

Bowman, J.P., Sly, L.I., Nichols, P.D.,Hayward, A.C., 1993. Revised taxonomy of the methanotrophs: Description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the Group-I methanotrophs. Int J Syst Bacteriol 43, 735-753.

Bruns, A., Cypionka, H.,Overmann, J., 2002. Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central Baltic Sea. Appl Environ Microbiol 68, 3978-3987.

Cleland, D., Krader, P., McCree, C., Tang, J.,Emerson, D., 2004. Glycine betaine as a cryoprotectant for prokaryotes. J Microbiol Methods 58, 31-38.

Davey, H.M., 2011. Life, Death, and In-Between: Meanings and Methods in Microbiology. Appl Environ Microbiol 77, 5571-5576.

Davey, H.M.,Hexley, P., 2011. Red but not dead? Membranes of stressed *Saccharomyces cerevisiae* are permeable to propidium iodide. Environ Microbiol 13, 163-171.

De Paoli, P., 2005. Biobanking in microbiology: From sample collection to epidemiology, diagnosis and research. FEMS Microbiol Rev 29, 897-910.

Dedysh, S.N., 2009. Exploring methanotroph diversity in acidic northern wetlands: Molecular and cultivation-based studies. Microbiology 78, 655-669.

Emerson, D.,Wilson, W., 2009. Giving microbial diversity a home. Nat Rev Microbiol 7, 758-U758.

Epstein, S.S., 2009a. Microbial awakenings. Nature 457, 1083-1083.

Epstein, S.S. (2009b) General Model of Microbial Uncultivability. In *Uncultivated*

Microorganisms. Epstein, S.S. (ed): Springer Berlin / Heidelberg, pp. 131-159.

Fuller, B.J., 2004. Cryoprotectants: The essential antifreezes to protect life in the frozen state. Cryoletters 25, 375-388.

Green, P.N.,Woodford, S.K., 1992. Preservation studies on some obligately methanotrophic bacteria. Lett Appl Microbiol 14, 158-162.

Hanson, R.S.,Hanson, T.E., 1996. Methanotrophic bacteria. Microbiol Rev 60, 439-471.

Hoefman, S., van der Ha, D., De Vos, P., Boon, N.,Heylen, K., 2011a. Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria. Microb Biotechnol.

Hoefman, S., van der Ha, D., De Vos, P., Boon, N.,Heylen, K., 2011b. Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria. Microb Biotechnol. doi: 10.1111/j.1751-7915.2011.00314.x.

Hubalek, Z., 2003. Protectants used in the cryopreservation of microorganisms. Cryobiology 46, 205-229.

Janssens, D., Arahal, D.R., Bizet, C.,Garay, E., 2010. The role of public biological resource centers in providing a basic infrastructure for microbial research. Res Microbiol 161, 422-429.

Joint, I., Muhling, M.,Querellou, J., 2010. Culturing marine bacteria - an essential prerequisite for biodiscovery. Microb Biotechnol 3, 564-575.

Joux, F.,Lebaron, P., 2000. Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. Microbes Infect 2, 1523-1535.

Jules, M., Guillou, V., Francois, J.,Parrou, J.L., 2004. Two distinct pathways for trehalose assimilation in the yeast *Saccharomyces cerevisiae*. Appl Environ Microbiol 70, 2771-2778.

Kaprelyants, A.S.,Kell, D.B., 1993. Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. Appl Environ Microbiol 59, 3187-3196.

Lennon, J.T.,Jones, S.E., 2011. Microbial seed banks: the ecological and evolutionary implications of dormancy. Nat Rev Microbiol 9, 119-130.

Mazur, P., 1984. Freezing of living cells: mechanisms and implications. Am J Physiol 247, C125-C142.

Morgan, C.A., Herman, N., White, P.A.,Vesey, G., 2006. Preservation of micro-organisms by drying; A review. J Microbiol Methods 66, 183-193.

Nichols, D., Lewis, K., Orjala, J., Mo, S., Ortenberg, R., O'Connor, P. et al., 2008. Short peptide induces an "uncultivable" microorganism to grow in vitro. Appl Environ Microbiol 74, 4889-4897.

Oliver, J.D., 2005. The viable but nonculturable state in bacteria. J Microbiol 43, 93-100.

Oliver, J.D., 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol Rev 34, 415-425.

Pegg, D. (2007) Principles of Cryopreservation. In *Cryopreservation and Freeze-Drying Protocols*. Day, J., and Stacey, G. (eds). Totowa, New Jersey: Humana Press, pp. 39-58.

Porter, J., Edwards, C.,Pickup, R.W., 1995. Rapid assessment of physiological status in *Escherichia coli* using fluorescent probes. J Appl Bacteriol 79, 399-408.

Seki, S.,Mazur, P., 2009. The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure. Cryobiology 59, 75-82.

Siaterlis, A., Deepika, G.,Charalampopoulos, D., 2009. Effect of culture medium and cryoprotectants on the growth and survival of probiotic lactobacilli during freeze drying. Lett Appl Microbiol 48, 295-301.

Smith, D., 2003. Culture collections over the world. Int Microbiol 6, 95-100.

Song, J., Oh, H.M.,Cho, J.C., 2009. Improved culturability of SAR11 strains in dilution-to-extinction culturing from the East Sea, West Pacific Ocean. FEMS Microbiol Lett 295, 141-147.

Stahl, D.A.,Wagner, M., 2006. The knowledge explosion in environmental microbiology offers new opportunities in biotechnology. Curr Opin Biotechnol 17, 227-228.

Streeter, J.G., 2003. Effect of trehalose on survival of *Bradyrhizobium japonicum* during desiccation. J Appl Microbiol 95, 484-491.

Tindall, B. (2007) Vacuum-Drying and Cryopreservation of Prokaryotes. In *Cryopreservation and Freeze-Drying Protocols*. Day, J., and Stacey, G. (eds). Totowa, New Jersey: Humana Press, pp. 73-98.

USDA-FSIS 2008 Most Probable Number Procedure and Tables. S.W. Washington, D.C.

Wang, Y.Y., Claeys, L., van der Ha, D., Verstraete, W.,Boon, N., 2010. Effects of chemically and electrochemically dosed chlorine on *Escherichia coli* and *Legionella beliardensis* assessed by flow cytometry. Appl Microbiol Biotechnol 87, 331-341.

Whitesides, M.D.,Oliver, J.D., 1997. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Appl Environ Microbiol 63, 1002-1005.

Xu, H.S., Roberts, N., Singleton, F.L., Attwell, R.W., Grimes, D.J.,Colwell, R.R., 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb Ecol 8, 313-323.

Chapter IX

Efficient cryopreservation protocol enables accessibility of a broad range of ammonia-oxidizing bacteria

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Author Contributions:

SH designed the experiments. SH performed the experiments, analyzed the data and wrote the paper. APR provided the AOB strains. ES helped with the experiments. KH, APR and PDV commented on the manuscript.

SUMMARY

Long-term storage of the fastidious Ammonia-Oxidizing Bacteria has proven difficult, which limits their public availability and results in a loss of cultured biodiversity. To enable their accessibility to the scientific community, an effective protocol for cryopreservation of ammonia-oxidizing cultures at -80°C and in liquid nitrogen was developed. Long-term storage could be achieved using 5% DMSO as cryoprotectant, preferably in a cryoprotective preservation medium containing ten-fold diluted trypticase soy broth and 1% trehalose. As such, successful activity and growth recovery was observed for a diverse set of ammonia-oxidizing cultures

INTRODUCTION

The lithoautotrophic ammonia-oxidizing bacteria (AOB) utilize ammonia and carbon dioxide as sole energy and carbon source, respectively. They catalyze the first step in the nitrification process, converting ammonia to nitrite, and are therefore, along with the ammonia-oxidizing archaea (Könneke et al., 2005), key organisms in the global nitrogen cycle. AOB are widely distributed in nature and, although their isolation procedure is time-consuming and laborious, numerous pure cultures have been isolated from various habitats (Koops and Pommerening-Röser, 2001). Despite being a well-studied and functionally important group of bacteria, sporadic reports on the storage of AOB mostly focus on only a few strains (Koops and Möller, 1992; Tokuyama, 1994; Satoh et al., 2004; Koops et al., 2006), and developed procedures are not generally applicable within this functional guild. As a result, most researchers in the field reside to periodic sub-cultivation of their AOB strains. This is not only very time-consuming but also enhances the risk of mutations and loss of biological material, due to contamination or reduced fitness over time. Indeed, several fully described AOB species are no longer extant (Skerman et al., 1980; Koops et al., 2006). The lack of suitable preservation protocols is a direct cause of the poor accessibility of AOB cultures for the scientific community - only three different AOB strains are available in public culture collections (based on a search of the World Federation for Culture Collections database (http://www.wfcc.info/wdcmdb/)) - but also severely limits commercial use and patent applications. In addition, scientific research on AOB and specifically the description of novel species is also hampered, since it requires the deposition of the type strain in two different public culture collections (Lapage et al.,

1992). Strictly speaking, only two AOB species, namely *Nitrosomonas europaea* and *Nitrosolobus multiformis* currently meet these criteria, while researchers in the field are able to show the existence of at least 14 other AOB species, which are usually represented by a number of well-characterized strains (Koops and Pommerening-Röser, 2005).

It is clear that a long-term, high-quality and generally applicable preservation protocol for AOB is necessary. Recently, an elaborate screening of different preservation protocols on a wide diversity of methane-oxidizing bacteria yielded a general cryopreservation protocol suitable for stable and long-term storage (Hoefman et al., 2012b). The simple protocol applied the innate cryoprotective effects of carbon-rich media, which are often overseen in preservation procedures of oligotrophic microorganisms, used DMSO (that outperformed the widely used glycerol) as cryoprotectant and was able to reverse dormancy induced by preservation (Hoefman et al., 2012b). AOB are physiologically related to methane-oxidizing bacteria (Hanson and Hanson, 1996) and contain similar key enzymes and intracytoplasmic membranes, the conserved integrity of which might be crucial for successful storage. Therefore, the goal of this study was to evaluate the above-mentioned cryopreservation protocol for its general applicability using a physiologically and phylogenetically diverse set of eight AOB cultures from terrestrial, brackish and marine origins (Table IX-1).

EXPERIMENTAL PROCEDURES

Four cryopreservation conditions were tested: cultures were preserved with 5% DMSO as cryoprotectant at -80°C and in liquid nitrogen either in standard growth medium (further referred to as DMSO conditions) or growth medium supplemented with 0.3% trypticase soy broth and 1% trehalose (further referred to as DMSO/TT conditions). For DMSO/TT conditions, the TT preservation medium was used for (i) cultivation prior to storage, (ii) preservation during storage (with 5% DMSO) and (iii) resuscitation after storage. AOB strains (Table IX-1) were cultured in medium I for terrestrial strains (Krümmel and Harms, 1982), medium II for brackish strains (Koops et al., 1976) or medium III which is a modification of II containing 750 mL L^{-1} of 0.2 µm filtered seawater for marine strains (Table IX-1). Seawater (salinity of 30-34 psu) was collected from the North Sea at 3 m depth, 5 to 20 km off the Belgian coast. In parallel, strains were cultured in the same media supplemented with TT (media I/II/II-TT). Cultures were incubated at 28°C in Erlenmeyer flasks (150 mL medium). It should be noted that *N. communis* Nm 2, *N. ureae* Nm 10 and *Nitrosopira* sp. Nsp 1 were not tested for preservation using DMSO/TT. *N. communis* Nm 2 was not tested using DMSO/TT because the original culture tested positive for heterotrophic contamination prior to this study (if TT would be consumed by heterotrophs prior to preservation, this would bias its effect as a protectant during storage). *N. ureae* Nm 10 and *Nitrosopira* sp. Nsp 1 were not tested using DMSO/TT since these strains did not show any activity in the preservation medium. As ammonia oxidation acidifies the media, the pH was adjusted using 10% $NaHCO_3$ upon color shift of the cresol red pH indicator. Colorimetric methods were used for monitoring of ammonia oxidation (Taylor et al., 1974) and nitrite production (Griess, 1879) in the different media. When approximately 10 mM ammonia was consumed in both standard and preservation media, the cells were harvested for preservation. A similar amount of cells based on OD_{600nm} measurements as used for inoculation of standard incubations were added to CryoVials™ (Nunc) filled with 5% DMSO at 4°C and stored at -80°C or in liquid nitrogen for six months. Resuscitation after six months was performed in duplicate. Cultures were thawed at 37°C, DMSO was immediately removed to reduce toxicity and cultures were resuscitated in fresh medium (I/II/III or I/II/III-TT). Details on the preservation procedure are described elsewhere (Heylen et al., 2012b; Hoefman et al., 2012b). To evaluate the success of preservation, activity measurements were performed in standard growth media (I/II/III) on resuscitated cultures similar as prior to preservation. Ammonia oxidation activity correlated well with

nitrite production measurements of all cultures before preservation (Figure IX-1). Therefore, after preservation, nitrite levels were only determined at the start of incubation (all samples were below detection limit, data not shown) and at the last ammonia oxidation measurement point for each culture (Table IX-2). Growth of active cultures was confirmed by OD_{600nm} measurements and indirectly by observation of cell pellets used for DNA extraction. Before and after preservation, authenticity and purity of the AOB cultures were verified by partial 16S rRNA gene sequence analysis (Hoefman et al., 2012a) and absence of growth on trypticase soy agar, respectively.

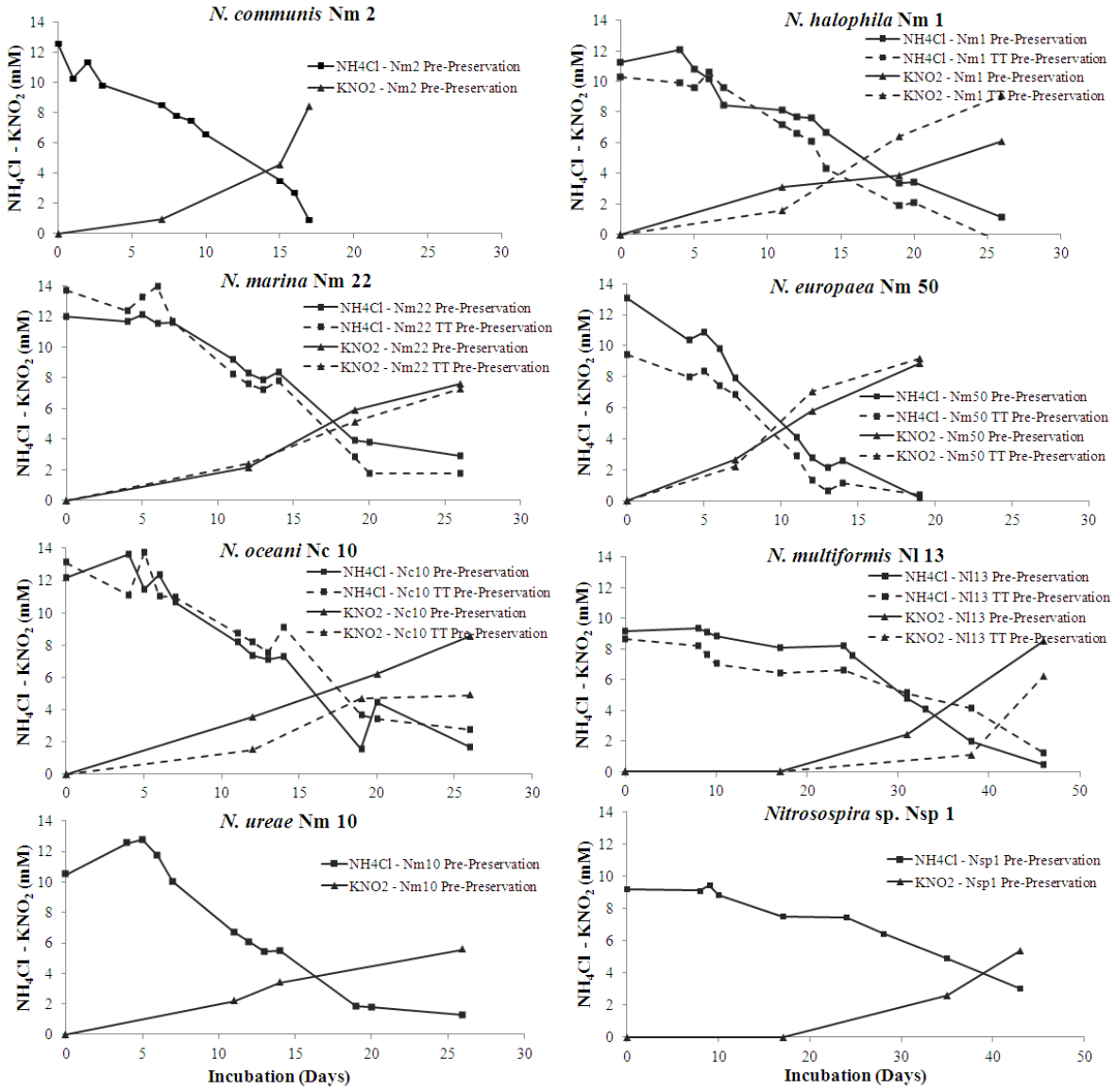

Figure IX-1: Correlation between consumption of ammonia (squares) and production of nitrite (triangles) of AOB strains cultivated in standard growth medium (solid line) and TT preservation medium (dashed line) before preservation.

RESULTS & DISCUSSION

All AOB strains could be successfully preserved, with seven out of eight cultures showing similar ammonia oxidation rates as before preservation under at least one test condition (Figure IX-2; Table IX-1). Success was limited for *N. ureae* Nm 10 showing no or reduced activity with DMSO in liquid nitrogen or at -80°C, respectively. Reduced ammonia oxidation rates were observed after preservation using DMSO for one of the duplicate measurements of *N. marina* Nm 22 at -80°C, of *N. multiformis* Nl 13 in liquid nitrogen, of *N. halophila* Nm 1 both at -80°C and in liquid nitrogen and of *N. ureae* Nm 10 at -80°C. Even after a prolonged incubation of 140 days, no ammonia oxidation (or growth) was observed after preservation using DMSO for one of the duplicate measurements of *N. communis* Nm 2 at -80°C, of *N. halophila* Nm 1 both at -80°C and in liquid nitrogen, of *N. ureae* Nm 10 at -80°C and for both measurements of *N. ureae* Nm 10 in liquid nitrogen. Interestingly, reduction or loss of activity was never apparent for a culture preserved using DMSO/TT (although only five out of eight strains could be tested with DMSO/TT). This success could probably be attributed to the use of the carbon-rich preservation medium. In addition, these observations concur with previous results on methanotrophic bacteria, where use of the same carbon compounds reduced the induction of a viable but non-culturable state in a large fraction of the preserved cells (Hoefman et al., 2012b). During the pre-preservation growth in TT medium, *N. multiformis* Nl 13 got contaminated with a heterotroph later identified as *Bacillus* sp. Nevertheless, the strain was successfully preserved using DMSO/TT at -80°C and in liquid nitrogen with clear activity recovery (Figure IX-2, Table IX-2) and confirmed authenticity of *N. multiformis* Nl 13 after preservation.

Table IX-1: Overview of preservation results in standard growth and TT preservation medium using DMSO or DMSO/TT at -80°C or in liquid nitrogen of eight AOB strains with corresponding species name and cultivation conditions (medium I was used for terrestrial strains, medium II for brackish strains and medium III for marine strains). The AOB form two monophyletic groups within the *Betaproteobacteria* and *Gammaproteobacteria*, respectively. The species-rich betaproteobacterial group is subdivided in seven phylogenetic lineages well reflected by ecophysiological properties (Koops et al., 2006). For each strain, the taxonomic position (*Betaproteobacteria* or *Gammaproteobacteria*, and lineage within *Betaproteobacteria*) is listed.

Strain	Taxonomic position: Proteobacteria / Lineage	Cultivation	DMSO		DMSO/TT	
			-80°C	Liquid nitrogen	-80°C	Liquid nitrogen
Nitrosomonas communis Nm 2	*Beta / N. communis*	I; 28°C	+/-	+	Nt	Nt
Nitrosomonas halophila Nm 1	*Beta / N. europaea*	II & II-TT; 28°C	+/-	+/-	+	+
Nitrosomonas marina Nm 22	*Beta / N. marina*	III & III-TT; 28°C	+/-	+	+	+
Nitrosomonas europaea Nm 50	*Beta / N. europaea*	I & I-TT; 28°C	+	+	+	+
Nitrosococcus oceani Nc 10	*Gammaproteobacteria*	III & III-TT; 28°C	+	+	+	+
Nitrosolobus multiformis Nl 13	*Beta / Nitrosospira*	I & I-TT; 28°C	+	+/-	+	+
Nitrosomonas ureae Nm 10	*Beta / N. oligotropha*	I; 28°C	+/-	-	Nt	Nt
Nitrosospira sp. Nsp 1	*Beta / Nitrosospira*	I; 28°C	+	+	Nt	Nt

+: Similar activity and growth after preservation as before preservation; +/-: Reduced or no activity of at least one of the duplicate measurements after preservation; -: No activity and no growth after preservation; Nt: Not tested

Table IX-2: Nitrite concentrations (mM) at the final measured time-point following cultivation of the independent duplicate data sets (left and right) of the AOB strains after 6 month preservation using DMSO and DMSO/TT at -80°C and in liquid nitrogen. Average values are not shown since final nitrite levels from the duplicates were potentially obtained at different time-points. Nitrite production (or absence of) correlated well with ammonia oxidation results.

Strain	DMSO		DMSO/TT		DMSO		DMSO/TT	
	-80°C	Liquid nitrogen	-80°C	Liquid nitrogen	-80°C	Liquid nitrogen	-80°C	Liquid nitrogen
N. communis Nm 2	BDL	10.6	Nt	Nt	7.81	10.6	Nt	Nt
N. halophila Nm 1	BDL	BDL	5.90	8.24	9.83	9.87	9.65	6.65
N. marina Nm 22	6.80	10.9	10.6	12.3	7.52	7.74	11.5	10.8
N. europaea Nm 50	6.44	8.03	4.77	6.27	9.26	9.40	9.24	9.32
N. oceani Nc 10	9.60	10.1	6.00	10.9	8.08	10.7	9.36	11.2
N. multiformis Nl 13	7.95	11.0	6.18	8.00	8.39	8.67	10.8	10.2
N. ureae Nm 10	8.76	BDL	Nt	Nt	BDL	BDL	Nt	Nt
Nitrosospira sp. Nsp 1	2.66[a]	10.9	Nt	Nt	11.1	10.4	Nt	Nt

a: Early cultivation stop due to technical error; BDL: Below Detection Limit; Nt: Not tested

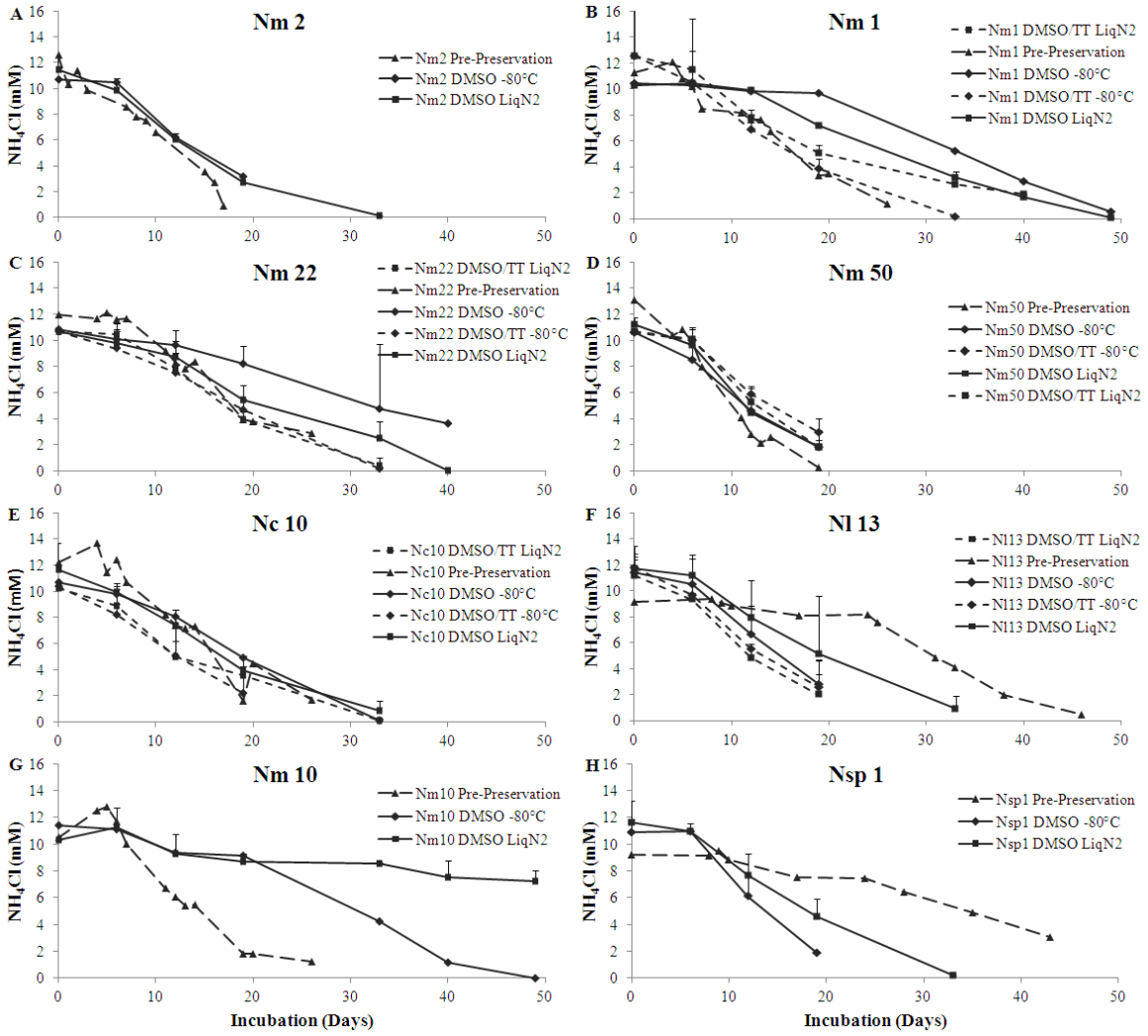

Figure IX-2: Average (n=2) ammonia consumption after 6 month preservation using DMSO and DMSO/TT at -80°C and in liquid nitrogen ($LiqN_2$) of *N. communis* Nm 2 (A), *N. halophila* Nm 1 (B), *N. marina* Nm 22 (C), *N. europaea* Nm 50 (D), *N. oceani* Nc 10 (E), *N. multiformis* Nl 13 (F), *N. ureae* Nm 10 (G) and *Nitrosospira* sp. Nsp 1 (H). All cultures post-preservation were grown in standard growth media, including the cultures which were preserved and cultivated before preservation in TT media. The following is displayed per strain, (i) ammonia consumption before preservation in standard growth medium (Pre-Preservation, long-dashed line, triangles) and after preservation using (ii) DMSO/TT (short-dashed line) or (iii) DMSO (solid line) either (iv) in liquid nitrogen (squares) or (v) at -80°C (diamonds). One-sided error bars are shown. *N. communis* Nm 2, *N. ureae* Nm 10 and *Nitrosospira* sp. Nsp 1 could not be preserved using DMSO/TT. When there was no activity after preservation for one of the duplicate measurements, the culture where no activity was observed is not shown, which was the case for *N. communis* Nm 2 using DMSO at -80°C, *N. halophila* Nm 1 using DMSO at -80°C and in liquid nitrogen and *N. ureae* Nm 10 using DMSO at -80°C. Since duplicate experiments were performed serially and independently, final measurements can occur at different time-points for some duplicates, for which only one measurement is available at the last time-point.

It remains unclear whether micro-organisms are best preserved in widely available electronic freezers at -80°C or in well-monitored liquid nitrogen containers (up to -196°C), which are mostly limited to specialized labs. The latter is considered more stable, since liquid water does not exist below -135°C and cells that survived cooling to such

temperatures remain intact indefinitely (Mazur, 1984). At -80°C, traces of unfrozen water can still exist decreasing stability over time. Perhaps this explains why the activity recovery of strains *N. multiformis* Nl 13 and *N. ureae* Nm 10 preserved with DMSO was more variable at -80°C than in liquid nitrogen. The actual cooling and warming rates, both crucial factors in cryopreservation (Heylen et al., 2012b), were measured in both set-ups (Figure IX-3) and clearly indicated a faster cooling rate in liquid nitrogen than at -80°C, while the warming rates were very similar. This difference in cooling rates could explain the more stable preservation with DMSO at -80°C compared to liquid nitrogen for *N. marina* Nm 22 and *N. communis* Nm 2, since rapid cooling can increase the chance of intracellular ice formation, leading to cell death (Mazur, 1984; Heylen et al., 2012b). Although the effect of freezing temperature and cooling rate is clearly strain dependent, AOB recovery can potentially be further improved by first freezing cells at -80°C followed by rapid transfer to liquid nitrogen. All AOB strains stored with DMSO/TT were preserved successfully at -80°C and in liquid nitrogen with similar activity rates before and after preservation, which again suggests a reduced variability in activity recovery after storage in TT preservation medium.

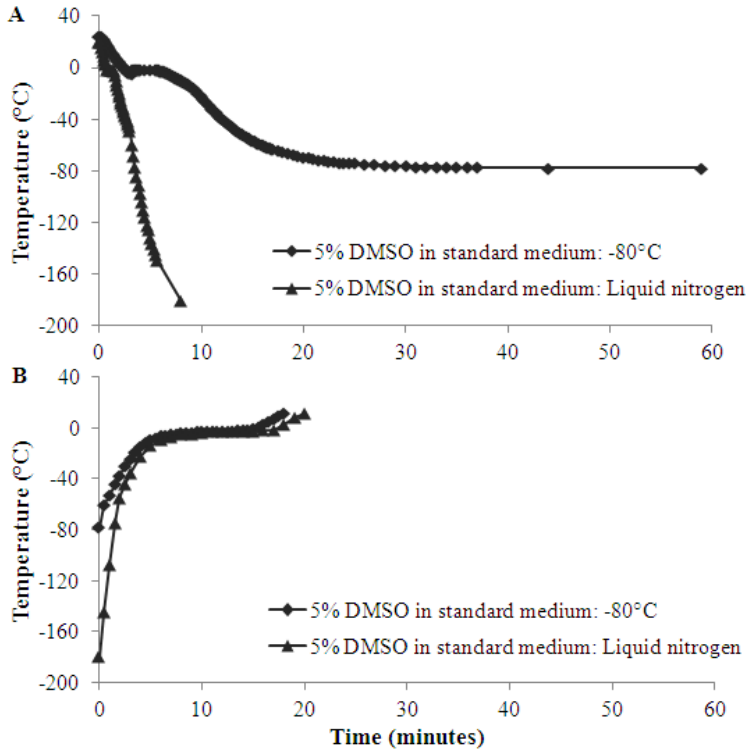

Figure IX-3: Cooling (top) and warming (bottom) temperature-time profiles of a sample at -80°C (squares) and in liquid nitrogen (triangles). While the rate of thawing is similar at -80°C and in liquid nitrogen, the rate of freezing is much slower at -80°C. Measurements were performed with a Fluke 52II type K thermometer in Nunc CryoVials™ filled with 1 mL of 5% DMSO in standard growth medium.

The observations from this study support the concept of significantly improving cryopreservation of fastidious and/or oligotrophic microorganisms by addition of the TT preservation medium. Currently, the influence of the time point of carbon addition during the preservation procedure as well as the choice of carbon compounds remains to be elucidated. Our results suggest that this could be strain dependent. Pre-preservation incubation of *N. ureae* Nm 10 and *Nitrosospira* sp. Nsp in TT medium inhibited activity and thus preservation in TT medium was not considered. Since these strains are known to be salt sensitive (Koops et al., 2006), the addition of TT (which contains extra salts) probably induced salt stress inhibiting growth, which could potentially be avoided by a different choice of carbon compound or by TT addition at the time of preservation. It has been reported that adding trehalose during growth can be much more effective than addition at the time of preservation (Streeter, 2003), and that in this way methane-oxidizing cultures can be preserved most successfully using DMSO/TT (Hoefman et al., 2012b). However, the addition of TT along with DMSO without pre-incubation can also improve activity recovery compared with DMSO without any TT (Heylen et al., 2012a). This alternative might be viable for the preservation of AOB that do not grow in TT, provided that the short exposure to TT before preservation is not harmful for the cells, which should be confirmed experimentally. Based on the above observations, the following scheme for long-term cryopreservation of AOB is proposed: if the AOB culture can oxidize ammonia in TT preservation medium, cryopreservation using 5% DMSO in TT preservation medium at -80°C or in liquid nitrogen is recommended. If not, preservation of AOB using DMSO without TT also has a high success rate, although with a higher variability in activity recovery.

In conclusion, we demonstrated for the first time that long-term cryopreservation of a diverse set of AOB can be efficiently achieved. This protocol is easily applicable by laboratories equipped with -80°C freezers and/or liquid nitrogen containers. These results also add to the growing evidence that most fastidious micro-organisms can be successfully cryopreserved based on a combination of DMSO as cryoprotectant and the TT preservation medium.

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REFERENCES

Griess, P., 1879. Bemerkungen zu der abhandlung der H.H. Weselsky und Benedikt "Ueber einige azoverbindungen." Chem. Ber 12, 426-428.

Hanson, R.S.,Hanson, T.E., 1996. Methanotrophic bacteria. Microbiol. Rev 60, 439-471.

Heylen, K., Ettwig, K., Hu, Z.Y., Jetten, M.,Kartal, B., 2012a. Rapid and Simple Cryopreservation of Anaerobic Ammonium-Oxidizing Bacteria. Appl. Environ. Microbiol 78, 3010-3013.

Heylen, K., Hoefman, S., Vekeman, B., Peiren, J.,De Vos, P., 2012b. Safeguarding bacterial resources promotes biotechnological innovation. Appl. Microbiol. Biotechnol 94, 565-574.

Hoefman, S., van der Ha, D., De Vos, P., Boon, N.,Heylen, K., 2012a. Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria. Microb. Biotechnol 5, 368-378.

Hoefman, S., Van Hoorde, K., Boon, N., Vandamme, P., De Vos, P.,Heylen, K., 2012b. Survival or revival: long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria. PLoS One 7, e34196.

Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B.,Stahl, D.A., 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437, 543-546.

Koops, H.-P., Harms, H.,Wehrmann, H., 1976. Isolation of a moderate halophilic ammonia-oxidizing bacterium, *Nitrosococcus mobilis* nov. sp. Arch. Microbiol 107, 277-282.

Koops, H.-P.,Möller, U. (1992) The Lithotrophic Ammonia-Oxidizing Bacteria. In *The Prokaryotes, second ed*. Balows, A. (ed). New York: Springer Verlag, pp. 2625-2637.

Koops, H.-P.,Pommerening-Röser, A., 2001. Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiol. Ecol 37, 1-9.

Koops, H.-P.,Pommerening-Röser, A. (2005) The lithoautotrophic ammonia-oxidizing bacteria. In *Bergey's Manual of Systematic Bacteriology, second ed*. Garrity, G.M., Williams, S.T., Staley, J.T., Brenner, D.J., Holt, J.G., Boone, D.R. et al. (eds). New York: Springer Verlag.

Koops, H.-P., Purkhold, U., Pommerening-Röser, A., Timmermann, G.,Wagner, M. (2006) The Lithoautotrophic Ammonia-Oxidizing Bacteria. Chapter 3.2.12. In *The Prokaryotes, third ed*, pp. 778-811.

Krümmel, A.,Harms, H., 1982. Effect of organic matter on growth and cell yield of ammonia-oxidizing bacteria. Arch. Microbiol 133, 50-54.

Lapage, S., Sneath, P., Lessel, E., Skerman, V., Seeliger, H.,Clark, W. 1992 International Code of Nomenclature of Bacteria: Bacteriological Code, 1990 Revision. Washington (DC): ASM Press.

Mazur, P., 1984. Freezing of living cells - mechanisms and implications. Am. J. Physiol 247, C125-C142.

Satoh, K., Tanaka, T., Oguro, Y., Takahashi, R.,Tokuyama, T., 2004. Improvement of preservation method for ammonia-oxidizing bacteria by freeze-drying. J. Soil. Sci. Plant Nutr 50, 777-781.

Skerman, V.B.D., McGowan, V.,Sneath, P.H.A., 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol 30, 225-420.

Streeter, J.G., 2003. Effect of trehalose on survival of *Bradyrhizobium japonicum* during desiccation. J. Appl. Microbiol 95, 484-491.

Taylor, S., Ninjoor, V., Dowd, D.M.,Tappel, A.L., 1974. Cathepsin B2 measurement by sensitive fluorometric ammonia analysis. Anal. Biochem 60, 153-162.

Tokuyama, T., 1994. A method for long-term preservation of a chemoautotrophic ammonia-oxidizing bacterium, *Nitrosomonas* using silica gel powder. Bull. Jpn. Soc. Microb. Ecol 9, 119-123

Chapter X

Influence of carbon and DMSO concentration on the successful preservation of nitrite-oxidizing bacteria

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Redrafted from:

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Author Contributions:

BV, KH and SH designed the experiments. ES provided the NOB strains. BV performed the experiments. BV, KH and SH analyzed the data. BV and KH wrote the paper. ES, SH and PDV commented on the manuscript.

SUMMARY

Nitrite-oxidizing bacteria are key members of the global nitrogen cycle but their study is hampered by their limited availability in culture, mostly due to laborious cultivation procedures and the lack of stable preservation methods. Here, we demonstrated that long-term cryopreservation of nitrite-oxidizing bacteria is possible by successfully applying a simple and rapid protocol on strains belonging to five different genera. Optimal preservation conditions were strain-dependant but a general cryopreservation protocol using 10% dimethyl sulfoxide with or without ten-fold diluted trypticase soy broth allowed successful preservation of all tested strains.

INTRODUCTION

Nitrite-oxidizing bacteria (NOB) are obligate or facultative lithoautotrophic bacteria assimilating CO_2 as their main carbon source and oxidizing nitrite as their sole energy source. NOB perform the second step of the nitrification process, the conversion of nitrite to nitrate. By doing so they remove the toxic nitrite from the environment, which is harmful for living organisms (Philips et al., 2002) and convert it to nitrate that acts as a major nitrogen source for many organisms. NOB are fastidious and slow growing microorganisms, and consequently their isolation and cultivation is a time-consuming process. As a result, only few representatives of this functional guild have been validly described. These are phylogenetically affiliated to the *Alpha-*, *Beta-*, *Gammaproteobacteria* and the deep-branching phyla *Nitrospirae* and *Nitrospinae* (Ehrich et al., 1995; Spieck and Bock, 2005; Alawi et al., 2007; Lucker et al., 2013). Recently a new member was added to this guild belonging to the widespread phylum *Chloroflexi* (Sorokin et al., 2012).

The conventional way to preserve and safeguard NOB is the continuous subcultivation and storage at a temperature of 17°C. However, this approach increases the risk of contamination and loss of authenticity over time, requires periodic maintenance, time and physical space, and is therefore impractical for large-scale culture collections (Monaghan et al., 1999). The lack of a simple, high quality preservation protocol for members of this functional guild increases the possibility of strains being lost for future research and application (Heylen et al., 2012b). Well-defined freezing protocols - freezing in liquid nitrogen with hatefi buffer - for nitrite-oxidizing bacteria have been proposed in the past

(Spieck and Bock, 2005), but were not really picked up by the scientific community. This has led to the current situation of only few NOB species being publically available (http://www.wfcc.info/wdcmbd). A fast, simple and high-quality method without the use of costly equipment would provide significant benefits for NOB maintenance, allowing better access of the scientific community, which will undoubtedly stimulate further research. A recent study on a wide diversity of methane-oxidizing bacteria demonstrated the effectiveness of a simple cryopreservation protocol, applying (i) the innate cryoprotective effects of carbon rich media, which are often overseen in preservation procedures of oligotrophic microorganisms, and (ii) the use of dimethyl sulfoxide (DMSO) that outperformed the widely used glycerol as a cryoprotectant (CPA) (Hoefman et al., 2012). This easy method already proved successful for the preservation of other oligotrophic microorganisms, namely aerobic and anaerobic ammonium oxidizers (Heylen et al., 2012a; Hoefman et al., 2013) and nitrite-dependent anaerobic methane oxidizers (K. Heylen, K. Ettwig, M. Jetten, B. Kartal, unpublished data). However, in the initial screening of cryopreservation conditions (Hoefman et al., 2012) only one carbon-rich medium (ten-fold diluted trypticase soy broth supplemented with 1% trehalose) was tested in combination with one fixed concentration of DMSO (5%). The effect of trypticase soy broth and trehalose as well as the cryoprotective effects of other carbon compounds with various DMSO concentrations remain unknown for other oligotrophic bacteria but can potentially also improve their preservation success.

Because preservation success cannot be extrapolated with acceptable level of certainty, even within closely related strains, it should be experimentally demonstrated for each organism of interest. Therefore we here aimed at assessing the above-mentioned "established" cryopreservation protocol for an additional functional guild, namely the nitrite-oxidizing bacteria. In addition, we wanted to evaluate the influence of different carbon-rich media and various concentrations of DMSO on the preservation of NOB and ideally determine suitable preservation conditions for all (tested) members of this functional group.

EXPERIMENTAL PROCEDURES

Nitrite oxidizing strains

Six different NOB strains representing six different species and five different genera were included in this study. Three non-marine strains, *Nitrobacter vulgaris* AB1 from sewage, *Candidatus* Nitrospira defluvii A17 from activated sludge and *Candidatus* Nitrotoga arctica 6680 from permafrost soil, and three marine strains, *Nitrococcus mobilis* 231, *Nitrospina gracilis* 3/211 and *Nitrospira marina-like* bacterium strain Ecomares 2.1, were cultivated in mineral salt medium and marine medium respectively (Spieck and Lipski, 2011). These were all pure cultures, except for *Candidatus* Nitrotoga artica, which was highly enriched. All strains were grown at a pH of 7.4-7.6, a temperature of 28°C and a nitrite concentration of 3 mM, with exception of the strain *Nitrotoga arctica* 6680, which was grown at a temperature of 15°C and a nitrite concentration of 0.3 mM.

Parameters for success rate evaluation

Colorimetric methods were used for monitoring of nitrite oxidation (Griess, 1879) and nitrate production (Cataldo et al., 1975) in the different media. Both activity measures correlated very well (data not shown); only nitrite oxidation data is shown further. Growth was monitored via optical density at 600 nm, while ATP values obtained with BacTiter-GloTM Microbial Cell Viability assay (Promega) were used as a measure for viability. Specific activity measured as nitrite oxidation was demonstrated to be a reliable proxy for both growth and viability for all cultures as data from simultaneous monitoring of all three parameters showed strong positive Pearson correlation values (Table X-1). Based on these data and because of high doubling times of NOB, only nitrite oxidation activity values were used for preservation evaluation.

The lag phase induced by preservation defined as the time point at which significant nitrite oxidation occurred (determined as the point when nitrite oxidation was equal to five times the standard deviation of the technical error on the Griess test) was also determined. Total protein content was measured with the bicinchoninic acid (BCA) assay (Pierce, US), according to manufacturer's instructions and using bovine serum albumin as a standard. Purity checks were performed periodically by plating on nutrient rich media (trypticase soy agar). To ensure the purity and authenticity of each culture, 16S rRNA gene sequence analysis was performed periodically.

Growth on carbon-rich media

All NOB strains were screened for growth on different carbon-rich media. Screening was miniaturized in microtiter plates, to allow a high throughput testing of a large number of carbon-rich media. Following media were investigated: (1) trypticase soy broth (TSB), (2) ten-fold diluted TSB, (3) ten-fold diluted TSB supplemented with 1% trehalose, (4) 1% yeast extract, (5) 0.5% yeast extract, (6) 1% malt extract, (7) 0.5% malt extract, (8) 1% skimmed milk, (9) 1% trehalose, (10) 5% trehalose and (11) 1% sucrose. All carbon-rich media were prepared by adding the carbon compounds to the standard (mineral salt or marine) medium (Spieck and Lipski, 2011). For each strain the standard medium was included as a growth control. Also, a blank was incorporated for each carbon media to compensate for NO_2 fluctuations due to evaporation.

Pre-preservation growth, cryopreservation and resuscitation

Prior to preservation, NOB strains were grown in standard medium and three carbon rich media (i.e. ten-fold diluted TSB, ten-fold diluted TSB supplemented with 1% trehalose, and 1% sucrose). Starting with an OD value of 0.001, cultures were grown for two months, and starved via depletion of nitrite to enter stationary phase. Biomass was concentrated via centrifugation (8000 rpm, 15min) to a final volume of 1 ml, and washed two times with fresh growth medium. Samples were taken from each culture to determine biomass. A total of 29 preservation conditions were applied per strain by varying the pre-preservation growth medium (standard vs carbon rich), the preservation medium (standard vs carbon-rich), the concentration of DMSO as cryoprotective agent (0, 1, 5, 10%). The sole cryoprotective effect of carbon-rich media was only tested in combination with pre-preservation growth in these media. In addition, the cryoprotective buffer, hatefi (consisting of 100 mL distilled water, 0.6 g TRIS, 22.6 sucrose, 0.015 g histidine, pH 7.5) was tested as CPA; it was used as a 100% solution, so without addition of preservation medium (Spieck and Lipski, 2011). Addition of DMSO was performed at 4°C to decrease toxicity, after which cultures were immediately preserved. Each preservation condition was prepared in duplicate. Subsequently all six NOB strains were subjected to long-term cryopreservation in liquid nitrogen (-196°C). Cultures were stored in Cryotube™ vials (Nunc, Denmark) and were placed in the gas phase just above the liquid phase. The cryopreserved NOB strains were resuscitated after a preservation period of eight months. After preservation biomass was thawed quickly at 37°C in an incubator and upon thawing, immediately transferred to eppendorf tubes and centrifuged (8,000 rpm, 15 min) at 4°C to

minimize the toxic effect of DMSO. NOB biomass was washed twice with an equal volume of fresh standard medium (without nitrite) to ensure the removal of DMSO residues, resuspended in an equal volume of fresh standard medium after which sodium nitrite was added.

RESULTS & DISCUSSION

High-quality and stable long-term cryopreservation of nitrite-oxidizing bacteria during eight months was successful for all six tested strains representing five different genera. A total of twenty-nine different conditions were tested on both non-marine (*Nitrobacter vulgaris* AB1, *Candidatus* Nitrospira defluvii A17 and *Candidatus* Nitrotoga arctica 6680) and marine strains (*Nitrococcus mobilis* 231, *Nitrospina gracilis* 3/211 and *Nitrospira marina*-like bacterium strain Ecomares 2.1. further designated as *Nitrospira* sp. Ecomares 2.1.), as we intended to determine a generally applicable protocol for all nitrite oxidizers regardless of phylogenetic affiliation and/or origin. Suitable cryopreservation conditions were strain-dependent. But, in general, most strains resuscitated surprisingly well, with only short lag phases of a couple of days, and showed mainly small, non-significant differences in their specific activity recovery after preservation in different conditions (used as a proxy for survival rate of biomass; compared to unpreserved control samples). Only cryopreservation of *Nitrospira* sp. Ecomares 2.1. and *Nitrospina gracilis* 3/211, most notably two marine strains, proved to be more challenging, however efficient cryopreservation was still successful in several of the applied conditions.

The only previously described cryoprotective agent used for these bacteria, Hatefi buffer consisting of sucrose and histidine (Spieck and Bock, 2005), was only tested and proven successful on a limited number of strains from the genus *Nitrobacter* and *Nitrospira* and has thus far not been widely applied. Therefore, it was compared to the golden standard among cryoprotective agents dimethyl sulfoxide (DMSO), used in various concentrations. The choice of CPA, Hatefi versus DMSO, or the concentration of DMSO made little to no significant difference in the specific activity recovery of five out of six strains (Figure X-1) while within-strain variation of lag phase duration was also limited (data not shown). Although between-strain results are tricky to compare due to differences in growth rates and final biomass obtained, it was clear that *Nitrospira* sp. Ecomares 2.1. recovered similarly from all applied preservation conditions but that the recovered specific activity was significantly lower than the four other strains. *N. gracilis* 3/211 only resuscitated

successfully when preserved with 10% DMSO, albeit a lag phase of almost 16 days.

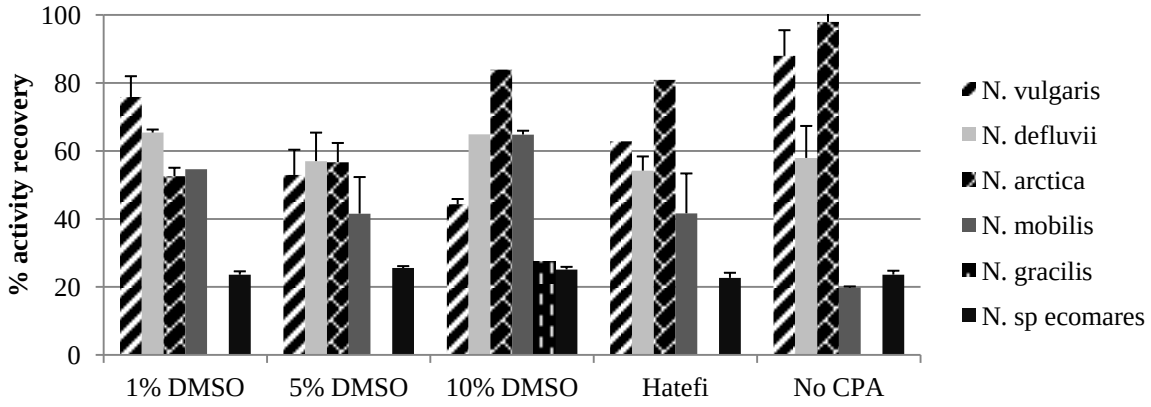

Figure X-1. The cryoprotective effect of different CPA's indicated as average % activity recovery compared to a control sample (n=2). NOB strains were grown in standard mineral medium and subsequently preserved in mineral medium with the addition of one of the CPA's indicated. The preservation condition with Hatefi was performed in 100% Hatefi solution, and thus differs from the other preservation conditions. Standard deviations for each preservation condition are indicated by one-sided error bars. Absence of error bars refers to singular measurements (n=1) and is indicative for unstable preservation conditions.

Surprisingly, all strains besides *N. gracilis* 3/211 resuscitated well after cryopreservation without any cryoprotectant added to the culture (Figure X-1) and without a significant increase of the lag phase (data not shown). *Candidatus* Nitrotoga arctica 6680 even reached a significantly higher recovery (97%) without cryoprotectant. The fact that this is a cold adapted NOB isolated from permafrost soil from the Siberian arctic with seasonal freezing and thawing cycles, and has an optimum growth temperature of 10°C might have contributed to this feature (Alawi et al., 2007). Bacteria have already been shown to respond to low temperatures by the expression of cold induced proteins (Ermolenko and Makhatadze, 2002), which can function as important freeze protection mechanisms. Similar results were obtained previously with enrichment cultures (Heylen et al., 2012a) but were then mainly attributed to the additional protective power of exopolysaccharides in existing flocs. These observations suggest that the toxic effect of addition of CPA, which can vary among compounds used but is an issue when using DMSO, can impede successful preservation more that the freezing itself. Although DMSO is considered toxic, the threshold between protective versus toxic effect is strongly dependent of the organism of interest. Some bacteria can tolerate high DMSO concentrations and are even capable of growing in media containing 20-45% (Fedorka-Cray et al., 1988). Nevertheless, care should be taken when using any potentially toxic CPA. The strain-dependent influence of concentration of DMSO as well as the success of cryopreservation without any protective

additive demonstrates that simple tests or evaluation of small adaptations to established protocols are worthwhile to perform.

All NOB strains known to date are obligate or facultative chemolithoautotrophs that are able to fix CO_2 as carbon source and oxidize NO_2 to gain energy. Therefore, these cultures are always grown in a mineral medium containing no additional carbon or nutrient sources. We hypothesized that this standard practice, beneficial to avoid contamination, can be at the basis of the problematic preservation of specific NOB strains, as carbon compounds can beneficially affect the (cryo)preservation of microorganisms (Hubàlek, 2003; Morgan et al., 2006; Heylen et al., 2012a; Hoefman et al., 2012). As is the case for other CPA, depending on the organism, the carbon compounds either are taken up by the cell to avoid intracellular ice formation, only penetrate the periplasmic space where they stabilize the cell membrane during ice formation by interaction with the polar head groups of the phospholipids (i.e. disaccharides such as trehalose and sucrose) (Rudolph and Crowe, 1985), or remain extracellular and only protect the cells from external ice formation (i.e. polymers with high molecular weight such as proteins or polysaccharides) (Hubàlek, 2003; Heylen et al., 2012b). Therefore, in combination with hatefi or DMSO at various concentrations, improvements of preservation success through use of carbon-rich media, either as preservation medium or pre-preservation growth medium, were also assessed.

Firstly, growth of NOB strains in eleven carbon-rich media was verified because this would suggest no or very limited toxicity and potential carbon uptake (although this was not verified) and thus indicate its suitability for further testing as preservation medium. Carbon compounds were selected based on previous studies (Hubàlek, 2003; Fuller, 2004; Hoefman et al., 2012). Specific activity rates of the strains in the carbon-rich media were monitored over a period of four weeks (Table X-2) and six carbon-rich media supported growth of all strains. *Candidatus* Nitrotoga arctica 6680 was most sensitive to the addition of carbon-rich nutrients in the medium. Because of logistic reasons only three carbon-rich media - addition of 1% sucrose, tenfold diluted TSB, and tenfold diluted TSB with 1% trehalose - were selected for further testing as preservation and pre-preservation growth medium, because they already proved to be successful for cryopreservation of other oligotrophic bacteria (Heylen et al., 2012a; Hoefman et al., 2012) and because sugars are natural occurring CPAs (Hubàlek, 2003). In addition, trehalose is used as a compatible solute by *Nitrobacter* (Spieck, unpublished data).

Secondly, these carbon-rich media were used as preservation medium in combination with various concentrations of DMSO as cryoprotective agent (Hatefi buffer was only tested as

a 100% solution, not in combination with these preservation media). For the non-marine strains, which were already efficiently preserved with or without DMSO, addition of carbon to DMSO did not significantly alter the activity recovery after cryostorage. *Nitrospira* sp. Ecomares 2.1. only achieved an activity recovery of around 20% using no CPA or only DMSO in mineral medium. But addition of either tenfold diluted TSB without or with 1% trehalose did significantly increase activity recovery for all three DMSO conditions (Figure X-2), up to around 80%, while the latter condition also drastically decreased the lag phase from about sixteen days to six days in 10% DMSO (data not shown). These results correlate well with previous observations of improved growth (i.e. higher cell yield and shorter generation time) of this strain in the presence of low amount of organics (Spieck, unpublished data). For *N. gracilis* 3/211, obtained results are unfortunately less reliable because of unsuccessful resuscitation of replicates from several preservation conditions. However, the results confirmed the preference for 10% DMSO and also suggested a potential beneficial influence of sucrose and to a lesser extended of tenfold diluted TSB with trehalose, allowing use of other DMSO concentrations (data not shown). Additional preservation experiments for unequivocal confirmation were not performed due to the duration of these experiments and the proven suitability of 10% DMSO for preservation.

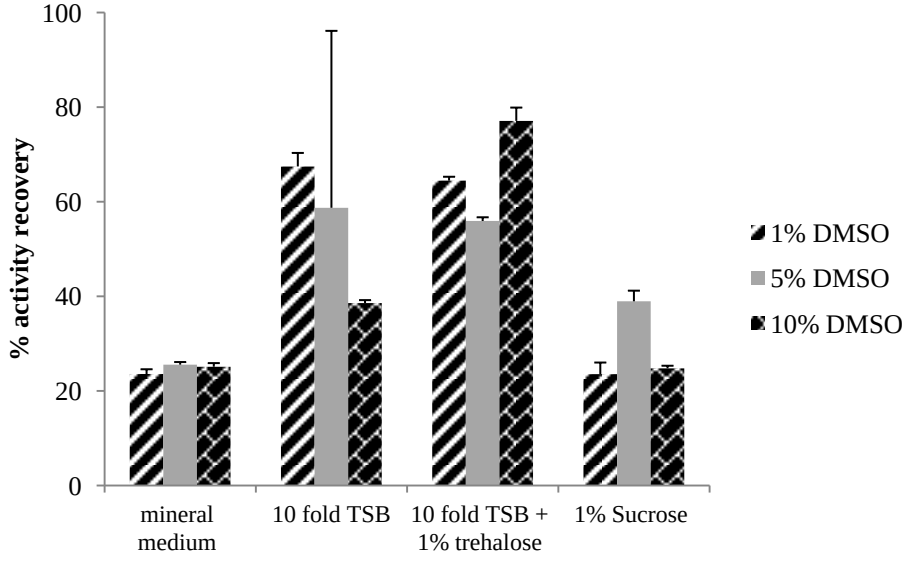

Figure X-2. The combined cryoprotective effect of carbon rich media in combination of a CPA is depicted for the strain *Nitrospira* sp. Ecomares 2.1. Growth was performed in mineral medium, followed by the preservation in a carbon rich medium (x-axis) in combination with 1, 5 or 10% DMSO as CPA. Graph indicates the average (n=2) percentage of growth recovery compared to a control sample. Variation is depicted as one sided error bars. Large error bars are a result of large variations in the slopes of the replicates.

Finally, the three carbon-rich media were used as pre-preservation growth medium as well as preservation medium, both with or without CPA. This was done to improve the intracellular uptake of the carbon during growth and allow a better cryoprotective effect than mere addition before preservation. However, despite the preliminary screening in carbon-rich media, some cultures showed unstable growth in some of the selected carbon-rich media, which sometimes resulted in a complete inhibition of activity (data not shown). Only for *Nitrospira* sp. Ecomares 2.1. these type of preservation conditions led to a significant improvement of preservation success, with activity recovery of around 100% (Figure X-3) and a lag phase of less than one day (data not shown). Interestingly, all strains could also be successfully preserved when using carbon-rich media as sole CPA during preservation (after an initial growth in these carbon rich media).

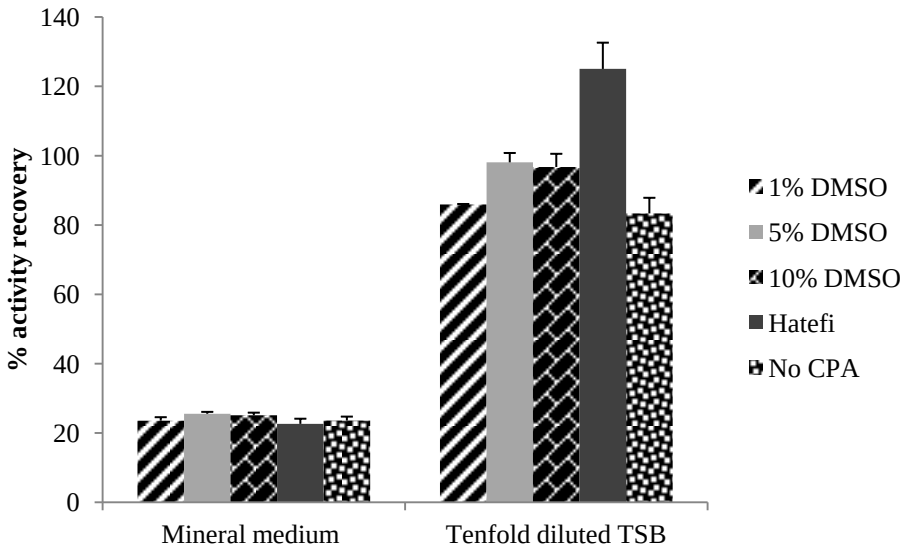

Figure X-3. The combined cryoprotective effect of pre-preservation growth in a ten-fold diluted TSB medium and subsequently preservation compared to growth and preservation in mineral medium, both in combination with a CPA, for *Nitrospira* sp. Ecomares 2.1. Other carbon rich media (TT and sucrose) were not tested for this strain since pre-preservation growth failed. The preservation condition with Hatefi was performed in 100% Hatefi solution, and thus differs from the other preservation conditions. The preservation success is indicated as the average (n=2) percentage of growth recovery compared to a control sample (y-axis). Standard deviations are depicted as one sided error bars. Absence of error bars indicates no or very small variations.

Table X-1. Pearson correlation values between measurements for nitrite oxidation, growth and viability monitored simultaneously for all cultures grown in different carbon rich media.

Growth medium	***N. vulgaris*** AB1			***N. mobilis* 231**			***Candidatus* N. defluvii A17**			***Nitrospira. sp* Ecomares 2.1.**			***N. gracilis* 3/211**			***Candidatus* N. arctica 6680**		
	NO_2 vs OD	**NO_2 vs ATP**	**OD vs ATP**	**NO_2 vs OD**	**NO_2 vs ATP**	**OD vs ATP**	**NO_2 vs OD**	**NO2 vs ATP**	**OD vs ATP**	**NO_2 vs OD**	**NO_2 vs ATP**	**OD vs ATP**	**NO_2 vs OD**	**NO_2 vs ATP**	**OD vs ATP**	**NO_2 vs OD**	**NO_2 vs ATP**	**OD vs ATP**
Mineral medium	0.955	0.817	0.762	0.957	0.900	0.866	0.879	0.909	0.660	0.967	0.632	0.529	0.957	0.932	0.929	0.986	0.574	0.550
Tenfold diluted TSB	0.965	0.813	0.794	0.966	0.937	0.747	-	-	-	0.980	0.900	0.811	0.986	0.990	0.930	-	-	-
Tenfold diluted TSB + 1% trehalose	0.911	0.705	0.691	0.980	0.936	0.896	-	-	-	-	-	-	0.940	0.979	0.911	-	-	-
1% sucrose	0.946	0.832	0.742	0.983	0.975	0.940	0.851	0.921	0.847	-	-	-	0.917	0.834	0.699	0.810	0.762	0.911

Table X-2. Overview of the relative growth success of different NOB strains in different carbon rich media compared to standard medium. All carbon-rich media are denoted by the carbon compound added to the standard (mineral salt or marine) medium. Growth success is indicated as % activity rate compared to the activity rate of the strain in standard mineral medium. Media in bold were selected for further testing of long-term preservation of NOB.

Carbon rich media	***N. vulgaris*** AB1	***Can.* N. defluvii A17**	***Can.* N. arctica 6680**	***N. mobilis* 231**	***N. gracilis* 3/211**	***Nitrospira* sp. Ecomares 2.1**
TSB	100	14.8	0	29.9	94.1	43.1
1/10 TSB	**100**	**100**	**34.7**	**69.5**	**57.7**	**65.8**
1/10 TSB+1%trehalose	**100**	**100**	**44.6**	**45.0**	**67.2**	**67.6**
Yeast extract 1%	100	23.3	0	30.0	86.3	64.7
yeast extract 0.5%	100	24.8	0	29.1	83.2	57.7
malt extract 1%	41.9	23.3	0	54.2	123.7	56.2
malt extract 0.5%	64.4	32.9	17.3	37.1	129.3	71.2
skimmed milk 1%	100	50	74.4	94.1	126.1	54.3
trehalose 1%	100	100	8.4	100	94.1	60.4
trehalose 5%	100	100	0	100	82.9	73.4
sucrose 1%	**100**	**100**	**13.4**	**100**	**93.6**	**66.6**

Table X-3. Overview of the best preservation conditions for the different NOB strains. All carbon-rich media are denoted by the carbon compound added to the standard (mineral salt or marine) medium. Growth success is indicated as the average (n=2) % activity rate compared to the activity rate of the strain in standard mineral medium. Lag phase is indicated as the average (n=2) increase in time (expressed in days) compared to a non-preserved control sample.

Strain	Preservation condition (growth medium/preservation medium + CPA)	% Activity recovery (± STDEV)	Lag phase (± STDEV)
***Nitrobacter vulgaris* AB1**	Ten-fold diluted TSB / Ten-fold diluted TSB +1% DMSO	121 (6)	0.000 (0.000)
	Ten-fold diluted TSB / Ten-fold diluted TSB + no CPA	116 (5)	0.107 (0.099)
	Ten-fold diluted TSB + 1% trehalose (TT) / TT+ 1% DMSO	114 (1)	0.044 (0.009)
***Nitrococcus mobilis* 231**	Mineral medium / 1% sucrose +10% DMSO	76.5 (1.7)	0.331 (0.102)
	Ten-fold diluted TSB / no preservation medium + Hatefi	71.5 (3.9)	0.000 (0.000)
	1% sucrose / 1% sucrose +5% DMSO	69.8 (15.9)	0.007 (0.009)
***Candidatus* Nitrotoga arctica 6680**	Mineral medium / Ten-fold diluted TSB +1% DMSO	109.7 (8.0)	0.407 (0.576)
	Mineral medium / Ten-fold diluted TSB +5% DMSO	104 (6.4)	0.097 (0.137)
	Mineral medium / Mineral medium + no CPA	97.9 (3.1)	5.14 (2.19)
***Candidatus* Nitrospira defluvii A17**	Mineral medium / Ten-fold diluted TSB +1% trehalose +10% DMSO	72.0 (0.1)	0.688 (0.486)
	Mineral medium / 1% sucrose +10% DMSO	68.9 (5.9)	1.30 (0.89)
	Mineral medium / Mineral medium +1% DMSO	65.4 (0.9)	1.90 (1.19)
***Nitrospira* sp. Ecomares *2.1*.**	Ten-fold diluted TSB / no preservation medium + Hatefi	125 (7.6)	0.439 (0.620)
	Ten-fold diluted TSB / Ten-fold diluted TSB + 5% DMSO	98.0 (2.7)	0.144 (0.079)
	Ten-fold diluted TSB / Ten-fold diluted TSB + 10% DMSO	96.0 (3.8)	0.103 (0.126)
***Nitrospina gracilis* 3/211**	Mineral medium / Ten-fold diluted TSB + 10% DMSO	33.8 (12.2)	13.2 (0.6)

Our observations confirm our hypothesis that carbon-rich media as preservation medium and/or pre-preservation growth medium can be beneficial to improve cryostorage of specifically difficult to maintain marine strains, as demonstrated for *N. gracilis* 3/211 and *Nitrospira* sp. Ecomares 2.1. They are also in agreement with earlier studies reporting a better preservation when carbon sources are added during growth and/or preservation (Streeter, 2003; Heylen et al., 2012a; Hoefman et al., 2012; Hoefman et al., 2013). Nevertheless, carbon addition can also be detrimental for some sensitive oligotrophic organisms and preliminary tests should always be carried out before final application of a preservation condition.

In conclusion, we demonstrated that long-term cryopreservation of NOB is possible using a simple and rapid protocol. Based on obtained results we propose the use of 10% DMSO as CPA with or without ten-fold diluted trypticase soy broth as preservation medium and storage in liquid nitrogen as a standard procedure to store nitrite-oxidizing bacteria. However the most optimal preservation condition is strain dependant (Table X-3) and preliminary tests should always be included to confirm the preservation success. Additionally, the two candidatus strains included in this study can be deposited in public culture collections allowing valid description.

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REFERENCES

Alawi, M., Lipski, A., Sanders, T., Pfeiffer, E.M.,Spieck, E., 2007. Cultivation of a novel cold-adapted nitrite oxidizing betaproteobacterium from the Siberian Arctic. ISME J 1, 256-264.

Cataldo, D.A., Haroon, M., Schrader, L.E.,Youngs, V.L., 1975. Rapid Colorimetric Determination of Nitrate in Plant-Tissue by Nitration of Salicylic-Acid. Commun. Soil Sci. Plant Anal. 6, 71-80.

Ehrich, S., Behrens, D., Lebedeva, E., Ludwig, W.,Bock, E., 1995. A new obligately chemolithoautotrophic, nitrite-oxidizing bacterium, Nitrospira moscoviensis sp. nov. and its phylogenetic relationship. Arch Microbiol 164, 16-23.

Ermolenko, D.N.,Makhatadze, G.I., 2002. Bacterial cold-shock proteins. Cell Mol Life Sci 59, 1902-1913.

Fedorka-Cray, P.J., Cray, W.C., Jr., Anderson, G.A.,Nickerson, K.W., 1988. Bacterial tolerance of 100% dimethyl sulfoxide. Can J Microbiol 34, 688-689.

Fuller, B.J., 2004. Cryoprotectants: the essential antifreezes to protect life in the frozen state. CryoLetters 25, 375-388.

Griess, P., 1879. Bemerkungen zu der abhandlung der H.H. Weselsky und Benedikt “Ueber einige azoverbindungen". Chem. Ber. 12, 426-428.

Heylen, K., Ettwig, K., Hu, Z., Jetten, M.,Kartal, B., 2012a. Rapid and simple cryopreservation of anaerobic ammonium-oxidizing bacteria. Appl Environ Microbiol 78, 3010-3013.

Heylen, K., Hoefman, S., Peiren, J., Vekeman, B.,De Vos, P., 2012b. Safeguarding Bacterial Resources promotes Biotechnological Innovation. Appl. Microbiol. Biotechnol. 94, 565–574.

Hoefman, S., Van Hoorde, K., Boon, N., Vandamme, P., De Vos, P.,Heylen, K., 2012. Survival or revival: long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria. Plos One In Press.

Hoefman, S., Pommerening-Roser, A., Samyn, E., De Vos, P.,Heylen, K., 2013. Efficient cryopreservation protocol enables accessibility of a broad range of ammonia-oxidizing bacteria for the scientific community. Res Microbiol In Press.

Hubàlek, Z., 2003. Protectants used in the cryopreservation of microorganisms. Cryobiology 46, 205-229.

Lucker, S., Nowka, B., Rattei, T., Spieck, E.,Daims, H., 2013. The Genome of *Nitrospina gracilis* Illuminates the Metabolism and Evolution of the Major Marine Nitrite Oxidizer. Front Microbiol 4, 27.

Monaghan, R., Gagliardi, M.,Streicher, S. (1999) Culture preservation and inoculum development. In *Manual of industrial microbiology and biotechnology*. Demain, A., and Davies, J. (eds). Washington, DC: ASM, pp. 29-48.

Morgan, C.A., Herman, N., White, P.A.,Vesey, G., 2006. Preservation of micro-organisms by drying; A review. Journal of Microbiological Methods 66, 183–193.

Philips, S., Laanbroek, H.,Verstraete, W., 2002. Origin, causes, and effects of increased nitrite concentrations in aquatic environments. Rev Environ Sci Biotechnol 1, 115-141.

Rudolph, A.S.,Crowe, J.H., 1985. Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline. Cryobiology 22, 367-377.

Sorokin, D.Y., Lucker, S., Vejmelkova, D., Kostrikina, N.A., Kleerebezem, R., Rijpstra, W.I. et al., 2012. Nitrification expanded: discovery, physiology and genomics of a nitrite-oxidizing bacterium from the phylum Chloroflexi. ISME J 6, 2245-2256.

Spieck, E.,Bock, E. (2005) The lithoautotrophic nitrite-oxidizing bacteria. In *Bergey’s Manual of Systematic Bacteriology, vol. 2*. Garrity, G.M., Brenner, D.J., Krieg, N.R., and Staley, J.T. (eds).

New York: Springer-Verlag, pp. 149-153.

Spieck, E.,Lipski, A. (2011) Cultivation, growth physiology and chemotaxonomy of nitrite-oxidizing bacteria. In *Methods in Enzymology*: Elsevier.

Streeter, J.G., 2003. Effect of trehalose on survival of *Bradyrhizobium japonicum* during desiccation. Journal of Applied Bacteriology 95, 384-491.

Reflection & Discussion

Many bacteria can be stored successfully for long periods either by freezing or freeze-drying. Usually, heterotrophs that grow easily in the lab are also easy to preserve. In contrast, for many bacteria that appear more difficult to cultivate, i.e. fastidious organisms, the available literature also reported their long-term storage to be problematic. In Chapter VIII, we showed that this is not necessarily the case. It appeared that not the storage as such caused poor recovery of MOB culturability, but rather the inability to resuscitate the cultures properly. All MOB type strains tested in Chapter VIII as well as all MOB isolated in this study, could be easily cryopreserved and resuscitated using 5% DMSO as cryoprotectant and 1% trehalose in 10-fold diluted TSB (TT) as preservation medium. The results for MOB formed the basis for the subsequent successful cryopreservation of aerobic ammonia-oxidizing bacteria (Chapter IX), nitrite-oxidizing bacteria (Chapter X), anaerobic ammonium-oxidizers (Heylen et al., 2012a) and nitrite-dependent anaerobic methane oxidizers (Heylen K, Ettwig K, Jetten M, Kartal B, unpublished data). Therefore, it can be concluded that the cryopreservation protocol is widely applicable to a variety of fastidious organisms. Since preservation success can still be organism-specific, it remains necessary to demonstrate the success of the method for other functional groups of fastidious organisms or for novel isolates within tested groups.

We want to advocate the routine incorporation of preservation tests in environmental or applied microbiological research. Although this will require some effort of the researcher, a set of guidelines were formulated to facilitate the process of establishing custom-made preservation protocols (Heylen et al., 2012b). In our opinion, the rewards of indefinite storage of all your strains greatly outweigh the effort needed to assess if the strains can be easily cryopreserved. Moreover, once a reliable preservation procedure is demonstrated for taxa originally known as very difficult to handle and preserve, public culture collections will be more prone to accept the deposit of these strains. For example, culture collections often do not to accept AOB and NOB strains because of their fastidious nature, which greatly hampers AOB and NOB research, and the description of novel taxa in particular (A. Pommerening-Röser and E. Spieck, personal communication). By demonstrating the

success of our cryopreservation protocol, the BCCM/LMG Bacteria collection now accepts deposits of MOB, AOB and NOB from other researchers. In this regard, it is vital that the depositor and the culture collections work closely together to realize a proper transfer of handlings and knowledge because successful preservation includes more than storage alone as shown in Chapter VIII. Public collections generally have thousands of strains in their holdings and can thus not be specialized in the cultivation of all possible organisms. As it appears that most cells survive storage procedures, the emphasis of preserving fastidious organisms should shift from survival of cells to revival. Because of this, the role of the depositor should become more prominent: instead of merely sending the strains, he/she should help the collection in detail how his/her strains are best cultivated. We believe that this alone can greatly improve a successful deposit into a collection, as cells that survive storage will resuscitate more easily under optimal growth conditions. Nonetheless, certain strains might still be difficult to preserve. In this case, the applied cryoprotectant solution can be optimized as explored in Chapter X or culture collections could explore preservation methodology that proved successful for many eukaryotic cells. For example, the cooling rate during freezing is crucial. In Chapter IX, it was shown that this rate is very rapid in liquid nitrogen containers, which potentially results in tremendous cell injury regardless of the added cryoprotectant. Hence, vitrification techniques, which totally avoid ice formation, and/or the use of controlled temperature devices for freezing cells at more optimal cooling rates, might proof vital for successful long term preservation of even the most difficult bacteria. As an easy starting point, it should be assessed whether recovery can be enhanced by initially freezing the cells at for example -80°C due to the slower cooling rate, followed by immediate transfer to liquid nitrogen containers.

In addition, together with the Laboratory of Microbial Ecology and Technology (LabMET) Ghent University, we are currently evaluating whether our standardized cryopreservation protocol can also store mixed cultures successfully. Here, it will be evaluated if such co-cultures retain their function as well as their community structure following long-term storage. Successful preservation of mixed cultures is highly desired, since several microbial processes, either naturally or applied in biotechnology, are not performed by individual cells, but by consortia of different organisms. Moreover, in the case of obligate mutualism (e.g. anaerobic methane oxidation by some methane-oxidizing archaea with sulphate reducing bacteria) or in the case of highly-enriched fastidious cells that are notoriously hard to isolate into pure culture, the only way to store such organisms is via mixed culture preservation. Lastly, we want to emphasize that it was shown in Chapter

VIII that all MOB could be successfully lyophilized, combining sucrose and BSA as protectant and TT as preservation medium. Since lyophilization offers several advantages over cryopreservation such as ease of storage and transport, we encourage culture collections to also assess freeze-drying success of fastidious organisms.

REFERENCES

Heylen, K., Ettwig, K., Hu, Z.Y., Jetten, M.,Kartal, B., 2012a. Rapid and Simple Cryopreservation of Anaerobic Ammonium-Oxidizing Bacteria. Appl. Environ. Microbiol 78, 3010-3013.

Heylen, K., Hoefman, S., Vekeman, B., Peiren, J.,De Vos, P., 2012b. Safeguarding bacterial resources promotes biotechnological innovation. Appl. Microbiol. Biotechnol 94, 565-574.

Concluding Remarks

Currently, it has become widely accepted that human-induced emissions of greenhouse gases such as carbon dioxide, methane and nitrous oxide lead to global warming. This occurs both directly, through combustion of fuels, or indirectly, through stimulation of greenhouse gas production by microorganisms. Methane-oxidizing bacteria (MOB) are key organisms for the study of these indirect processes, as they link both the global carbon and nitrogen cycles, since these organisms consume methane but are also capable of detoxifying many nitrogen compounds without relying on these processes for growth. Moreover, these organisms have been shown to produce high-value products as well as bioremediate pollutants. Therefore, MOB have been studied intensively over the past 40 years by ecologists as well as technologists. Still, many of these organisms have been detected in nature, but were never successfully cultivated. This leaves a large part of their physiological diversity undiscovered.

The concept of this thesis was to improve our ability to efficiently (i) bring MOB into culture, (ii) optimize their culture conditions, (iii) study the physiology of the retrieved diversity and (iv) store MOB by freezing or drying to enable their accessibility at all times.

The research led to the rapid isolation by miniaturized extinction culturing of 17 genetically distinct MOB (Type Ia, Ib and II MOB) from a variety of ecosystems, several of which were assigned to novel taxa. Many strains were shown to be highly physiologically diverse, even between strains within a single genus or species. This strain dependency was observed at different levels, most notably in regard to their preference of medium composition, their interaction pattern in co-cultivation with heterotrophs, their tolerance to high nitrogen amendments, and their capability of detoxifying nitrogen sources. These findings open opportunities for future research such as the evaluation of (i) the ability of an individual organism in a community to ensure functional stability upon disturbance regardless of the higher level community structure, (ii) a large scale isolation campaign by miniaturized extinction culturing using many different isolation media, (iii)

additional physiological traits such as the bioremediation potential of fast-growing MOB strains by miniaturized screening, (iv) the mechanisms behind the observed co-cultivation interaction patterns of MOB and heterotrophs.

In addition, our studies showed that successful cryopreservation and lyophilization of MOB is possible. It was observed that many cells survived storage but could not be resuscitated. The addition of the cryoprotective TT medium, allowed to significantly revive cells from this viable but non-culturable state. Furthermore, the standardized cryopreservation protocol using DMSO as cryoprotectant and TT as preservation medium proved to be widely applicable for many fastidious organisms as the method allowed the successful preservation of nitrite oxidizers and aerobic and anaerobic ammonia oxidizers.

To conclude, I believe that many discoveries are to be made by culturing as much MOB strains as possible, regardless of whether they belong to novel or known taxa. Tools are available to rapidly bring these strains into culture, to preserve the retrieved biodiversity and to study many aspects of their physiology. As more strains are recovered, more answers will be provided on how these organisms function in the environment, and more opportunities will arise for their successful application in biotechnology.

Summary

Methane is an important greenhouse gas. Human-induced methane emissions contribute to global warming and climate change. Methanotrophs are microorganisms that can consume methane and utilize it as a carbon and energy source. It is of vital importance to understand what factors may positively or negatively impact the methane consuming activity of these organisms, in order to efficiently mitigate human-related methane emissions by the methanotrophs. Furthermore, many of these microbes have been shown to degrade major groundwater pollutants such as trichloroethylene or can convert methane into high-value products such as bio-plastics.

This study focused on the proteobacterial Methane-Oxidizing Bacteria (MOB), which have been found to oxidize methane in a wide variety of environments such as in rivers, lakes, ponds, marine environments, wetlands, rice paddies, groundwater and waste water. As with most groups of bacteria, there is a discrepancy between the diversity of MOB that is detected in nature, and the diversity that has been cultured in the lab, termed "the great plate count anomaly". However, several of these organisms have been brought into pure culture successfully, which allows the study of their physiology and their potential for biotechnology. Most of these studies have focused on a thorough characterization of a few reference strains. However, it remains unclear whether many of these findings can be extrapolated to other, even closely related, strains. Moreover, many MOB that were brought into culture were subsequently lost again, in part due to a lack of efficient long-term preservation protocols, which is most unfortunate given the amount of effort required to isolate these organisms. In this study, several of the above mentioned aspects were addressed.

Firstly, a diverse set of MOB type strains were preserved successfully. Preservation induced a viable but non-culturable state in many cells. Significant revival from this state was observed by using 1% trehalose in 10-fold diluted TSB (TT) as preservation medium. In this way, all strains could be cryopreserved without a significant loss in culturability using TT in combination with 5% DMSO as cryoprotectant. Moreover, all strains could be

successfully lyophilized using 20% sucrose and 10% BSA as lyoprotectant in combination with TT. Furthermore, the standardized protocol appeared widely applicable as a diverse set of ammonia and nitrite oxidizing bacteria were successfully cryopreserved based on the results obtained for the methanotrophs.

Secondly, miniaturized extinction culturing proved successful in rapidly isolating a diverse set of 17 methanotrophic strains from the three major types of proteobacterial MOB (Type Ia, Ib and II) from different environments. Subsequent miniaturized cultivation experiments proved useful as a simple tool to extract valuable growth information for many methanotrophic strains tested in parallel. These tests revealed that many closely related strains differed profoundly in respect to their nitrogen metabolism, their growth medium preference and their interaction pattern when co-cultivated with heterotrophs. This finding has implications for ecology studies that use molecular tools with a low taxonomic resolution and thus observe contradictory patterns which might be explained by the strain dependent nature of many characteristics of the methanotrophs. Several strains showed enhanced growth rates and cell yields depending on the medium, and some strains in particular revealed a tolerance to high nitrogenous additions. These fast-growing strains with an innate tolerance to harsh conditions might prove potent candidates for biological methane mitigation in situations where other MOB would fail to grow. Additionally, several strains belonged to novel taxa which are formally described in the thesis: *Methyloparacoccus murrellii* isolated from pond water in South Africa and Japan and *Methylomonas lenta* isolated from manure and a denitrification tank in Belgium.

In conclusion, complementary with in-depth studies that thoroughly characterize reference strains, it also appears useful to screen many strains in parallel for desired features, as closely related methanotrophs can still widely differ physiologically. The use of miniaturized cultivation proved reliable in obtaining such information and in isolating the dominant MOB from a variety of MOB enrichments. Novel isolates can then be successfully stored by either cryopreservation or lyophilization to ensure that novel biodiversity does not get lost and to allow future application of their biotechnological potential.

Samenvatting

Methaan is een belangrijk broeikasgas. Methaanemissies die veroorzaakt worden door menselijke activiteiten versterken de opwarming van de aarde en de klimaatverandering. Methanotrofen zijn micro-organismen die methaangas kunnen consumeren en gebruiken als koolstof –en energiebron. Het is dus zeer belangrijk om de factoren te begrijpen die zowel een positieve als negatieve impact kunnen hebben op de methaan consumerende activiteit van deze organismen, zodat methaanemissies mogelijks kunnen gemanaged worden. Ook kunnen vele methanotrofen belangrijke grondwater polluenten afbreken zoals trichlooretheen of kunnen ze methaan omzetten naar hoogwaardige producten zoals bio-plastics.

Dit onderzoek spitste zich toe op de proteobacteriële methaan-oxiderende bacteriën (MOB). Deze bacteriën zijn actief in vele natuurlijke omgevingen zoals in rivieren, meren, vijvers, mariene milieus, moerasgebieden, rijstvelden, grondwater maar ook in afvalwater. Zoals bij vele andere groepen van bacteriën, is er een groot verschil tussen de methanotrofe diversiteit die wordt gedetecteerd in de natuur en de diversiteit die reeds in laboratoria werd onderzocht op basis van de geïsoleerde reinculturen. Dit wordt de "great plate count anomaly" genoemd. Toch zijn microbiologen er in geslaagd om een beperkt aantal methanotrofen in laboratoria te kweken, wat toelaat om de fysiologie en potentieel voor biotechnologische toepassingen te bestuderen. In de meeste van deze studies werd enkel een beperkt aantal referentie stammen grondig onderzocht. Echter, het blijft onbekend of de resultaten van deze studies kunnen doorgetrokken worden naar andere, zelfs nauwverwante, stammen. Bovendien zijn veel in cultuur gebrachte methanotrofen verloren gegaan, onder meer door een gebrek aan efficiënte lange-termijn bewaarmethodes, wat zeer jammer is gelet op de hoeveelheid tijd nodig voor het isoleren van deze stammen. In dit proefschrift werden enkele van de hierboven vermelde aspecten behandeld.

Ten eerste werd een diverse groep van MOB type stammen succesvol bewaard. Bij veel cellen induceerde het bewaarproces een stadium waarbij cellen nog leefden maar niet meer konden groeien. Zo'n cellen konden significant ontwaakt worden wanneer 1% trehalose in 10-voudig verdund TSB (TT) werd gebruikt als bewaarmedium. Zo konden alle methanotrofen succesvol bewaard worden via invriezen zonder significante afsterving van

cultiveerbare cellen met gebruik van het TT bewaarmedium in combinatie met 5% DMSO als cryoprotectans. Ook konden alle methanotrofen succesvol gevriesdroogd worden waarbij 20% sucrose en 10% BSA als lyoprotectans werd gebruikt in combinatie met TT. De methode via invriezen bleek breed toepasbaar te zijn aangezien ook ammoniak en nitriet oxiderende bacteriën succesvol konden bewaard worden.

Ten tweede bleek de geminiaturiseerde methode op basis van hoge verdunningen succesvol in het efficiënt isoleren van een diverse groep methanotrofen uit de drie gekende types van proteobacteriële MOB (Type Ia, Ib, II). Nadien bleek het cultiveren via 96-well microtiter platen zeer efficient als eenvoudige methode om nuttige groei informatie te verkrijgen voor vele methanotrofen die in parallel werden getest. Uit deze testen kwam duidelijk naar voor dat zelfs zeer nauwverwante stammen sterk verschillend waren in hun stikstofmetabolisme, hun groeimedium voorkeur alsook in hun interactiepatroon in co-cultivatie met heterotrofen. Dit heeft implicaties voor ecologische studies die gebruik maken van moleculaire methodes met een lage taxonomische resolutie, en dus zo tegenstrijdige patronen zien die mogelijks verklaard kunnen worden door de stamafhankelijke natuur van vele eigenschappen van de methanotrofen. Vele stammen vertoonden een betere groeisnelheid en celopbrengst afhankelijk van de groeimedia en sommige specifieke stammen vertoonden een sterke tolerantie tegen hoge stikstofgehaltes. Deze snelgroeiende methanotrofen met inherente tolerantie tegen ruwe omstandigheden zouden interessante kandidaten kunnen zijn om methaan als broeikasgas te verwerken in giftige omstandigheden voor andere methanotrofen. Enkele methanotrofe isolaten bleken tot nieuwe soorten te behoren, en deze werden uitvoerig beschreven in dit proefschrift: *Methyloparacoccus murrellii* geïsoleerd uit stilstaand water in Zuid-Afrika en Japan en *Methylomonas lenta* geïsoleerd vanuit mest en een denitrificatie tanker van een waterzuiveringsinstallatie in België.

Er kan worden geconcludeerd dat het nuttig is om zowel studies uit te voeren die diepgaand referentiestammen karakteriseren alsook studies die meerdere stammen tegelijkertijd screenen naar gewenste eigenschappen, omdat zelfs zeer nauwverwante stammen sterk kunnen verschillen. Het gebruik van geminiaturiseerde cultivering bleek betrouwbaar in het genereren van dergelijke informatie alsook in het isolaren van dominante methanotrofen uit methanotrofe aanrijkingen. Nieuwe isolaten kunnen nadien efficiënt bewaard worden zowel via invriezen als via vriesdrogen zodat de stammen voor een lange termijn bewaard blijven voor verdere studie en het exploreren van biotechnologische toepassingen.

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Curriculum vitae

Personalia

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Educational Background

2009 – Present	PhD – LM-UGent, Faculty of Science, Ghent University. Dissertation: From Nature to Nurture: Isolation, Physiology and Preservation of Methane-Oxidizing Bacteria. Promotors: Prof. Paul De Vos, Prof. Peter Vandamme, Dr. Kim Heylen
2006 – 2007	Ghent University, Ghent, Belgium Degree Master of Science in Applied Microbial Systematics, achieved with great honour; Thesis: Development of a Real-Time PCR method for detection of spore-forming bacteria in milk
2002 – 2006	Provincial Industrial Hogeschool (PIH), Kortrijk, Belgium Degree Master Industrial Engineer Chemistry option Biochemistry, achieved with great honour; Thesis: Optimization of Disinfection Methods in Aquaculture
2002	Koninklijk Atheneum Kortrijk Degree Secondary Education Science-Mathematics

Professional Experience

2007 – 2008	Ablynx NV, Zwijnaarde: Bioanalytic Assay Developer
2008 – 2009	Ablynx NV, Zwijnaarde: Pharmacokinetic Modeler

Specialist Courses

2011 Interscience NV, Breda, NL
Course: Gas Chromatography
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Scientific Output

A1 Publications

Hoefman S, Pommerening-Röser A, Samyn E, De Vos P, Heylen K (2013). Efficient cryopreservation protocol enables accessibility of a broad range of Ammonia-Oxidizing Bacteria for the scientific community. Research in Microbiology, 164 (4), 288-292.

Hoefman S, Van Hoorde K, Boon N, Vandamme P, De Vos P, Heylen K (2012). Survival or revival: long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria. PLoS ONE 7(4): e34196. doi:10.1371/journal.pone.0034196.

Hoefman S*, van der Ha D*, De Vos P, Boon N, Heylen K (2012). Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria. Microbial Biotechnology, 5(3), 368–378. *Contributed Equally

Hoefman S, van der Ha D, Boon N, Vandamme P, De Vos P, Heylen K (2013). Customized media based on miniaturized screening improve growth rate and cell yield of methane-oxidizing bacteria of the genus *Methylomonas*. Submitted.

Stock M*, **Hoefman S***, Kerckhof FM, Boon N, De Vos P, De Baets B, Heylen K, Waegeman W (2013). Exploration and prediction of interactions between methanotrophs and heterotrophs. Submitted. *Contributed Equally

Hoefman S, van der Ha D, Boon N, Vandamme P, De Vos P, Heylen K (2013). Versatile and strain-dependent nitrogen metabolism within the genus *Methylomonas*. Submission pending.

Hoefman S, van der Ha D, Iguchi H, Yurimoto H, Sakai Y, Boon N, Vandamme P, Heylen K, De Vos P (2013). *Methyloparacoccus murrellii* gen. nov., sp. nov., a novel methanotroph isolated from pond water in South Africa and Japan. Submission pending.

Hoefman S, Heylen K and De Vos P (2013). *Methylomonas lenta* sp. nov., a novel methanotroph isolated from manure and a denitrification tank in Belgium. Submission pending.

Heylen K, **Hoefman S**, Vekeman B, Peiren J, De Vos P (2011). Safeguarding Bacterial Resources promotes Biotechnological Innovation. Appl Microbiol Biotechnol, 94(3), 565-574.

van der Ha D, **Hoefman S**, Boeckx P, Verstraete W, Boon N (2010). Copper enhances the activity and salt resistance of mixed methane-oxidizing communities. Appl Microbiol Biotechnol, 87(6), 2355-63.

van der Ha D, **Hoefman S**, Isaacs M, De Vos P, Cowan K, Boon N (2013). Facultative ponds harbor methane-oxidizing communities supported by algal oxygen production. Submitted.

Vekeman B, **Hoefman S**, De Vos P, Spieck E, Heylen K (2013). Influence of carbon and dimethyl sulfoxide concentration on the successful preservation of nitrite-oxidizing bacteria. Submitted.

van der Ha D, Vanwonterghem I, **Hoefman S**, De Vos P, Boon N (2012). Selection of associated heterotrophs by methane-oxidizing bacteria at different copper concentrations. Antonie van Leeuwenhoek, 103(3), 527-37.

Verhagen P, De Gelder L, **Hoefman S**, De Vos P, Boon N (2011). Planktonic versus biofilm catabolic communities: importance of the biofilm for species selection and pesticide degradation. Appl Environ Microbiol, 77(14), 4728-35.

van der Ha D, Gorteman D, Ho A, **Hoefman S**, Schneider B, De Vos P, Boon N (2013). Selection and characterization of a methane oxidizing consortium resistant to high ammonium loads. Submitted.

Ganendra G, De Muynck W, Ho A, **Hoefman S**, De Vos P, Boeckx P, Verstraete W, Boon N (2013). Atmospheric methane removal by methanotrophic bacteria immobilized on porous building materials. Submitted.

C3 Publication

Hoefman S, Boon N, De Vos P, Heylen K (2010). Protecting the fragile: preservation of methanotrophic bacteria. Cryobiology 61(3). p.367-367.

Oral Presentation

Hoefman S, Vekeman B, Van Hoorde K, Vandamme P, Boon N, Heylen K, De Vos P. "Protecting the Fragile": long-term preservation of fastidious organisms. Oral Presentation: PhD Symposium Faculty of Science 2012, Gent, Belgium.

Hoefman S, Boon N, De Vos P, Heylen K. "Protecting the Fragile": Preservation of Methanotrophic Bacteria. Oral Presentation: Cryo 2010, Annual Meeting for Cryobiology, Bristol, UK.

Poster Presentations

Hoefman S, Vandamme P, De Vos P, Heylen K. Methanotrophic Resource Management: Rapid isolation, miniaturized characterization and long-term preservation of fast-growing methane-oxidizing bacteria: BSM 2012, Brussels, Belgium

Hoefman S, Vandamme P, De Vos P, Heylen K. Miniaturized isolation and medium optimization for efficient selection of fast-growing *Methylomonas* cultures: Molecular Basis of Microbial One-Carbon Metabolism (GRC/GRS) 2012, Lewiston, Maine, USA

Hoefman S, Van Hoorde K, Boon N, Vandamme P, De Vos P, Heylen K. Survival or revival: Long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria: BSM 2011, Brussels, Belgium.

Hoefman S, Boon N, Vekeman B, Verstraete W, De Vos P, Heylen K. Prerequisite for MRM: preservation of valuable microorganisms: MRM Symposium 2011, Ghent, Belgium.

Vekeman B, **Hoefman S**, Spieck E, Boon N, De Vos P, Heylen K. Long-term preservation of nitrite-oxidizing bacteria: ICoN2-ENC Meeting 2011, Nijmegen, The Netherlands.

Hoefman S, De Vos P, Heylen K. Polyphasic Characterization of Methanotrophic Bacteria Isolated by Rapid Liquid Culturing: FEMS 2011, Geneva, Switzerland.

Hoefman S, De Vos P, Heylen K. Efficient Isolation Procedure for Methane-Oxidizing Bacteria: NVvM 2011, Arnhem, The Netherlands.

Hoefman S, De Vos P, Heylen K. Challenging "The Great Plate Count Anomaly": Isolation of Methanotrophic Bacteria: BSM 2010, Brussels, Belgium.

Hoefman S, De Vos P, Heylen K. Challenging the Great Plate Count Anomaly: Cultivation-based Studies of Methanotrophic Bacteria: ISME 2010, Seattle, USA.