# Growth Characterization and Transcriptomics of Methanotrophic Bacteria as Effected by Carbon and Nitrogen Sources

by

Catherine Tays

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## Abstract

While industrial activities have shaped our modern world and lifestyles, one of their many important environmental effects is the significant increase in methane emissions and the resultant atmospheric methane concentrations. A common byproduct of many industries, methane is often burned off as waste or simply released to the atmosphere indiscriminately. This is undesirable as methane is a potent greenhouse gas, second only to carbon dioxide in significance to global warming, and current levels are more than double the pre-Industrial Revolution levels. As our climate continues to change rapidly, it is more pressing than ever to pursue mitigation and remediation efforts.

Methanotrophs are a specialized class of microorganisms that derive both their carbon and energy from methane. Their ecological impact is huge, playing a major role in the regulation of the methane cycle, and serving as the only biological methane sink. Taxonomically, they spread across the tree of life, however one of the largest groups currently known and studied is the aerobic, proteobacterial methanotrophs, also known as the methane oxidizing bacteria (MOB). These bacteria have long been well-known for their incredible potential in the field of biotechnology, bioremediation, and bioconversion. The benefit of MOB-based methane bioconversion is two-fold: mitigation of undesirable methane released from industry as well as production of a vast inventory of green, value-added products to be sold for profit.

Despite their hundred-year history in culture, much remains to be understood about MOB, and these gaps in our knowledge hamper attempts to adopt large-scale industrial methanotroph technologies. This includes even fundamental questions about culturing, down to identifying optimal carbon and nitrogen conditions for growth, arguably the two most vital nutrients to cell function. This uncertainty can manifest in undesirable outcomes for bioprocesses, including slow or inhibited growth and diversion of cell resources away from desired bioproducts. The main carbon sources of interest for industry are methane and methanol, the latter also being a common

industrial byproduct. In terms of nitrogen, both ammonium and nitrate have been investigated for growth, and both are common industrial N-forms, including use as agricultural fertilizers. It is well documented that both C and both N forms can strongly affect methanotroph growth, possessing unique carbon fixation pathways and diverse nitrifying and denitrifying pathways that affect N-metabolism.

There are complicating factors when discussing optimal growth conditions for aerobic proteobacterial MOB. In fact, this group can be further divided into separate types based on taxonomic and physiological differences: the gammaproteobacterial MOB and the alphaproteobacterial MOB. As such, this work begins with a survey of the physiologies of 5 MOB strains, representing two gamma-MOB and three alpha-MOB, in four C-N growth conditions to determine the effects of C source, N source, and combined C-N conditions on growth. In the following chapters studies, two MOB demonstrating unique physiologies were subject to further transcriptomic analysis, allowing insight into the global gene regulation occurring in these strains during growth in each C-N combination. These works take strides in broadening our understanding of how C and N sources affect MOB, and what conditions are best for ensuring efficient, stable growth suitable for bioindustrial implementation. They also highlight how the carbon and nitrogen central assimilation pathways interact in these methanotrophs, and that a more holistic view is required to build optimized bioprocesses around these bacteria. These are necessary steps towards the establishment of best possible growth outcomes, supporting both economical development and environmentally-responsible industrial activities.

## Preface

Part of this thesis has been previously published and some chapters will be submitted for publication. Additional acknowledgements are provided at the end of each specific chapter.

Chapter 3 was published as Tays, C., Guarnieri, M.T., Sauvageau, D., and Stein, L.Y. (2018) "Combined Effects of Carbon and Nitrogen Source to Optimize Growth of Proteobacterial Methanotrophs" in *Frontiers in Microbiology* **9**(2239). doi:10.3389/fmicb.2018.02239. As the primary author, I was responsible for conceptualization, designing, performing, and analyzing experiments, bioinformatics analysis, and writing the manuscript. Dr. Michael Guarnieri is a researcher at National Renewable Energy Lab (NREL) in Colorado, USA, who was responsible for the lipid and small organic acid data collection and analysis, and contributed to the preparation and composition of the manuscript. Dr. Dominic Sauvageau and Dr. Lisa Stein were the supervisory authors who contributed in all stages from conceptualization, data interpretation, advice, and manuscript composition.

Chapter 4 and Chapter 5 will be submitted for publication as Tays, C., Orata, F., Sauvageau, D., and Stein, L.Y. "Comparative transcriptomics of *Methylomicrobium album* BG8 in different carbon and nitrogen growth conditions", and Tays, C., Orata, F., Sauvageau, D., and Stein, L.Y. "Comparative transcriptomics of *Methylocystis* sp. Rockwell in different carbon and nitrogen growth conditions". As the primary author, I was responsible for conceptualization, designing, performing and analyzing experiments, bioinformatics analysis, and writing the manuscript. Dr. Fabini Orata contributed to bioinformatics analysis and preparation of the manuscript. Dr. Dominic Sauvageau and Dr. Lisa Stein were the supervisory authors who contributed in all stages from conceptualization, data interpretation, advice, and manuscript composition.

*This thesis is dedicated to the generations that follow.*

*May we leave them a better Earth than we found.*

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## List of Abbreviations

ACP: acyl carrier protein

Alpha-MOB: alphaproteobacterial methane oxidizing bacteria

AMO: ammonia monooxygenase

AMS: ammonia mineral salts

ANME: anaerobic methanotrophic archaea

ANOVA: analysis of variance

AOB: ammonia oxidizing bacteria

AOM: anaerobic oxidation of methane

ATP: adenosine triphosphate

BLAST: Basic Local Alignment Search Tool

C source: carbon source

C1: one carbon

cDNA: complementary DNA

COG: clusters of orthologous groups

DGE: differential gene expression

DIET: direct interspecies electron transfer

DNA: deoxyribonucleic acid

DOXP: 1-deoxy-D-xylulose 5-phosphate

ED: Entner–Doudoroff

EMBL: European Molecular Biology Laboratory

EMP: Embden–Meyerhof–Parnas

FADH: formate dehydrogenase

FAE: formaldehyde activating enzyme

FAME: fatty acid methyl esters

FDR: false-discovery rate

Gamma-MOB: gammaproteobacterial methane oxidizing bacteria

GAP: glyceraldehyde-3-phosphate

GC:FID: gas chromatography: flame ionization detection

GDH: glutamate dehydrogenase

GOGAT: glutamine-oxoglutarate amidotransferase or glutamate synthase

GS: glutamine synthetase

$H_4$MPT: tetrahydromethanopterin

HAO: hydroxylamine dehydrogenase

HPLC: high pressure liquid chromatography

HPS: hexulosephosphate synthase

ICM: intracytoplasmic membrane

KDPG: 2-keto-3-deoxy-6-phosphogluconate

KEGG: Kyoto Encyclopedia of Genes and Genomes

MDH: methanol dehydrogenase

MEP: methylerythritol 4-phosphate

MHC: multiheme cytochrome c

MMO: methane monooxygenase

MOB: methane oxidizing bacteria

MVA: mevalonate

N source: nitrogen source

NADH: nicotinamide adenine dinucleotide

NCBI: National Centre for Biotechnology

N-DAMO: nitrite-dependent anaerobic methane oxidation

NMS: nitrate mineral salts

OD: optical density

OMZ: oxygen minimum zone

PCA: principal component analysis

PEP: phosphoenolpyruvate

PHA: polyhydroxyalkanoate

PHB: polyhydroxybutyrate

PHI: phosphohexulose isomerase

PKT: phosphoketolase

PLFA: phospholipid fatty acid

pMMO: particulate methane monooxygenase

PQQ: pyrroloquinoline quinone

pXMO: divergent particulate methane monooxygenase

RNA: ribonucleic acid

RNA-Seq: whole transcriptome shotgun sequencing

RPKM: reads per kilobase million

RuBisCo: ribulose-1,5-bisphosphate carboxylase/oxygenase

RuMP: ribulose monophosphate

SCF: self-cycling fermentation

sMMO: soluble methane monooxygenase

SRB: sulfate reducing bacteria

TCA: tricarboxylic acid

THF: tetrahydrofolate

TPM: transcripts per million

# 1. Introduction

## 1.1. Motivation and Context

Methanotrophs, or methane-oxidizing bacteria (MOB), are a specialized class of bacteria characterized by their use of methane as their sole source of carbon and energy. Ubiquitously distributed in the environment, they are hugely important in the regulation of the global methane cycle (Hamer, 2010; Murrell, 2010). Methane is the second most potent greenhouse gas, with global warming potential between 25-85 times that of carbon dioxide (EPA, 2019; Jackson, Solomon et al., 2019). As industrialization took off around the world in the 18$^{th}$ century, dramatic effects – many of which continue to this day – could be observed and measured as a consequence, including the release of methane into the atmosphere as a byproduct of many processes. Unfortunately, emissions from modern industries are only increasing, with 2018 showing record concentrations of methane concentrations in the atmosphere, 2.5 times greater than pre-industrial levels. Clearly, strategies that address increasing methane concentrations in the atmosphere are more necessary than ever, but human technologies have yet to match the demand (Jackson et al., 2019).

Fortunately, methanotrophs have long been recognized for their potential in the field of industrial biotechnology. As well as the direct potential for methane abatement (Cantera, Bordel et al., 2019), they've long been known to be powerful agents of bioremediation of such pollutants and toxic compounds like chloroethylene (Jiang, Chen et al., 2010; Wendlandt, Stottmeister et al., 2010). Recently however, what is also of interest is the bioproduction of value-added products from methane. As methane is often released from industry as offstream gas – both a waste and an environmental pollutant –, its biotransformation via methanotrophs can be both environmentally-friendly and economically sound, turning lost revenue into profitable outcomes (Cantera et al., 2019; Hwang, Nguyen et al., 2018; Pieja, Morse, & Cal, 2017).

Methane is also highly desirable as feedstock for bioprocesses, in that its low cost mitigates one of industrial biotechnology's most critical drawbacks: the cost of feedstock. In fact, in some cases, feedstock can account for up to 50% of the total cost of a bioprocess, and reducing these costs drastically improves the economic prospects of bioproduction (Rostkowski, Criddle, & Lepech, 2012). While methane is the natural substrate of MOB, methanol too can be used as a growth substrate. Also produced as a waste product of many industries, this allows for even more opportunities to utilize methanotrophs in eco-friendly, economical technologies (Mockos, Smith et al., 2008). Methanol, unlike methane, also has the benefit of being liquid at normal temperature and pressure, allowing for easier transport and feeding strategies (K. Khosravi-Darani, Z.-B. Mokhtari et al., 2013). Both carbon sources, often low-value waste, are therefore being pursued as valuable potential feedstocks for MOB-focused biotechnologies.

While the economic and environmental benefit may be enticing, much work remains to be completed to design optimal, efficient processes for methanotroph-based biorefineries. Media optimization is a chief concern, affecting both growth and bioproduction capability of these bacteria (Hoefman, van der Ha et al., 2014; Sundstrom & Criddle, 2015). The current standard medium and growth conditions for methanotrophs was first described by Whittenbury and colleagues (1970) decades ago. However, fundamental questions remain about the selection of even two of the most central, basic components of these growth conditions: carbon, in the form of methane or methanol, and nitrogen, in the form of ammonium or nitrate. Uncertainties remain with regards to which of each is preferred, and how much these preferences can be generalized amongst methanotrophs at large.

Methanotrophs, being carbon fixers with a vast potential of nitrifying and denitrifying pathways, are strongly affected by both carbon and nitrogen sources. They can be hindered by growth in ammonium, a more energetically favourable N source, due to oxidation of ammonium by the same enzyme they rely on for methane oxidation (Stein, 2018). Methanol, a more energetically

favourable C source, has long been associated with growth defects at high concentrations due to toxicity (Murrell, 2010). Even further, the combined effects of these C and N sources has yet to be well established, as most nutrient-focused MOB literature examines either C or N sources, not accounting for the interconnection of regulation in all cells, let alone these highly specialized bacteria.

This thesis will address this gap in knowledge by directly investigating how methanotrophs react to and grow in different combinations of C and N sources. This knowledge will support future refinements of media and growth conditions, as well as outlining how strain-specific these characteristics are. A number of industrially-promising MOB strains are assayed and optimal conditions found, and the underlying regulatory changes that dictate these behaviours are assessed. By adding to the current knowledge-base of methanotroph physiology and regulation, these studies not only further our understanding of these vital biogeochemical effectors, but also lay critical groundwork for future process optimization and refinement, aiding in the development of superior, informed bioprocesses that benefit both society and the environment.

## 1.2. Aims of this Work

### 1.2.1. Growth and Optimization

To be industrially relevant, a biotechnology should be as efficient as possible, both from a processing and an economic perspective. This was addressed in Chapter 3 by determining optimal conditions via batch culture assessment and optimization for the purpose of growth of methanotrophic cultures and biomass for use in subsequent production schemes. As carbon and nitrogen are two of the most important factors in methanotroph culturing, and indeed critically influential in most bacterial culturing, their impact on growth must be assessed. This is specifically targeted towards identifying differences inherent to growth on industrially-relevant forms of carbon and nitrogen: methane and methanol, and ammonium and nitrate.

Also key to this work is the understanding that each species of methanotroph that may be of interest for future industrial biotechnology strategies will likely have individual growth behaviour profiles. In effect, one strain does not represent all strains, certainly for the wider class of methanotrophs, and even within the smaller subsection of proteobacterial MOB. Interestingly, unique physiologies emerge and should be treated as such, without falling into an oversight of over-generalizing. These aspects were captured by careful, comparative growth assays aimed at identifying particular profiles and assessing individually what they represent in a strain of interest.

### 1.2.2. Transcriptomics

Analysis of the RNA of the cell is another important facet of this work, allowing a more complete knowledge of the functioning of the cell, both in normal growth stages and for the eventual application to bioproduction schemes. Two strains were analyzed in this way, *Methylomicrobium album* BG8 (Chapter 4) and *Methylocystis* sp. Rockwell (Chapter 5), demonstrating how cells grown in different combinations of carbon-nitrogen sources responded transcriptionally to provide insight into regulation and potential pathway fluxes.

Because of the huge importance of both carbon and nitrogen to growth in methanotrophs, global transcriptional analysis under the four combinations of carbon and nitrogen sources are of special interest. These analyses should provide useful regulatory and growth optimization information, and may also bring to light how other metabolites of industrial interest are affected or induced by the growth conditions, providing both a better understanding of the process of growth in methanotrophs, and allowing for the discovery of other possible streams of research in terms of optimization and commercialization.

## 1.3. Hypothesis

The work explained in this thesis involved a number of areas of microbiological research, each pursued with individual expectations. However, these separate aspects followed an

overarching theory or hypothesis with respect to the overall goal of this work: understanding and optimizing the growth of methanotrophs. These are as follows:

i) It is expected that each of the methanotroph strains investigated will vary in terms of growth rate, lag time in batch, final cell density, cell yield per carbon, and differential preference for carbon-nitrogen source conditions. These differences can be quantified and optimal conditions can be identified, including suitability of strains for different processes.

ii) These differential preferences will result in differential RNA expression patterns between conditions, indicating diverse intracellular functioning due to dissimilar growth conditions, both between strains and between conditions.

iii) The differences in RNA transcription will in some way relate to the differential growth seen at the culture level, and will indicate future directions for growth process optimization, highlighting stress, activation of particular pathways, and potential targets for bioproduction.

Overall, these expectations can be synthesised into one hypothesis for the project at large: through careful applications of both classical cell physiology and more novel transcriptomics research, growth and regulation patterns beneficial to applications in bioindustry can be induced. This will be pursued through control of carbon and nitrogen sources`, guided by and optimized with principals of process development and assessment. Critically, the knowledge gained from one strain will not be treated as immediately representative of all MOB.

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# 2. Literature Review

## 2.1. A Brief Summary of the Methanotrophic Paradigm

### 2.1.1. Methanotrophy and its Significance

*Overview of Methanotrophy*

Methane oxidizing bacteria (MOB), also commonly referred to as methanotrophs, are microorganisms first described over 100 years ago. Their discovery is generally attributed to Sohngen, who isolated a bacterium on methane in 1906, naming it *Bacillus methanicus* (Söhngen, 1906), followed by a report in 1910 about the role of methane in organic life (Söhngen, 1910). Near simultaneously however, 1905 saw Kaserer report a similar observation (Kaserer, 1905), and ten years later a description of the physiology of methane-oxidizing bacteria was published (Münz, 1915). By 1949, it was reported that these bacteria were quite common in the environment, and physiological investigations were well underway (Hutton & ZoBell, 1949).

Methanotrophs are defined by their capacity to use methane as their sole source of carbon and energy, synthesizing all their intracellular carbon-containing compounds from methane gas (Murrell, 2010). Methanotrophs are a subset of a broader class of bacteria known as methylotrophs, which grow on compounds with no carbon-carbon bonds, but including multi-carbon substrates like trimethylamine. The additional specialization of methanotrophs arises from the obligate nature of the lifestyle, and the strict necessity of growth on specifically single-carbon substrates such as methane and sometime methanol (Murrell, 2010; Trotsenko & Murrell, 2008).

While considered ubiquitous in the environment, certain environments do favour higher proportions of methanotrophs, often in relation to methane-sources like methane-producing archaeal methanogens – this includes rice paddies, upland and forest soils, freshwater, marine water, and sediments (Murrell, 2010). Growing on an inorganic, gaseous form of carbon, the MOB

are notable for their highly specialized carbon fixation pathways; the serine cycle and the ribulose monophosphate (RuMP) pathways. These were first described by Lawrence and colleagues (Lawrence, Kemp, & Quayle, 1970) and rely on formaldehyde as a key intermediate in carbon metabolism (Trotsenko & Murrell, 2008). This lifestyle leaves methanotrophs unique amongst trophic categories, and their reliance on relatively low energy biochemical pathways means that in general they are known to be relatively slow growing (Murrell, 2010; Trotsenko & Murrell, 2008).

When first discovered, and for many decades following, methanotrophs were considered strictly aerobic, Gram-negative organisms (Whittenbury et al., 1970). Certainly, hundreds of species fitting this description have been cultured and isolated, and the number of genomes sequenced in this category is notable – though descriptions of their taxonomy and classification remain a work in progress (Orata, Meier-Kolthoff et al., 2018). However, more recent evidence points to more varied taxonomic classifications and even specific metabolisms.

For instance, the recent discovery of MOB in the phylum *Verrucomicrobia* points to a new branch in the field of methanotrophy, with characteristics quite unlike the classically envisioned methanotroph lifestyle. These microbes are extreme acidophiles, isolated from a variety of extremely challenging environments, quite unlike any MOB isolated and described until that time (Dunfield, Yuryev et al., 2007; Islam, Jensen et al., 2008; Pol, Heijmans et al., 2007). As well as this extremophile classification, they were found to possess lesions in the pathway of methane uptake and carbon assimilation to cell biomass. Thereafter, it was found that they employed the Calvin-Benson-Bassham pathway for carbon fixation instead, relying on a distinct form of enzyme RuBisCO (Khadem, Pol et al., 2011). This was a novel mode of life for MOB at the time of its discovery, outside the traditional serine and RuMP pathways.

Even beyond the paradigm of strictly bacterial life, other forms of methanotrophic lifestyles also exist. One of the most significant, ecologically, is sulfate-reducing bacteria (SRB) coupled with

anaerobic methanotrophic archaea (ANME), which are biologically methanogenic Archaea "operating in reverse", consuming methane (Strous & Jetten, 2004). Anaerobic oxidation of methane (AOM) was discovered by Reeburgh in 1976, and the microbes involved in this process were described at the turn of the millennium; this opened many doors in methane oxidation research, and redefined our understanding of the pervasiveness of this lifestyle and the impact it has on the ecosystem (Cui, Ma et al., 2015; Reeburgh, 1976). This metabolism relies on the transfer of electrons from the ANME partner to the SRB through direct interspecies electron transfer (DIET), including use of multiheme cytochrome c proteins (MHCs), allowing an otherwise thermodynamically impossible metabolism to occur in the ANME partner (Timmers, Welte et al., 2017).

Later, a consortium completing nitrite-dependent anaerobic methane oxidation was also found to occur, adding yet more streams of methane-oxidation research to the field (Cui et al., 2015; Raghoebarsing, Pol et al., 2006). ANME were thereafter found to complete nitrate-based AOM by delivering electrons to a membrane-bound nitrate reductase, and metal-dependent AOM was then proposed (Timmers et al., 2017). Since their relatively recent discovery, these different forms of specialized commensal microorganisms have been found to contribute massively to methane oxidation, and therefore mitigation, in the oceans (Conrad, 2009; Cui et al., 2015; Strous & Jetten, 2004). In fact, it is estimated that most methane produced in deep sea sediments is oxidized by ANME before it seeps out into the atmosphere; and without this process atmospheric methane levels would be up to 60% higher than current values (Conrad, 2009).

Another notable example of AOM that must be discussed is the NC10 phylum identified in 2010, which marked the first evidence for independent anaerobic methane oxidation driven by nitrite reduction in the process of denitrification, termed N-DAMO. These microorganisms are taxonomically distinct from the classical aerobic proteobacterial methanotrophs, and their discovery marked the realization that this lifestyle could function even in environments thought

impossible for the MOB paradigm (Ettwig, Butler et al., 2010; Stein, 2018). Linkages to denitrification have subsequently been found in proteobacterial methanotrophs as well (Kits, Campbell et al., 2015; Kits, Klotz, & Stein, 2015); certainly demonstrating that many more discoveries are yet to be made in the field of non-aerobic methane oxidation, and especially the metabolism of nitrogen species (Stein, 2018). Clearly, these more recently discovered versions of methanotrophy are rapidly redefining our understanding of the field, and further research continues to broaden our view of how methanotrophs operate *in vivo* and what contributions they make to biogeochemical cycles.

*Significance*

After carbon dioxide, methane is the second most important anthropogenic greenhouse gas, absorbing infrared radiation and having a global warming potential 84 times more potent than $CO_2$ in the first 20 years after emission on a molar basis, and ~28 times more potent after the fist century (Jackson et al., 2019). Atmospheric levels of methane have been rising alarmingly over the last 200 years. Though this trend slowed in the 1990's (Breas, Guillou et al., 2002), claims soon after emerged that indicated rising levels of methane once more (Rigby, Prinn et al., 2008), and in 2018 global methane concentrations passed 1860 ppb for the first time, more than 2.5 times the pre-industrial level (Jackson et al., 2019).

The biogeochemical significance of methanotrophs is then clear. Though chemical processes account for the majority of global methane sinks (Breas et al., 2002; Conrad, 2009), aerobic methanotrophs are responsible for mitigating 80-90% of the methane produced in some environments before it reaches the atmosphere (Frenzel, Rothfuss, & Conrad, 1992) and methanotrophs in general, both aerobic and anaerobic, play a large role in controlling atmospheric methane concentrations (Conrad, 2009). While this work will focus only on aerobic MOB, it should be emphasized that the previously mentioned AOM is equally important to the biogeochemistry of

methane flux balance (Conrad, 2009). Both lifestyles therefore play a noticeable role in the sequestration of methane and in the cycling of carbon through the methane cycle, balancing the effect of anaerobic methanogenic Archaea and mitigating greenhouse gas effects (Conrad, 2009; Smith, Trotsenko, & Murrell, 2010).

While the natural methane cycle does produce methane, anthropogenic contributions to methane emission are yet more pressing, and current mitigation strategies fall short of accounting for the scale of the problem (Jackson et al., 2019). Industries such as energy, transportation, and agriculture directly release large amounts of methane (Conrad, 2009), and less direct effects exist as well. Notably, land use and management has a large effect on soil capacity to enable methane capture and oxidation; a high degree of deforestation currently results in much less methane being oxidized by methanotrophs globally than might otherwise be possible in afforested or reforested lands (Tate, 2015). The question of better understanding the already functioning natural methane sink of methanotrophs is thus more pressing than ever.

### 2.1.2. Aerobic Proteobacterial Methanotrophs

*Aerobic Methanotrophy*

While the NC10, *Verrucomicrobia*, and ANME modes of life outlined previously are certainly vital contributors to the overall picture of methanotrophy in biogeochemical cycles, this work will focus on the aerobic proteobacterial methanotrophs. Due to its earlier discovery, aerobic methanotrophy remains likely the most thoroughly studied form of methanotrophy. It is a fairly rare physiology, and all known species currently exist within one of five distinct taxonomic groups within the Proteobacteria. It has been proposed that each such grouping arose from an independent horizontal gene transfer event of the methane monooxygenase genes, these being the critical and archetypal characteristic of these bacteria (Osborne & Haritos, 2018). While many intricacies of their metabolism and function remain elusive, there are a number of physiological behaviours that

have been described in detail. For instance, it has been noted that the preferred ratio of oxygen to methane availability in typical aerobic methanotrophs is approximately 1.5-1.7 (Amaral & Knowles, 1995; Joergensen & Degn, 1983; Leak & Dalton, 1986b).

Oxygen is required by these organisms for two critical reasons. Firstly, it is integral to the activation of methane by the methane monooxygenase enzymes and, secondly, oxygen is required for the functioning of oxidative respiration. This is true whether oxygen is captured from the aerobic environment surrounding the cells or generated from enzymatic reactions by the bacterium. As such, oxygen concentration, availability, and microaerophilic/anaerobic adaptation have all been major focuses in methanotrophic research in the past, and continuing to this day (Amaral & Knowles, 1995; Bussmann, Rahalkar, & Schink, 2006; Chidambarampadmavathy, Karthikeyan et al., 2017; Ettwig et al., 2010; Graham, Chaudhary et al., 1993; Lee, Soni, & Kelley, 1996). Interestingly, it is becoming increasingly clear that this definition may be overly strict, even for methanotrophic bacteria long-considered obligate aerobes, as many strains are found to flexibly operate alternative respiration, like denitrification, or even fermentation for survival and growth (Kalyuzhnaya, Gomez, & Murrell, 2019; Kalyuzhnaya, Yang et al., 2013; Kits, Campbell, et al., 2015; Kits, Klotz, et al., 2015).

Aerobic respiration remains the most common and seemingly preferred physiology of these methanotrophs, however. It is well-acknowledged in the field that this oxidative metabolism parallels that of the ammonia oxidizing bacteria (AOB), to which the MOB are evolutionarily related, owing to a shared evolutionary history between the enzymes used to oxidize their substrates of choice, ammonia monooxygenase (AMO) and methane monooxygenase (MMO), respectively. This shared evolutionary history critically means that AMO and MMO are able to oxidize the opposite target substrate as well as the intended one, *i.e.* MMO will oxidize ammonium, and AMO methane, if present (Culpepper & Rosenzweig, 2012; Holmes, Costello et al., 1995; Khadka, Clothier et al., 2018;

Osborne & Haritos, 2018; Stein & Klotz, 2011; Tavormina, Orphan et al., 2011). This point will become salient in a later discussion of nitrogen metabolism in the MOB.

*Types of Proteobacterial Methanotrophs*

The subsection of aerobic proteobacterial methane oxidizers can be further divided into two main categories, based on a number of physiological and biochemical factors. Taxonomically, they are distinct and are now referred to primarily by these classes in the literature: the gammaproteobacterial methanotrophs (gamma-MOB; classically termed Type I) and alphaproteobacterial methanotrophs (alpha-MOB; classically termed Type II). One of the primary differences is in carbon assimilation pathways: through the ribulose monophosphate (RuMP) pathway in gamma-MOB and through the serine pathway in alpha-MOB. Some other distinguishing features are: the possibility of cyst-like resting stages in the gamma-MOB and the formation of exospores or lipoidal cysts in the alpha-MOB; fatty acid chain lengths of 16 carbons in gamma-MOB and 18 carbons in alpha-MOB; higher G+C content in gamma-MOB DNA vs. alpha-MOB DNA (Murrell, 2010).

Further, alpha-MOB and gamma-MOB proteobacterial methanotrophs demonstrate different preferred nutrient concentrations, or responses to the concentrations. Classically, it has been reported that gamma-MOB growth excels in N-sufficient, high oxygen, low methane environments, while alpha-MOB thrive in the opposite case (Amaral, Archambault et al., 1995; Amaral & Knowles, 1995; Graham et al., 1993). More recently, it has emerged that gamma-MOB species actually grow well under high methane concentrations as well, coming to dominate over the alpha-MOB (Duan, Reinsch et al., 2017). Alpha-MOB have also been found to be more active than gamma-MOB in low, atmospheric methane concentrations, leading to the suggestion that dominance is perhaps ecosystem specific (Knief & Dunfield, 2005; Knief, Kolb et al., 2006). Clearly,

our understanding of the preferences and ecological niches of these different groups continues to evolve.

In mixed communities, it is certainly true that factors other than methane, including nitrogen species and copper-iron ratio (Chidambarampadmavathy et al., 2017), are important in determining competitive dominance, community size and structure (Bussmann, Pester et al., 2004; Duan et al., 2017). As it plays a major role in MOB growth, the specific effects of nitrogen will be explored further later in this review, specifically with regard to nitrate and ammonium as N sources.

Once again, oxygen should also be specifically highlighted. Oxygen mixing ratios have been shown to influence successful MOB isolation and culturing, but not in a linear manner (Bussmann et al., 2004), and the ratio of oxygen to methane is key, as different ratios allow for cultivation of different methanotroph species (Bussmann et al., 2006). In general, areas of high oxygen concentrations tend to be dominated by gamma-MOB while lower oxygen concentrations favour alpha-MOB (Shukla, Pandey, & Mishra, 2013). For example, high oxygen zones of the root surface in rice paddies were found to support gamma-MOB, while the alpha-MOB dominated lower oxygen zones further from the roots in the rhizosphere, where, coincidentally, methane is more plentiful (Shrestha, Abraham et al., 2008).

An interesting view into oxygen-dependent community structure lies in oxygen minimum zones (OMZ), areas of very scarce oxygen in the oceans, which are the largest source of marine methane into the atmosphere (Bertagnolli & Stewart, 2018). While aerobic methanotrophs are naturally not highly abundant in OMZ, community studies have identified the presence of both gamma-MOB and alpha-MOB in rare amounts, with gamma-MOB appearing to dominate (Chronopoulou, Shelley et al., 2017; Torres-Beltrán, Hawley et al., 2016). It is suggested that

gamma-MOB in OMZ might be supported by excreted $O_2$ from photosynthetic community members, and aided by their ability to denitrify as well as respire aerobically (Bertagnolli & Stewart, 2018).

Returning to structure, a major division between alpha-MOB and gamma-MOB is also made in the type of intracytoplasmic membranes (ICM) present in the species. These ICM are present in all methanotrophs, granting this class of bacteria to contain unusually high average amounts of lipid per cell, and to be very highly structured (Kalyuzhnaya et al., 2019). The lipids that compose ICM are also highly identifiable as molecular markers, given their specificity, with particular C18-type lipids characteristic of the alpha-MOB and specific C16 lipids signalling the gamma-MOB (Bodelier, Gillisen et al., 2009; Bowman, Skerratt et al., 1991; Fang, Barcelona, & Semrau, 2000). These lipids and the ICM they constitute are critical to facilitating regular methanotroph metabolism regardless of type, as they are the site of methane oxidation, hosting the pMMO enzyme. However, the form that they take is distinct by group; ICM are arranged in disks throughout the cell in the gamma-MOB while in the alpha-MOB, ICM exist in paired layers around the cell's periphery (Trotsenko & Murrell, 2008).

### 2.1.3. The Methanotrophic Model Organisms in this Work

*Methylosinus trichosporium* OB3b

While the bacterium was originally isolated decades ago (Whittenbury et al., 1970), it is only recently that the genome of *M. trichosporium* OB3b (for "oddball strain 3b") has been sequenced. It was found to be 4.9Mb long with over 4000 predicted protein-encoding genes (Heil, Lynch et al., 2017; Stein, Yoon et al., 2010). This strain serves as a model alphaproteobacterial methanotroph, and many studies have been conducted based around the functioning of this strain. It is often used as a comparison or reference in the literature in studies of novelties in methanotroph physiology, owing to its well-characterized physiology (Kalyuzhanaya, Yang et al., 2013; Matsen, Yang et al., 2013).

*Methylocystis* sp. Rockwell (ATCC 49242)

Though not a model organism, the alpha-MOB *Methylocystis* sp. Rockwell is another methanotroph that has been subjected to full genome sequencing, establishing it as a promising new strain of focus in the field of MOB research. It contains over 4600 predicted protein-coding genes, in a genome of 4.6 Mbp in size (Stein, Bringel et al., 2011). Little work focusing on this strain is currently available in the literature, though it has been used as comparison to more highly studied strains of *Methylocystis* (Dam, Dam et al., 2013).

*Methylocystis* sp. WRRC1

While also not a model organism, the alpha-MOB *Methylocystis* sp. WRRC1 has recently been subjected to full genome sequencing. Again, given its relatively recent sequencing, little work on this strain is currently available, although it has been used in a study of polyhydroxybutyrate-co-hydroxyvalerate production, showing industrial potential worth investigating (Cal, Sikkema et al., 2016).

*Methylomicrobium album* BG8

Another methanotroph with a completely sequenced genome – 4.49Mb in size and almost 4000 predicted protein coding genes (Kits, Kalyuzhnaya et al., 2013) – is *M. album* BG8, a gamma-MOB that was isolated alongside *M. trichosporium* OB3b decades ago (Whittenbury et al., 1970). Over time since its isolation, it has undergone numerous changes in nomenclature, resulting in a variety of names in the literature including *Methylobacter albus*, *Methylomonas albus*, and *Methylomonas alba* (Kits et al., 2013). Like *M. trichosporium* OB3b, it has been used in many physiological and genetic studies as a representative strain (Hanson & Hanson, 1996).

*Methylomonas denitrificans* FJG1

The gamma-MOB *Methylomonas denitrificans* FJG1 has been subjected to full genome sequencing, finding a genome of size 2.5 Mbp, encoding over 4500 protein-coding genes (Orata, Kits, & Stein, 2018). Little work about this strain is currently available, although it has been used as a model of denitrification in aerobic methanotrophs (Kits, Klotz, et al., 2015).

## 2.2. Metabolism in Aerobic Proteobacterial Methanotrophs

### 2.2.1. Carbon Metabolism in Methanotrophs

*Methane Oxidation*

Methane oxidation in MOB is accomplished by sequential transformation of methane to carbon dioxide via the intermediates methanol, formaldehyde, and formate. The first enzyme is methane monooxygenase (MMO), which exists in two forms: the membrane-bound, copper-centred particulate form (pMMO) and the cytosolic, iron-centred soluble form (sMMO). pMMO is found in the genome in the operon *pmoCAB*, while sMMO is found in *mmoXYZ* (Hakemian & Rosenzweig, 2007; Kenney, Sadek, & Rosenzweig, 2016; Larsen & Karlsen, 2016). While pMMO is present in almost all isolated MOB, save *Methylocella palustris* (Dedysh, Liesack et al., 2000) and other *Methylocella* species, sMMO is present in fewer isolates, its distribution not universal even within species (Kaluzhnaya et al., 2001). pMMO is considered the dominant form of the enzyme, however, and those bacteria encoding both forms exhibit a strong preference for its expression over sMMO (Semrau, Jagadevan et al., 2013). Both forms of MMO catalyze the initial oxidative attack of methane, creating methanol. Expression is largely copper-dependent – which will be returned to later – with sMMO synthesized in response to low copper concentrations (Culpepper & Rosenzweig, 2012; Murrell, McDonald, & Gilbert, 2000; Murrell & Smith, 2010).

While it is generally accepted that MMO comes in two forms, recent discoveries actually point to the importance of a third form, termed pXMO. Identified by Tavormina et al (2011), it is

found in the *pxmABC* operon of the genome, notably different in gene order than pMMO, to which it is evolutionarily related. This enzyme has so far only been identified in gamma-MOB, and appears to be expressed only in low oxygen environments (Kits, Campbell, et al., 2015; Kits, Klotz, et al., 2015; Tavormina et al., 2011).

After MMO, methanol is further oxidized to formaldehyde by the next enzyme in the pathway, the periplasmic methanol dehydrogenase (MDH) MxaFI. MxaFI is a calcium-dependent pyrroloquinoline quinone (PQQ)-linked enzyme, universally distributed in the alpha- and gamma-MOB, and was long thought to be the primary form of methanol oxidation in these strains (Anthony, 2004; Smith et al., 2010). Recently however, a novel methanol dehydrogenase, XoxF, has been identified and investigated in a number of strains. This enzyme possesses a lanthanide centre and was found to be more dominant than MDH in methanol oxidation in some species. XoxF is now the subject of much research and its role, distribution, and significance will likely become increasingly clear in the years to come (Chu & Lidstrom, 2016; Farhan Ul Haque, Kalidass et al., 2015; Gu, Farhan Ul Haque et al., 2016; Skovran, Palmer et al., 2011).

In the next step, formaldehyde is oxidized to formate by a number of possible pathways. This is considered a branchpoint in methanotroph physiology, towards either assimilation or energy generation. In model 'Type X' strain *Methylococcus capsulatus* Bath (having a "metabolic mosaic" of primary carbon metabolism, including an active Calvin-Benson-Basshom cycle and sMMO, which distinguishes it from the classic Type I and Type II classifications) cytochrome-linked formaldehyde dehydrogenase (FADH) tends to dominate when pMMO is expressed (Zahn, Bergmann et al., 2001). However, alpha- and gamma-MOB rely primarily on other intermediate pathways. First, the tetrahydrofolate (THF) pathway enzymes which, due to their reversibility, can be used to regulate the fate of formaldehyde either towards assimilation or oxidation to formate, depending on the needs of the cell (Vorholt, 2002). Another important pathway from formaldehyde is the tetrahydromethanopterin ($H_4$MPT) pathway, which can also lead to formate, or to

assimilation, as required by the cell (Vorholt, 2002), particularly for alpha-MOB. Formate, thus, is increasingly considered a branchpoint as well, and perhaps the true critical branchpoint for the serine cycle, which allows for assimilation of formaldehyde by alpha-MOB (Crowther, Kosály, & Lidstrom, 2008).

In the last step of methane oxidation, formate is oxidized to carbon dioxide by $NAD^+$-dependent formate dehydrogenase (FDH), which is present in all extant methanotrophs (Smith et al., 2010; Trotsenko & Murrell, 2008). It has been estimated that 40% of the carbon uptake by MOB is directed towards assimilation, while the other 60% encompasses the energy requirements of the organism (Bodelier & Laanbroek, 2004). This proportion can vary however for a number of reasons. First, the relative efficiencies of the assimilatory pathways for formaldehyde leads to overall more efficient growth for gamma-MOB compared to alpha-MOB (Hanson & Hanson, 1996; Smith et al., 2010; Trotsenko & Murrell, 2008). As well, there are considerations with regards to carbon and nitrogen sources, such as use of methane vs. methanol or ammonium vs. nitrate (Bodelier & Laanbroek, 2004; Leak & Dalton, 1986b; van Dijken & Harder, 1975).

*Carbon Assimilation*

As previously mentioned, carbon assimilation in methanotrophs can follow two distinct pathways: in gamma-MOB, the ribulose monophosphate (RuMP) pathways, and in alpha-MOB, the serine pathway, to incorporate formaldehyde into biomass. In both cases, formaldehyde, an intermediate of methane oxidation, is used as the starting molecule to form intermediates of central carbon metabolism, which can then be further metabolised and assimilated into cell mass (Anthony, 1978; De Vries, Kues, & Stahl, 1990; Quayle, 1980).

In the RuMP pathway, formaldehyde is first fixed with ribulose-5-phosphate by the enzyme hexulosephosphate synthase (HPS) to form hexulose-6-phosphate, a very unstable intermediate. It is then converted by phosphohexulose isomerase (PHI) into fructose-6-phosphate. The second part

of the RuMP pathway sees this molecule converted to 2-keto-3-deoxy-6-phosphogluconate (KDPG), which is cleaved by KDPG aldolase to produce pyruvate and glyceraldehyde-3-phosphate (GAP). Finally, rearrangement of GAP and fructose-6-phosphate allows regeneration of ribulose-5-phosphate (Lawrence et al., 1970; Trotsenko & Murrell, 2008). This pathway was first described by Quayle and colleagues (Johnson & Quayle, 1965; Kemp & Quayle, 1967; Strom, Ferenci, & Quayle, 1974).

The second carbon assimilation pathway in methanotrophs is the serine pathway, which was also proposed and described first by Quayle and colleagues (Lawrence et al., 1970). In this pathway, serine is formed by reaction of formaldehyde and glycine, catalyzed by the enzyme hydroxymethyltransferase. Transamination of serine follows, using glyoxylate as the amino group acceptor, releasing glycine and producing hydroxypyruvate. The enzyme hydroxypyruvate reductase converts this to glycerate and addition of phosphate from ATP by glycerate kinase produces phosphoglycerate which is then isomerized to phosphoenolpyruvate (PEP), using two enzymes unique to methanotrophs with the serine pathway (Anthony, 1978; Dijkhuizen, Levering, & de Vries, 1992; Quayle, 1980; Strom et al., 1974; Trotsenko & Murrell, 2008).

Next, PEP is carboxylated to oxaloacetate by fixation of carbon dioxide, and reduction to malate follows via malate dehydrogenase, in reactions similar to many heterotrophic bacteria. The subsequent formation of malyl co-enzyme A occurs through catalysis by malate thiokinase and cleavage by hydroxypyruvate reductase. These two enzymes are also unique to serine pathway-utilizing microorganisms. Finally, glyoxylate and acetyl-CoA, the major product of this pathway, are formed by malyl-CoA lyase. (Anthony, 1978; Dijkhuizen et al., 1992; Hanson & Hanson, 1996; Quayle, 1980; Strom et al., 1974; Trotsenko & Murrell, 2008).

The second part of the serine cycle pathway involves regeneration of glycine, the primary acceptor of formaldehyde and thus the entry point into the cycle. Originally, this was proposed to

occur through conversion of acetyl-coA via the glyoxylate cycle, however serine cycle MOB lacked a necessary enzyme, isocitrate lyase (Anthony, 1978; Dijkhuizen et al., 1992; Quayle, 1980; Strom et al., 1974; Trotsenko & Murrell, 2008). In some facultative methylotrophs not containing this key enzyme, the ethylmalonyl-CoA (EMC) pathway was found to be used in place of the glyoxylate cycle, and thus is was proposed that this may also be true for obligate serine-using methanotrophs (Erb, Berg et al., 2007; Trotsenko & Murrell, 2008). This was later confirmed to be accurate and remains the current understanding of how serine-cycle bacteria regenerate glyoxylate for transamination back into glycine (Kalyuzhanaya et al., 2013; Kalyuzhnaya et al., 2019).

*Other Considerations in Carbon Metabolism*

In addition to formaldehyde incorporation by methanotrophs, a mention must also be made of carbon dioxide assimilation. Due to the differences between alpha- and gamma-MOB outlined above, the amounts of carbon in cell biomass derived from carbon dioxide varies between the two types: only 5-15% of carbon biomass in gamma-MOB and up to 50% in alpha-MOB. Anapleurotic carbon dioxide fixation is, however, accomplished in both types by the enzyme PEP carboxylase (Shishkina & Trotsenko, 1986). Furthermore, some methanotrophs possess and utilize the Calvin-Benson-Bassham cycle for $CO_2$ fixation, notably the extremophilic Verrucomicrobia and NC10 phyla, a pathway quite distinct from methane fixation (Kalyuzhnaya et al., 2019; Khadem et al., 2011; Rasigraf, Kool et al., 2014).

Nevertheless, a major dissimilarity between the two pathways of formaldehyde assimilation is energy yield and expected growth rates of the two types of MOB. Biochemically, the RuMP pathway used by the gamma-MOB, based on calculations of enzymatic efficiency and ATP yield, results in yields that are on average 20% greater than those achieved by growth using the serine pathway, as in alpha-MOB (van Dijken & Harder, 1975). As such, and as noted in the summary of growth rates presented by Kalyuzhnaya et al. in a recent review (2019), gamma-MOB have long

been noted to demonstrate faster, more efficient growth than the alpha-MOB, in line with predicted energy requirements.

The role of the Embden–Meyerhof–Parnas (EMP) pathway and the role of fermentation in gamma-MOB should also be mentioned as this juncture. Historically, the Entner–Doudoroff (ED) pathway was considered to be the active glycolytic pathway in methanotrophs. Recently however, it was shown that the EMP pathway was dominant for pyruvate generation in gamma-MOB, significantly improving theoretical energetic efficiency (Kalyuzhnaya et al., 2013). This was also paired with the discovery that gamma-MOB consume methane at low-oxic conditions via fermentation, which could have many implications not only on the bioenergetics of cell growth, but also on the understanding of the role of methanotrophs in the environment (Kalyuzhnaya et al., 2013). Our understanding of methanotroph carbon fixation and assimilation will likely continue to evolve rapidly in the coming years.

### 2.2.2. Nitrogen Metabolism in Methanotrophs

*Nitrogen Fixation*

Much like carbon metabolism, understanding nitrogen metabolism associated with methanotrophy is vital to attaining a complete representation of the physiology and biochemistry of MOB. For every mole of carbon required by methanotrophs, 0.25 moles of nitrogen must also be used (Bodelier & Laanbroek, 2004), a fairly significant requirement. It was accepted for a long time that only alpha-MOB methanotrophs were capable of fixing dinitrogen ($N_2$) (Hanson & Hanson, 1996), referred to as diazotrophy, through use of the nitrogen fixation gene *nifH* (Stein, 2018). However, reports emerged that gamma-MOB are also capable of fixing $N_2$ (Auman, Speake, & Lidstrom, 2001; Boulygina, Kuznetsov et al., 2002; Dedysh, Ricke, & Liesack, 2004). Indeed, five genera across both the alpha-MOB and gamma-MOB have been linked to $N_2$-fixation capability, as does a member of the Verrucomicrobia phylum, demonstrating that this is a widespread trait

amongst MOB, though research remains open on the topic of the extent and regulation of such diazotrophy (Stein, 2018).

*Nitrogen Assimilation*

In terms of assimilation of nitrogen, MOB type again plays a role. The gamma-MOB primarily employ reductive amination of pyruvate or α-ketoglutarate to assimilate ammonium. Conversely, alpha-MOB use the glutamate cycle, also known as the GS-GOGAT system, relying on the activity of glutamine synthetase (GS) and glutamine-oxoglutarate amidotransferase or glutamate synthase (GOGAT) (Trotsenko & Murrell, 2008). However, Type I methanotrophs were also found to switch strategies depending on ammonium availability, relying on reductive amination in cases of ammonium surplus – such as growth in ammonia-spiked medium – and on the glutamate cycle when grown under ammonium limitation – including nitrogen-fixing conditions and growth on nitrate-containing medium (Murrell & Dalton, 1983). The genome of *M. capsulatus* Bath contains genes for four different predicted ammonium transporters, pointing to the importance of ammonium as a nitrogen source in at least some methanotrophs (Ward, Larsen et al., 2004).

*Ammonium Inhibition*

As mentioned above, a notable trait of methanotrophs lies in shared evolutionary history of the MMO and AMO enzymes, which allows for the binding of ammonium by MMO, creating a situation of competitive inhibition and thereby preventing methane oxidation, in effect inhibiting growth (Holmes et al., 1995; Stein, 2018). Nitrogen can thus be considered a regulatory factor for the process of methane oxidation (Bodelier & Laanbroek, 2004; Stein, 2018). However, competitive inhibition is likely not the most significant outcome of ammonia co-metabolism by MMO in most strains. Rather, this process results in the production of hydroxylamine, a highly toxic intermediate that many strains of methanotrophs cannot adequately overcome. Detoxification of this intermediate requires action of hydroxylamine dehydrogenase (HAO), which is indeed found in the

genomes of many methanotrophs (*haoAB*) (Stein, 2018), and upregulated in the presence of ammonium (Campbell, Nyerges et al., 2011). Some uncertainty remains still in the pathway following this enzymatic conversion, but those methanotrophs that encode HAO were found to be better nitrifiers than those that do not, and presence of HAO does predict ammonium-growth tolerance (Campbell et al., 2011; Nyerges, Han, & Stein, 2010; Nyerges & Stein, 2009; Stein, 2018).

The inhibitory effect of ammonium has been studied in a number of methanotroph strains, across types, proving it is not characteristic of only one taxonomic line. Lower rates of methane conversion in the presence of ammonium have been demonstrated repeatedly (Campbell et al., 2011; He, Chen et al., 2017; Mohammadi, Pol et al., 2017; Nyerges & Stein, 2009). It follows, then, that it has long been commonly noted in the literature that the presence of ammonium will inhibit the growth of methanotrophs and that nitrate in the medium or environment is preferable for the growth of methanotrophs, leading to its use as a standard in methanotroph culturing. This is seen in both observations of the environmental effects of ammonium fertilizers and in pure cultures of methanotrophs (Avrahami, Liesack, & Conrad, 2003; Bedard & Knowles, 1989; Bender & Conrad, 1995; Bosse, Frenzel, & Conrad, 1993; Bykova, Boeckx et al., 2007; Flessa, Pfau et al., 1996; Hu & Lu, 2015; Kim, Imori et al., 2012; King & Schnell, 1994a, 1994b; Mohanty, Bodelier et al., 2006; Reay & Nedwell, 2004; Schnell & King, 1994).

*Nitrate and Nitrite Inhibition*

While the inhibition of methanotrophs by ammonium may be well-known in the field, inhibition by nitrate and nitrite is less established. In fact, these effects have been described and explained only recently. In general, alpha-MOB like *Methylocystis* sp. Rockwell are not likely to possess nitrite- and nitric oxide reductases, unlike gamma-MOB which demonstrate a fairly high rate of occurrence. Under low oxygen conditions, these enzymes sequentially reduce nitrite to nitric oxide (NO) and to nitrous oxide ($N_2O$), allowing for detoxification, or even potentially respiratory

denitrification (Kits, Campbell, et al., 2015; Kits, Klotz, et al., 2015; Stein, 2018; Stein & Klotz, 2011). Under such circumstances, nitrate ($NO_3^-$) or nitrite ($NO_2^-$) can even provide a growth benefit. This is not a universal truth, however.

Like ammonium, high concentrations of nitrate lead to reduced methanotroph cell counts (Bussmann et al., 2004) and have previously been linked to reduced methane oxidation capacity in soil studies, inhibiting methane oxidation at low atmospheric concentrations (Reay & Nedwell, 2004). Further, nitrite inhibition has been noted in a number of strains; however the magnitude of the effect appears to vary strain by strain (King & Schnell, 1994a; Nyerges et al., 2010). *Methylocystis* sp. Rockwell is of key interest in this area. It is a strain notably well-adapted for growth in ammonium, and does possess HAO, but suffers from very poor growth in nitrite (Nyerges et al., 2010). HAO in methanotrophs favours production of nitric oxide (NO), not nitrite ($NO_3^-$), but this strain lacks a nitric oxide reductase, which facilitates the conversion of nitric oxide (NO) to nitrous oxide ($N_2O$). How nitrite fits into this pathway is not yet decisively resolved, but its potential for inhibitory effects are well noted (Campbell et al., 2011; Nyerges et al., 2010; Nyerges & Stein, 2009; Stein, 2018; Stein & Klotz, 2011).

Essentially, it is for this reason that, against popular knowledge, some strains actually prefer ammonium over nitrate for growth, both for assimilation purposes and for methane-linked nitrate/nitrite reduction. This complex interplay may explain the breadth of contradictory studies showing, for instance, that nitrate and ammonium amendment both (and separately) have either increased growth, had no effect, and negatively impacted methanotrophs in fertilizer studies (Bodelier & Laanbroek, 2004; Bodelier & Steenbergh, 2014; Singh & Strong, 2016).

As noted by Shrestha et al. (2010), these studies are done in different environments and on different communities, invariably with different component members. As the broad class of methanotrophs is comprised of many members with very distinct N source responses, it is perhaps

not surprising then to note a lack of cohesion in the current literature over nitrate/nitrite and ammonium effects. Both N sources are associated with a toxicity issue, the magnitude of which vary by strain, and therefore determine the preferential nitrogen growth condition (Stein, 2018). Importantly, these effects may not be predictable until the physiology is directly assessed.

### 2.2.3. Biotechnological Applications of Methanotrophs

*Bioremediation and Biocatalysis*

As the field of methanotroph research continues to evolve, emphasis is increasingly being placed on the biotechnological potential held by these bacteria; this facet is introduced or explored in a large number of the current reviews available in the field (Cantera et al., 2019; Dalton, 2005; Hanson & Hanson, 1996; Hwang et al., 2018; Kalyuzhnaya et al., 2019; Kalyuzhnaya, Puri, & Lidstrom, 2015; Karthikeyan, Chidambarampadmavathy et al., 2015; Kirschke, Bousquet et al., 2013; Lee, Hur et al., 2016; Murrell, 1992, 2010; Pieja et al., 2017; Reddy, Kim, & Song, 2013; Rostkowski et al., 2012; Smith et al., 2010; Strong, Kalyuzhnaya et al., 2016; Trotsenko & Murrell, 2008; Van Amstel, 2012; van der Ha, Nachtergaele et al., 2012; Wang & Dong, 2012; Wendlandt et al., 2010). The field opened in the 1970's with the discovery that the MMO enzyme of MOB was capable of product-forming biotransformation of a wide variety of substrates, organic and inorganic, including alkanes, alkenes, alicyclics, aromatics, ethers, heterocyclics, and ammonia (Colby, Stirling, & Dalton, 1977; Dalton, 1977; Dalton & Stirling, 1982), due to the non-specificity of the enzyme (Higgins, Best, & Scott, 1981).

Often, this vast potential is examined in terms of bioremediation, and especially the degradation of chlorinated organic wastes, such as trichloroethylene (Chang & Alvarez-Cohen, 1997; Chu & Alvarez-Cohen, 1998; Clapp, Regan et al., 1999; Tsien, Brusseau et al., 1989). Historically, methanotrophs were successfully employed in the production of single-cell protein (SCP) (Linton & Buckee, 1977; Wilkinson, Topiwala, & Hamer, 1974), proving their varied potential

in terms of industry and production. Modern interest mostly remains in the field of methanotroph-derived bioproducts: more recent studies in Denmark and Norway investigated the production of a value-added protein product, specifically amino-acid balanced feed for animals and fish (Smith et al., 2010).

*Environmental and Economic Benefits*

In general, the unique abilities of the MOB, based on the power of the MMO to catalyze co-metabolic reactions, have sustained research interest in the field, based on the environmental and economic significance of the possible applications of such technology (Cantera et al., 2019; Hwang et al., 2018; Smith & Dalton, 2004; van der Ha et al., 2012; Wendlandt et al., 2010). More specifically, there are reasons why methanotrophs in particular are the subject of such focus. First, the feedstock, often methanol in place of methane when growing methanotrophs industrially, is a relatively convenient carbon source; methanol is easily stored and transported, its price is not subject to large fluctuations, and it is relatively abundant and easy to produce (Dalton & Stirling, 1982; Hanson & Hanson, 1996; Higgins, Best, & Hammond, 1980; Large & Bamforth, 1988).

Importantly, even methane itself is an inexpensive and readily available substrate, which can only be utilized by this specialized class of bacteria. Furthermore, genetic manipulation methods have advanced sufficiently to engineer methanotrophs that can synthesize small-molecule products, beyond even the large amount of biotransformations that can be accomplished endogenously by the MOB (Khmelenina, Rozova et al., 2015; Puri, Owen et al., 2015; Smith et al., 2010).

In terms of environmental benefits, the idea of methane as a potent greenhouse gas (introduced earlier) must resurface. Sequestration of this pollutant is part of the ecological role of methanotrophs (Breas et al., 2002; Conrad, 2009). The successful operation of a methanotroph-derived production scheme would amplify this effect, reducing methane pollution by means of its

oxidation by organisms adapted to consume this specialized carbon substrate. The importance of such a measure should not be understated; in 2009 alone, the United States released approximately 15 billion tonnes of methane into the atmosphere, owing to the expensive cost of otherwise dealing with this gas (K. Khosravi-Darani, Z. B. Mokhtari et al., 2013). It follows that a strategy to both take advantage of a "waste product" and prevent environmental contamination is one that should be pursued.

### 2.2.4. Bioproducts of Interest

*Polyhydroxybutyrate (PHB)*

Polyhydroxybutyrate (PHB) is a molecule that has been identified and known in the scientific community for almost a century (Lemoigne, 1926), existing in the cell as sudanophilic, lipid-like inclusions whose synthesis was found to be a widespread adaptation of Gram-negative bacteria. A polyhydroxyalkanoate (PHA) that is stored in intracellular inclusions, it is a very common biological polymer produced in nature by microorganisms (Sudesh, Abe, & Doi, 2000). Inclusions of PHB are often not pure however, containing mixed amounts of other PHAs and proteins as well (Wallen & Rohwedde, 1974).

Notably for this work, PHB is able to be produced by methanotrophs (Smith et al., 2010). It was first identified in *Methylomonas methanica*, then known as *Pseudomonas methanica* (Kallio & Harrington, 1960). The purpose of the production of PHB by bacteria is to serve as a carbon and energy reserve in an attempt to enhance the survival of the bacteria in periods of nutrient limitation (Doudoroff & Stanier, 1959). Further, its presence in the cell frequently act as a retardant to the degradation of RNA and proteins in cells placed under situations of nutrient starvation stress. Essentially, this molecule provides a method for bacteria to store large amounts of reduced carbon without drastically affecting the osmotic balance of the cell itself (Anderson & Dawes, 1990).

Early studies making note of PHB biosynthesis in cells saw that PHB tends to accumulate in higher amounts if the carbon to nitrogen ratio increased, meaning a situation of carbon excess and nitrogen limitation (Macrae & Wilkinson, 1958). However, nitrogen is not the only nutrient affecting PHB biosynthesis: cultures grown in phosphate-, magnesium-, and sulfate-deficient conditions are found to produce PHB, though not iron-deficient cultures (Repaske & Repaske, 1976), and some oxygen-limited cultures also accumulate PHB (Ward, Rowley, & Dawes, 1977). PHB, therefore, is now understood to accumulate not only when nitrogen is limiting but in most situations where a bacterium has access to excess carbon but is lacking in some aspect of the complete complement of nutrients required for growth (Sudesh et al., 2000).

In methanotrophs, however, the importance of nitrogen metabolism cannot be ignored as it clearly plays a significant role in induction of PHB production. Studies comparing nitrogen availability and nitrogen species demonstrate large differences in total PHB accumulation based on this manipulation (Rostkowski, Pfluger, & Criddle, 2013). Nitrogen metabolism appears to serve as a major switch for methanotrophs, affecting both growth and specific metabolite biosynthesis, including PHB (Hanson & Hanson, 1996). Similarly, nitrogen-control of PHB production might be explained by a proposed theory that PHB serves as an electron sink or redox regulatory agent when less than ideal situations inhibit the cell, acting as an aspect of metabolism control of sorts (McDermott, Griffith et al., 1989; Senior & Dawes, 1971, 1973).

*Applications and Benefits of PHB*

When discussing the formation of value-added products, it is essential to also address what the applications of that product are, evaluating the practicality of the bioproduct and how it can be refined or incorporated into useable materials. For PHB, the immediate and important application is in the formation of biodegradeable bioplastics (Anderson & Dawes, 1990). PHB has similar physical properties, in terms of melting point and crystallinity, to some petrochemically-derived

plastics, including the common polypropylene, though some differences do exist: polypropylene shows better solvent resistance but PHB has superior resistance to weathering by UV light (Holmes, 1985).

One possible weakness of PHB as a product is its inherent stiffness and brittleness – greater than polypropylene. However this problem can be overcome by means of mixing polymers, forming a product containing both PHB and other types of PHA molecules. The addition of other PHA's, including 150 different monomers, to form copolymers allows for predictable customization of the final characteristics of the plastic, resulting in a wide range of possible properties (Steinbuchel & Doi, 2002). This allows for creation of a product with, for instance, increased thermal stability and tensile strength, as well as a number of characteristics that may be desirable for certain applications, such as increased water permeability, achieved through inclusion of certain additional secondary monomers of PHA (Hazer, Kilicay, & Hazer, 2012; Hoefer, Vermette, & Groleau, 2011).

The final consideration of PHB is the commercially relevant aspect of biodegradability, which should not be overlooked as the issues of plastic environmental pollution and its mitigation are continuing to gain attention. PHA, and therefore PHB, degradation occurs naturally in the environment, primarily based on microbial enzymatic activity (Anderson & Dawes, 1990; Martínez-Tobón, Gul et al., 2018), under a variety of conditions, including different temperatures and pH levels (Doi, Kanesawa et al., 1989; Doi, Kawaguchi et al., 1989). This biodegradation also has important medical applications as material for sutures, microcapsules, bone plates, and gauzes. Some such studies have already been done showing some success in this aspect (Hazer et al., 2012; Korsatko, Wabnegg et al., 1983; Korsatko, Wabnegg, & Korsatko, 1990). One notable aspect of this research has been a focus on drug delivery systems, using the degradation of PHB to time slow drug release (Zinn, Witholt, & Egli, 2001).

*Isoprenoids*

Isoprenoids, also known as terpenoids, are a class of organic molecules derived from terpene, comprised mostly of multicyclic compounds containing oxygen in their functional groups. They are often classed according to the number of five-carbon units that constitute the full chemical, and this broad spectrum of classification means that a majority of known organic compounds fall into this class (Li & Wang, 2016). These can include such compounds as sterols, pigments, flavours and aromatics, amongst many others (Chandran, Kealey, & Reeves, 2011; Schempp, Drummond et al., 2018). Critically for biotechnology, isoprenoids can be used as platform chemicals, or substances that can be produced and altered into any number of other useful products. This includes the growing market of biofuels (Gronenberg, Marcheschi, & Liao, 2013).

There are two main pathways that bacteria can use to produce isoprenoids: the mevalonate (MVA) pathway and the non-mevalonate pathway, also known as the methylerythritol 4-phosphate (MEP) pathway or the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway (Li & Wang, 2016). The melavonate pathways is considered the classical pathway, while the MEP/DOXP pathway was discovered more recently and found in bacteria and plants (Eisenreich, Bacher et al., 2004). In the mid 1980's, high amounts of isoprenoids were measured in methanotrophs and methylotrophs (Urakami & Komagata, 1986), identifying this class of bacteria as an interest in this field of bioproduction. Currently, interest is growing towards biosynthesis of isoprenoids via pathway engineering and overexpression in bacteria, as more efficient genetic tools become available. Regardless, cell growth optimization and bioenergetic balance remain pressing concerns in this field (Li & Wang, 2016).

*Lipids and Other Products*

As methanotrophs have long been known for their extremely high native production and maintenance of intracytoplasmic membrane (Demidenko, Akberdinl et al., 2017; Kalyuzhnaya et al.,

2019; Smith et al., 2010), it follows that modern efforts are turning to harvest this characteristic into biotechnology. Bacterial lipids can serve as valuable biofuels, with reduced emissions from their use and production alike (Lee et al., 2016). Precise mapping of the pathways involved in methanotrophs has been undertaken (Demidenko et al., 2017). However, developing this natural high expression into an efficient and economical bioprocess will require optimized performance conditions for growth, as well as potential further genetic optimization; work that is underway already (Fei, Smith et al., 2014; Henard, Smith, & Guarnieri, 2017; Lee et al., 2016).

Other products of interest are also being pursued and developed through methanotroph biorefinery processes and, in many cases, have been worked on for many years. These include single cell protein (Large & Bamforth, 1988; Pieja et al., 2017), specialty chemicals including specific amino acids, nucleotides, and enzymes (Large & Bamforth, 1988), as well as using biomass for bioremediation (Chu & Alvarez-Cohen, 1998; Jiang et al., 2010; Sullivan, Dickinson, & Chase, 1998). All aims require their own optimized process development, and economic interest exists for each (Cantera et al., 2019; Hwang et al., 2018; Jiang et al., 2010).

## 2.3. Considerations of Methanotroph Growth

### 2.3.1. Growth Conditions for Methanotroph Culturing

*Culturing Standards*

When considering culturing techniques, it is vital to first understand the growth requirements of the organism in question. Firstly, as aerobic microorganisms, proteobacterial methanotrophs can be expected to consume 4 g of oxygen for every 1 g of methane they require during the PHB production scheme. Similarly, the growth requirement for nitrogen can be estimated at 0.12 g per 1 g of biomass (Rostkowski et al., 2012), and one may expect growth yields of 0.345 g biomass/g methane (Leak & Dalton, 1986b). These inputs must all be carefully considered when optimizing growth of methanotrophic cultures.

The most common medium used to cultivate proteobacterial methanotrophs was originally described by Whittenbury and colleagues (1970) and is known as nitrate mineral salts (NMS) medium. This study also reported a very similar medium, differing only in nitrogen source, denoted as ammonia mineral salts (AMS). Following this publication, NMS has been accepted as a standard in the field of methanotrophic research (Murrell, 2010). This medium contains all the minerals and nutrients required for growth of methanotrophs, save the carbon source which is typically either methane or methanol and which is added separately. Included in this formula is: magnesium sulfate, calcium chloride, iron, and a trace element solution, all dissolved in distilled water. Agar may be added to result in a solid medium (Whittenbury et al., 1970).

Perhaps the most pivotal addition to the medium is the nitrogenous species. Nitrogen is typically added at the following amounts: 0.1% w/v potassium nitrate for NMS or 0.05% w/v ammonium chloride for AMS. Both equate to approximately 10 mM nitrogen in the medium, which is considered standard for methanotroph culturing. A phosphate buffer solution at a neutral pH is required to maintain favourable acidity, especially for the ammonium-containing medium (Whittenbury et al., 1970) as pH can affect the growth and rate of methane oxidation in methanotrophs; optimum values are usually quoted as falling between pH 6.8-7.65 (Bender & Conrad, 1995; Kelly & Wood, 2010; Whittenbury et al., 1970).

Copper is another important aspect of this medium; it is vital to the function of the organism as it has a major role in the functioning of the energy-requiring enzyme of methane oxidation, particulate MMO. Scarcity of copper can lead to different behaviours, including induction of sMMO over pMMO, referred to as the 'copper switch' (Fru, 2011; Leak & Dalton, 1986a; Semrau et al., 2013; Zahn & DiSpirito, 1996). Optimally, copper concentration should not exceed 4.3 mM to prevent inhibition of methane oxidation (Bender & Conrad, 1995). Classically, lower copper concentrations were known to favour alpha-MOB while higher concentrations favour gamma-MOB (Graham et al., 1993). Now, copper-based research tends to focus more on the link to

methanobactin, a copper-binding chalkophore produced by a wide number of methanotrophs, implicated in the 'copper switch' and affecting MOB growth. Different structures of this molecule have been discovered, and research is ongoing on what the implications and further methanobactin regulatory effects might be (DiSpirito, Semrau et al., 2016).

*Optimal Temperature*

Another aspect of culturing that must be considered is incubation temperature. A temperature of 30 degrees Celsius (or ambient temperature, from 25-30°C) is somewhat of a standard in this respect, having long been used in culturing studies (Kelly & Wood, 2010; Whittenbury et al., 1970). However, temperatures may vary depending on the specific physiology and native environment of the strain in question – e.g. strains isolated from the human body microbiome, whose optimal temperatures were found to be about 37°C (Anesti, McDonald et al., 2005; Anesti, Vohra et al., 2004). *M. capsulatus* Bath, previously introduced above, originally isolated from the thermal springs of Bath, England, also has a higher optimal temperature of 45°C (Kelly & Wood, 2010), though some sources claim 37°C to be more accurate (Soni, Conrad et al., 1998). Conversely, psychotrophic methanotrophic species also exist, some growing at colder water temperatures in the Antarctic ranging from 12°C-25°C (Moosvi, McDonald et al., 2005).

Overall, it is recommended that temperatures for incubation be tested in a range of 10°C ± the temperature of the environment at time of original isolation, as optimal temperatures may differ from those the organism was found in (Kelly & Wood, 2010). For example, returning to the study of the Antarctic psychotrophs, optimal temperatures were all found to be in the range of 25°C-30°C, more similar to the standard cited temperature for growth than the temperature from which they were isolated (Moosvi et al., 2005). Like growth, methane oxidation rates themselves are also affected by temperature. Though this process still occurs at temperatures as low as 0°C and as high as 35°C, maximal methane oxidation occurs around 25°C; rates at 0°C to 10°C are only 13-

38% of the maximal rates seen at the optimum of 25°C (Bender & Conrad, 1995; Dunfield, Knowles et al., 1993).

*Batch vs. Fed-Batch vs. Continuous Culture*

Three main methods of cultivation exist for the production of biomass: batch, fed-batch, and continuous culture. An intrinsic difference between these strategies lies in the state of the cell throughout culturing. Continuous culture relies on the concept of steady state, operating conditions remaining the same throughout the course of culturing. Batch and fed-batch cultures, being close variations on the same theme, experience less control of operating conditions in that these conditions are chosen and implemented at one time point in the growth cycle then allowed to vary as the culture grows (Betlem, Mulder, & Roffel, 2002; Brown, 2001). In other words, continuous culture implements fixed volume and substrate concentration while batch culture sees fixed volume but decreasing substrate concentration as it is used by the cells. Fed-batch is most useful for culturing under substrate-limited growth as it allows addition of the growth substrate when it is needed or depleted (Betlem et al., 2002).

To return to the topic of methanotrophs, both batch and continuous operations are used for the culturing of these bacteria, including for use in the production of some product of interest. A number of similarities and differences may be noticed between batch and continuous cultures. For example, copper inhibition concentrations are similar in both modes of operation. Conversely, carbon dioxide gassing during growth at higher densities was only important for continuous culture and not for batch culture (Park, Hanna et al., 1991; Park, Shah et al., 1992; Shah, Park et al., 1992; Shah, Hanna, & Taylor, 1996). A study of a methanotrophic mixed culture grown in both batch and continuous culture found that maximum growth rates in each condition were similar and that both operation schemes allowed for stable culturing (Lamb & Garver, 1980).

A final operation style that bears introduction is that of self-cycling fermentation (SCF), the method of induction and application of synchronized cell culture. Essentially, over multiple cycles of operation, cells grown using this method become more aligned in terms of their stage in the cell cycle. This means that the majority of cells double within a short timeframe. When this point is reached, a feedback control loop triggers the cycling procedure: harvesting one half of the culture then refilling the reactor with fresh medium back to the previous volume (Brown, 2001; Sauvageau, Storms, & Cooper, 2010).

SCF has been proven to have a number of helpful and economically significant effects on the culturing of cells. These include: 1) shortening of the cell cycle time; 2) very reliable and predictable cell cycles that do not vary between rounds of culturing; and 3) higher yields when applied to the production of some bioproducts of interest. This technique can be applied in a two-stage form, first grown in one reactor then harvested to a second reactor where bioproducts accumulates (Crosman, Pinchuk, & Cooper, 2002; Sauvageau & Cooper, 2010; Sauvageau et al., 2010; Storms, Brown et al., 2012). Though a previously attempt at producing PHB in methane-fed cultures proved unsuccessful (Marchessault & Sheppard, 1997).

### 2.3.2. Carbon and Nitrogen Effects

*Considerations of Different Carbon Sources*

Methanotrophs are usually grown with one of two one-carbon (C1) substrates: methane ($CH_4$), the natural substrate of methanotrophs, and methanol ($CH_3OH$), the first intermediate in the pathway of methane oxidation. When methane is used, the possible range of methane available to the culture can vary, from a 30:70 to a 50:50 methane to air ratio (Whittenbury et al., 1970) or up to values as high as two parts methane to one part air (Kelly & Wood, 2010). Growth yields of 0.345 g of biomass per every 1 g of methane supplied can be expected (Leak & Dalton, 1986b). Alternatively, growth of these organisms on methanol is typically completed at concentrations of

10-50 mM (Kelly & Wood, 2010), as higher concentrations can be inhibitory due to the toxicity effect of methanol on the cells (Leadbetter & Foster, 1958; Whittenbury et al., 1970).

When considering methane-based growth, one issue of significance is mass transfer limitations, referring to impedance of growth based on slow methane diffusion into the medium. This problem can be overcome by increased agitation of the medium during incubation. Agitation at a rate of rotation between 240-400 rpm has proven successful in this respect in continuous flow reactors with 2 L media volume, and 130-300 rpm in 1 L flasks on shaker tables. Higher speeds of rotation can result in culture inhibition, likely due to either mechanical shear or oxygen toxicity due to increased oxygen transfer into the medium (Graham et al., 1993).

Phospholipid fatty acid (PLFA) analysis and comparison of methane- and methanol-grown cells has previously demonstrated no difference in PLFA composition – which is vital to membrane maintenance – between these conditions. This showed that, at least with regards to fatty acid synthesis, these cultures were not influenced by this difference in carbon source (Guckert, Ringelberg et al., 1991; Nichols, Smith et al., 1985). Rather, as mentioned previously, the PLFA profile of a methanotroph has been considered a signature or ecological marker, differentiating this class of bacteria from others, and even from other types of methanotrophs. This principle still holds true in the field, though novel lipids have recently been found and incorporated into the known methanotroph lipid constituents (Bodelier et al., 2009; Duan et al., 2017; Fang et al., 2000).

Another point of interest is growth of these bacteria under methane limitation, yet another aspect of methanotroph culturing that is distinct between the two types of MOB. In a competition study between alpha- and gamma-MOB, imposition of methane stress resulted in a clear shift towards domination by the gamma-MOB *M. album* BG8 (Type I) over the alpha-MOB *M. trichosporium* OB3b (Type II). Further, when cells were supplied methanol and methane simultaneously, cell numbers increased four-fold for *M. album* BG8 and were decreased by half for

*M. trichosporium* OB3b (Graham et al., 1993). Though the published literature cites that these changes are not reflected in the phospholipid content of the cells, alpha-MOB and gamma-MOB clearly differ in their preference for and tolerance of different carbon sources.

*Considerations of Different Nitrogen Sources*

As was mentioned above, NMS, which includes nitrate as the only nitrogen source, is the most common medium used for growing methanotroph cultures. AMS, having ammonia as the sole nitrogen source, has historically been less favoured since it was originally noticed that cultures tend to grow more poorly in this medium (Leadbetter & Foster, 1958). This differential preference may be attributed to the previously discussed issue of MMO inhibition by ammonium, inhibiting growth of methanotrophs (Bender & Conrad, 1994b; Holmes et al., 1995). Because of this, and because nitrate is readily used as a nitrogen source in methanotrophs, growth with NMS medium is recommended first. The substitution of NMS for AMS medium (and therefore growth on ammonia) necessitates lower nitrogen concentrations, aiming to circumvent inhibition (Smith et al., 2010) as methane oxidation has been shown to be inhibited at ammonium concentrations of 5-22 mM. However, values below 5 mM ammonia have no negative effect on methane oxidation, even stimulating it to some extent – which is attributed to the availability of nitrogen as a growth factor for the cells (Bender & Conrad, 1995).

Once again, an inherent difference between the alpha-MOB and the gamma-MOB should be noted. In studies of mixed methanotrophic communities, differing input conditions to the reactor led to domination by either alpha- or gamma-MOB, depending on the limitation or abundance of available nitrogen species. Compared to alpha-MOB, the gammaproteobacterial methanotrophs appear to thrive under conditions of high nitrogen concentrations – either nitrate or ammonia. This is attributed to rapid nitrogen assimilation within the cell. In contrast, the alphaproteobacterial

methanotrophs thrive in conditions of low nitrogen concentrations – i.e. nitrogen limitation – and low oxygen levels (Graham et al., 1993; Noll, Frenzel, & Conrad, 2008).

It is also helpful to consider field studies. There is some disagreement in the literature with regards to effects of specific N-species amendment (Bodelier & Laanbroek, 2004). In a study conducted on using different fertilizer types as nitrogen source, analysis of abundance of methanotrophs found that the gamma-MOB generally dominate, except in situations of stress, in which the alpha-MOB proportion becomes larger (Shrestha et al., 2010). Relatedly, differences also exist in patterns of coping with different nitrogenous inhibitors: gamma-MOB are more sensitive to inhibition by ammonia and more tolerant to inhibition by nitrite ($NO_2^-$), another known inhibitor of methanotrophic growth. Alpha-MOB demonstrate the reverse pattern, being more tolerant to situations of ammonia inhibition than nitrite inhibition (Nyerges et al., 2010; Nyerges & Stein, 2009). This trait is likely related to the ability of gamma-MOB to growth hypoxically by fermentation and/or dissimilatory reduction of nitrate/nitrite (Kalyuzhnaya et al., 2013; Kits, Campbell, et al., 2015; Kits, Klotz, et al., 2015).

### 2.3.3. Regulation Studies in Methanotrophs

*Gene Expression in Methanotrophs*

Gene expression regulation in bacteria, or transcription, is a key process that forms part of the central tenet of biology – DNA is transcribed into RNA, which is translated into proteins that affect functions in the cell. Control of gene transcription serves as a key regulator in the cell, and the study of the profile of these transcribed genes is called transcriptomics. This relatively recent and rapidly developing field allows for insight into how a bacterial cell functions or responds to the stimuli and circumstances around it (Cavill, Jennen et al., 2015; Conesa, Madrigal et al., 2016). These analyses were previously accomplished primarily via microarray studies, but RNASeq, or next-

generation sequencing, has becoming drastically more affordable in recent years and data is being published at exponential rates (Liu, Li et al., 2012; Wang, Gerstein, & Snyder, 2009).

This is of course also true in methanotrophs, and a number of studies have been published in regards to expression and regulation in MOB, examining either single genes or pathways, and global or whole-genome regulation. These include transcriptomic profiles of *M. trichosporium* OB3b, an alpha-MOB, grown in batch (Matsen et al., 2013), *M. capsulatus* Bath, a gamma-MOB also growing in batch, favouring either pMMO or sMMO (Larsen & Karlsen, 2016), and a methanotroph, *Methylotenera mobilis*, in its natural habitat (Kalyuzhnaya, Beck et al., 2009). Other studies have been accomplished primarily in gamma-MOB, often aimed at better understanding and taking advantage of bioindustrial potential: *Methylomicrobium buryatense* 5GB1C was analysed for fermentation metabolism (Gilman, Fu et al., 2017), *M. buryatense* 5GB1 was examined for lipid production (Demidenko et al., 2017; Gilman, Laurens et al., 2015), and *Methylomicrobium alcaliphilum* 20Z was assessed for fermentation and EMP potential (Kalyuzhnaya et al., 2013).

One of the most characteristic elements of MOB transcriptomics is the categorically high levels of expression of the methane monooxygenase (MMO) operons, especially the particulate form *pmo*CAB. RNASeq analysis finds high expression of *pmo*CAB compared to all other operons; it is often, in fact, the most expressed operon by a wide margin (Dam, Dam et al., 2014; Matsen et al., 2013). This enzyme, pMMO, is considered constitutively expressed, unlike sMMO which tends to be expressed solely in low copper situations (Collins, Buchholz, & Remsen, 1991; Park et al., 1991).

At this point, it is relevant to mention the importance of copper as a regulator in methanotrophs. Given that pMMO requires copper in its active site (Rosenzweig, 2008), it is clear that this metal is critical for methanotroph function when growing on methane. As mentioned, low concentrations of copper lead to a drastic shift in expression from pMMO towards sMMO, if both are encoded in the genome (Nielsen, Gerdes, & Murrell, 1997). Other effects have been noted, including

production of methanobactin, which is a copper-binding peptide or chalkophore (DiSpirito et al., 2016; Semrau, DiSpirito, & Yoon, 2010). As well, differential expression of 137 genes was noted in a MOB strain when copper concentration induced sMMO-based growth instead of pMMO, demonstrating a wider effect than simply the MMO type (Larsen & Karlsen, 2016).

In recent years, the importance of other trace elements, including lanthanides, has come into focus, primarily tied to methanol dehydrogenase (Farhan Ul Haque et al., 2015; Gu et al., 2016). Like MMO, this next step of the methane oxidation pathway is also significantly expressed. The promoter for methanol dehydrogenase is so constitutive in activity that it is widely used as a promoter for genetic manipulation in methanotrophs, including to induce overexpression of lipid biosynthesis genes (Henard et al., 2017). Amendment of culture medium with lanthanides can shift the RNA profile into favouring the lanthanide-centred XoxF methanol dehydrogenase, a regulation which can lead to significant effects on growth rate, amongst others (Farhan Ul Haque et al., 2015; Gu et al., 2016).

*Carbon and Nitrogen Effects on Transcriptional Regulation*

In biology, carbon and nitrogen assimilation are tightly linked, and regulation of both are intertwined, affecting the obvious linkage of amino acid biosynthesis and more (Commichau, Forchhammer, & Stülke, 2006). Due to its importance in methanotrophs, discussion of carbon and nitrogen effects on gene regulation must mention again the transcription of methane monooxygenase. As mentioned above, while overall levels remain significant, growth in methane compared to methanol has been associated with increased transcription of methane monooxygenase, though expression of the pxm operon is seemingly uncorrelated (Nguyen, Kim, & Lee, 2019). This downregulation is logical as the physiological purpose of MMO, to oxidize methane, would not be required in a methanol-grown culture.

It has previously been explained that nitrogen source, specifically ammonium compared to nitrate, has significant effects on methane monooxygenase as a competitive inhibitor. In terms of transcription however, studies in *Methylocystis* sp. SC2 found that nitrate and ammonium, even high concentrations of the latter, did not induce differential expression of *pmo*CAB. However, ammonium was found to significantly differentially regulate overall gene expression, not just *pmo*CAB, resulting in clear shifts in global gene regulation when cells were switched from ammonium to nitrate (Dam et al., 2014). In other nitrogen-focused work, denitrification studies in *M. denitrificans* FJG1 (Kits, Klotz, et al., 2015) and *M. album* BG8 (Kits, Campbell, et al., 2015) found that nitrate-fed, low-oxygen conditions lead to expression of the alternative methane monooxygenase operon *pxm*ABC (Tavormina et al., 2011).

Other recent findings should also be noted. Gene expression is regulated by carbon source in *M. trichosporium* OB3b, with methanol leading to less expression of both methane oxidation and formaldehyde assimilation genes, though this study noted similar growth outcomes in both conditions (Haque, Gu et al., 2017). Separately, methane compared to ethanol resulted in distinguishable transcriptome profiles in another alpha-MOB, *Methylocystis* sp. SB2. The authors note that from the RNASeq data, the methane oxidation step to methanol was likely rate-limiting, and growth on methane resulted in increased expression of both the methane oxidation pathway and the serine cycle (Vorobev, Jagadevan et al., 2014).

Finally, in gamma-MOB *Methylomonas* sp. DH-1, growth on methane and methanol was examined, finding differential transcriptional response in C1 assimilation, secondary metabolite pathways, and oxidative stress. Upregulation of formaldehyde oxidation and assimilation and downregulation of the tricarboxylic acid (TCA) cycle when grown on methanol implies favouring of the former for NADH production, and use of the latter more for *de novo* biosynthesis, not for NADH (Nguyen et al., 2019). Paired with a strong physiological understanding of pathways, use of RNASeq

is enabling ever more insights into the regulation and functioning of these interesting and diverse bacteria.

### 2.3.4. Considerations of Transcriptomics and Analysis

*Comparison to Proteomics and Metabolomics*

Besides transcriptomics, other fields aim to quantifiably analyze cell function and metabolic state. There is the study of the total gene inventory in a species, genomics. This can provide insight into the potential of an organism and the roles it likely fulfills environmentally or otherwise (Binnewies, Motro et al., 2006). Also highly informative are the studies of total protein profiles and metabolite profiles, called proteomics and metabolomics, respectively. These provide insight into the current active state of the cells, which processes are active and which pathways are favoured. Like transcriptomics but unlike genomics, these are aimed at providing a snapshot view of function, not potential (Cavill et al., 2015; Vogel & Marcotte, 2012).

It is salient then, to discuss how the fields of transcriptomics, proteomics, and metabolomics intersect, and what can be gained from the pursuit of each. Primarily, it can be said that the intention, or what is being investigated, is different between transcriptomics and the other two; cellular regulation and response to conditions is inferred from the former, while behaviour and nutrient flux is inferred from the latter. Each of these areas of study, particularly supported by genomic knowledge, can add value to the total functional understanding of an organism of interest, and interest lies in integrating data from these fields (De Keersmaecker, Thijs et al., 2006).

Curiously, however, the profiles from within a single strain in identical conditions do not necessarily align to a high degree. It is known that metabolites and transcript pools do not directly map (Cavill et al., 2015). In methanotrophs and beyond, transcript profile also does not translate directly to protein profile (Fu, He et al., 2019; Vogel & Marcotte, 2012), though these two pools are integrated (Cavill et al., 2015). This likely implies the importance of post-translational regulation,

which exists in addition to the transcriptional regulation assayed by RNASeq, but is unaccounted for in the scheme of metabolomics, proteomics, transcriptomics, and genomics without a great deal of additional targeted work (Cavill et al., 2015; Fu et al., 2019).

*Technologies and Methodologies*

As mentioned previously, transcriptomic analysis now currently relies primarily on techniques referred to as RNASeq (Liu et al., 2012; Wang et al., 2009). Previously, hybridization-based techniques provided insight but relied upon a high degree of existing knowledge of the genome, had smaller dynamic range, and required complex standardization. Sanger-based sequencing followed, but was generally low-throughput and expensive. Finally, high-throughput next generation sequencing came into wide use in the 2000's and has become increasingly economical and thus accessible, resulting in a boom of new -omics research being completed and released in recent years (Wang et al., 2009). In RNASeq, the sample RNA is converted to a library of cDNA fragments, each with an adaptor attached. These fragments are then sequenced, either from one end (single-end sequencing) or both ends (paired-end sequencing) through a high-throughput sequencer, including systems developed by Illumina, Applied Biosystems, or Roche 454 Life Science (Liu et al., 2012; Wang et al., 2009).

Once the samples have been sequenced, what follows is data processing, which can be completed through any number of particular pipelines, each with benefits and drawbacks (Conesa et al., 2016). The central scheme however remains relatively constant. First, the data are quality controlled, including removal of any poor fidelity reads and trimming of ends, if necessary. Next, the reads are often mapped against a reference, previously sequenced genome assigning fragments to the part of the genome that they match. *De novo* mapping is also possible, but is more computationally intensive. After mapping, the reads must be quantified, counting how many fragments sequenced aligned with each gene or region of the genome (Conesa et al., 2016).

At this stage, a decision must be made in how reads will be normalized. This step is critical to ensure that, particularly in comparative studies, the data acquired from each separate sample or condition can be accurately compared to each other case. As samples can vary greatly in total reads sequenced, and genes vary naturally in total length, this step controls for unavoidable differences in parameters (Conesa et al., 2016).

One of the most prevalent methods of reporting expression at this point is reads per kilobase million (RPMK), which divides total reads mapped to a gene by the read length and the total number of reads in a sample. This was proposed to enable comparison between samples of varying sizes, *i.e.* different library sizes (Mortazavi, Williams et al., 2008). A new measure gaining traction in the transcriptomics field is transcripts per million (TPM), a refinement of RPKM that proposes to remove a bias inherent in the RPKM analysis, allowing for more truthful comparison between samples of different size (Wagner, Kin, & Lynch, 2012).

After reads have been aligned, comparison between conditions follows, which is often the aim of RNASeq studies. Some programs like CLC Genomics Workbench lean towards the use of the normalized data as input, including RPKM or TPM, while others, including DESeq2, likely the most widely used program, use the raw read data and normalize as part of the process (Love, Huber, & Anders, 2014). Regardless, it is this data that serves at the basis of physiological comparison and from which insights into differential gene expression (DGE) or regulation can be gleaned (Conesa et al., 2016).

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# 3. Combined Effects of Carbon and Nitrogen Source to Optimize Growth of Proteobacterial Methanotrophs

## 3.1. Abstract

Methane, a potent greenhouse gas, and methanol, commonly called wood alcohol, are common by-products of modern industrial processes. They can, however, be consumed as a feedstock by bacteria known as methanotrophs, which can serve as useful vectors for biotransformation and bioproduction. Successful implementation in industrial settings relies upon efficient growth and bioconversion, and the optimization of culturing conditions for these bacteria remains an ongoing effort, complicated by the wide variety of characteristics present in the methanotroph culture collection. Here, we demonstrate the variable growth outcomes of five diverse methanotrophic strains – *Methylocystis* sp. Rockwell, *Methylocystis* sp. WRRC1, *Methylosinus trichosporium* OB3b, *Methylomicrobium album* BG8, and *Methylomonas denitrificans* FJG1 – grown on either methane or methanol, at three different concentrations, with either ammonium or nitrate provided as nitrogen source. Maximum optical density (OD), growth rate, and biomass yield were assessed for each condition. Further metabolite and fatty acid methyl ester (FAME) analyses were completed for *Methylocystis* sp. Rockwell and *M. album* BG8. The results indicate differential response to these growth conditions, with a general preference for ammonium-based growth over nitrate, except for *M. denitrificans* FJG1. Methane is also preferred by most strains, with methanol resulting in unreliable or inhibited growth in all but *M. album* BG8. Metabolite analysis points to monitoring of excreted formic acid as a potential indicator of adverse growth conditions, while the magnitude of FAME variation between conditions may point to strains with broader substrate tolerance. These findings suggest that methanotroph strains must be carefully evaluated before use in industry, both to identify optimal conditions and to ensure the strain selected is appropriate for the process of interest. Much work remains in addressing the optimization of growth strategies for

these promising microorganisms since disregarding these important steps in process development could ultimately lead to inefficient or failed bioprocesses.

## 3.2. Introduction

Methane-oxidizing bacteria (MOB), or methanotrophs, oxidize single-carbon molecules, specifically methane, to be used as their sole carbon and energy source. Methanotrophs are widely distributed in the environment, from rice paddies to upland soils to marine environments, among others (Bender & Conrad, 1994a). Methanotrophic bacteria are taxonomically diverse and are found in the phyla Verrucomicrobiae (Dunfield et al., 2007), NC10 (Ettwig, van Alen et al., 2009), and Proteobacteria (Bowman, 2006; Kelly, McDonald, & Wood, 2014; Webb, Ng, & Ivanova, 2014). Within the Proteobacteria, which encompass the majority of currently cultured methanotrophs, MOB can be further classified as Alphaproteobacteria (Alpha-MOB and Type II) or Gammaproteobacteria (Gamma-MOB, Type I, or Type X), with each group having distinct physiological traits. Differentiating traits include their primary central carbon pathways (serine pathway in Alpha-MOB and ribulose monophosphate pathway in Gamma-MOB), orientation and distribution of intracytoplasmic membranes (ICMs), and composition of lipids in terms of fatty acid proportions (Hanson & Hanson, 1996).

Methane is the natural energy and carbon substrate of methanotrophs, the first molecule that is activated in their central oxidation pathway through the enzyme methane monooxygenase (MMO). MMO oxidizes methane to methanol, which is sequentially oxidized to carbon dioxide via formaldehyde and formate or incorporated at the level of formaldehyde into cell biomass (Hanson & Hanson, 1996). Though the pathway of methane oxidation to carbon dioxide is overall energy generating, the MMO enzyme requires energy in the form of two reducing equivalents (Hanson and Hanson, 1996). Methanotrophs can also grow exclusively on methanol and it has thus been investigated as an alternate carbon source for their culture. However, due to its toxicity, methanol

as a sole growth substrate generally results in lower yields, despite the apparently decreased energetic and oxygen demands of methanol-grown cultures (Best & Higgins, 1981; van Dijken & Harder, 1975; Whittenbury et al., 1970). An exception to poor growth on methanol is the Gamma-MOB strain *Methylomicrobium buryatense* 5B, which was shown to grow faster and to higher yields when grown on methanol in batch culture (up to a concentration 1.75 M) than on methane (Eshinimaev, Khmelenina et al., 2002). The related strain *M. buryatense* 5GB1 grew better on methane than on methanol in a bioreactor, but still demonstrated robust growth on methanol (Gilman et al., 2015), as did *Methylomicrobium alcaliphilum* 20Z (Akberdin, Thompson et al., 2018).

Aside from carbon source, most methanotrophs utilize either ammonium or nitrate as nitrogen sources for assimilation while some have the capacity to fix $N_2$. Theoretically, use of ammonium as a nitrogen source should be bioenergetically favorable compared to nitrate, given that it can be directly assimilated into cell biomass. However, the structural similarity between ammonium and methane leads to competitive inhibition of MMO enzymes and co-oxidation of ammonia to the cytotoxic products, hydroxylamine and nitrite (Nyerges & Stein, 2009). Toxicity and inhibition of methane oxidation by ammonium, hydroxylamine and nitrite vary significantly among methanotrophic strains (Nyerges & Stein, 2009). MOB that encode and express hydroxylamine dehydrogenase enzymes (HAO) with similarity to those found in ammonia-oxidizing bacteria can more easily overcome hydroxylamine toxicity derived from the oxidation of ammonia (Campbell et al., 2011). Yet these same strains, such as *Methylocystis* sp. Rockwell, can still be sensitive to nitrite toxicity (Nyerges et al., 2010). Then again, some methanotrophs encode and express nitrite and nitric oxide reductase enzymes that can detoxify nitrite and are thus less susceptible to these cytotoxic effects (Kits, Campbell, et al., 2015; Mohammadi et al., 2017; Stein & Klotz, 2011). The presence and expression of genes for overcoming toxic intermediates of nitrogen metabolism are not phylogenetically coherent among the MOB as the ability to oxidize ammonia

(i.e., nitrify) and/or reduce nitrogen oxides (i.e., denitrify) are fairly randomly distributed across MOB taxa (Stein & Klotz, 2011).

Because carbon (e.g., methane or methanol) and nitrogen (e.g., ammonium, nitrate, or N-limitation) sources have different effects on the physiology and growth of individual MOB strains, the optimization of growth medium has to be empirically determined for each isolate. For instance, a study comparing growth of the Alpha-MOB, *Methylosinus trichosporium* OB3b, and the Gamma-MOB, *Methylomicrobium album* BG8, revealed that *M. album* BG8 grew better on lower methane concentrations. Moreover, the combination of methanol and methane further enhanced growth of *M. album* BG8 over *M. trichosporium* OB3b, while *M. trichosporium* OB3b fared better than *M. album* BG8 under nitrate limitation due to its ability to fix $N_2$ (Graham et al., 1993). Another study showed that the Alpha-MOB *Methylocystis* sp. Rockwell grew significantly better with ammonium, rather than nitrate, as N source, whereas the Gamma-MOB *M. album* BG8 preferred nitrate and was uninhibited by high nitrite concentrations in the medium (Nyerges et al., 2010). A study of *Methylocystis* sp. SC2, showed no inhibition of growth activity with up to 30 mM ammonium, three times the standard amount in ammonium mineral salts (AMS) medium (Dam et al., 2014). Beyond growth implications, nitrogen source can also have other important implications for bioindustry. For example, nitrogen starvation serves as the most common trigger for inducing production of polyhydroxybutyrate (PHB), a carbon-based storage molecule which is a truly biodegradable polymer (Sundstrom & Criddle, 2015). Through different growth/limitation schemes, nitrogen limitation has resulted in high yields of PHB at high molecular weights; though these studies generally consider nitrogen source concentration and do not focus on nitrogen species (Kianoush Khosravi-Darani et al., 2013). This is especially relevant as techno-economic analyses favor ammonium as an N source; nitrate is a key cost driver in most bioconversion processes. As such, the growth and metabolic implications of nitrogen source are important considerations when evaluating strains for their bioindustrial potential.

The current study compares the effects of carbon source (methane or methanol) and nitrogen source (ammonium or nitrate) on growth rates and biomass yields of three Alpha-MOB and two Gamma-MOB under batch cultivation. The objectives of this study are to: (1) compare strain-to-strain variation in their carbon/nitrogen preference, (2) find preferred carbon/nitrogen combinations for each strain, and (3) determine whether changes in carbon/nitrogen sources affect the phospholipid fatty acid (PLFA) composition and/or abundance in representative strains of Alpha- and Gamma-MOB. Previous studies of strains of M. *buryatense* grown in methanol showed a significant reduction in fatty acid methyl esters (FAME) and visible reduction of ICMs (Eshinimaev et al., 2002; Gilman et al., 2015), which is logical as MMO enzymes housed in ICMs are not necessary for growth on methanol. Whether growth on methanol results in a compositional change in PLFAs remains understudied in MOB. The results of this study are useful to demonstrate the range of strain-to-strain variation in carbon/nitrogen preference among MOB toward optimized growth of strains with industrial potential.

## 3.3. Materials and Methods

### 3.3.1. Growth and Maintenance of Methanotrophic Bacteria

Five MOB isolates were selected to provide a wide comparative assessment of their growth characteristics on different carbon/nitrogen source combinations. Strains included three alpha-MOB: *Methylocystis* sp. Rockwell (ATCC 49242), *Methylocystis* sp. WRRC1 (gift from Mango Materials), and *Methylosinus trichosporium* OB3b; and two gamma-MOB: *Methylomicrobium album* BG8 (ATCC 33003) and *Methylomonas denitrificans* FJG1 (Kits, Klotz, et al., 2015).

Cultures were grown using either ammonium mineral salts (AMS) or nitrate mineral salts (NMS) medium (Whittenbury et al., 1970), containing either 10 mM ammonium chloride (AMS) or 10 mM potassium nitrate (NMS) as N source. For all growth experiments, Wheaton media bottles (250 mL) closed with butyl-rubber septa caps and filled with 100 mL medium, were used as

previously reported (Kits, Campbell, et al., 2015). The copper ($CuSO_4$) concentration in the final medium was 5 μM for all media formulations. The media were buffered to pH 6.8 through addition of 1.5 mL phosphate buffer (26 g/L $KH_2PO_4$, 33 g/L $Na_2HPO_4$) and inoculated with 1 mL (1%) of previously grown cultures that had been passaged once in identical conditions to each of the experimental conditions; as such, initial biomass at inoculation varied somewhat, reflecting the growth result of the inoculum culture.

Methane was provided via injection through a 0.22 μm filter-fitted syringe. 0.5, 2, or 2.5 mmol of methane were provided and the pressure was maintained at 1 atm by removing the equivalent amount of gas headspace via syringe prior to methane addition. To delay onset of hypoxia, the 2.5 mmol methane incubations were conducted under approximately 1.05 atm. In the appropriate experiments, 0.5, 1, or 2 mmol of pure HPLC-grade methanol were added and the cultures were kept at a pressure of 1 atm. All cultures were incubated at 30°C, the optimal growth temperature for all five strains, with shaking at 150 rpm. Experiments were performed with replication (n=3) for all conditions.

### 3.3.2. Analysis of Growth

To monitor growth, 500-μL samples were extracted from cultures via sterile syringe at regular intervals over lag, exponential, and stationary phases. Three replicates were grown and assayed for each condition such that standard deviations could be calculated. Growth was assessed using optical density (OD) measurements at 540 nm in a 48-well microplate (Multiskan Spectrum, Thermo Scientific). Growth rates were calculated from two points on the growth curve covering an interval of logarithmic growth using the following formula (Eq.1), where α = the growth rate constant, N = number of cells (herein defined by OD measurements), and t = time:

(Eq.1.) $$\alpha = \frac{\ln\left(\frac{N_T}{N_0}\right)}{(t_T - t_0)}$$

Growth yield was determined as the change in biomass (as measured by optical density) per mol of carbon source supplied. Optical density was selected as a growth metric due to its widespread use in industrial bioprocess monitoring. Optimal growth conditions were chosen by weighted evaluation of both growth rate and yield, as described in equation 2, with the highest resultant value selected as optimal:

(Eq.2.) $x = \left(0.25 \times \frac{yield}{max\ yield}\right) + \left(0.75 \times \frac{growth\ rate}{max\ growth\ rate}\right)$

Culture purity was assured through phase contrast microscopy and plating of culture on TSA/nutrient agar plates, where lack of growth demonstrated lack of contamination. Multivariate ANOVA was done using R Studio to identify contribution of factors to outcomes, as well as any interaction effects between factors.

Methane and oxygen were measured using a gas chromatograph with TCD detector (GC-TCD, Shimadzu; outfitted with a molecular sieve 5A and Hayesep Q column, Alltech). A 250-µL gas-tight syringe (SGE Analytical Science; 100 µL/injection) was used to extract and inject headspace samples. Injection and detection temperatures were 120°C and oven temperature was 90°C with current set to 90 mA, using helium carrier gas (Ultra High Purity, Praxair) at 200kPa. Gas concentrations were calculated using standard curves of known amounts of the respective pure gases (Praxair).

### 3.3.3. Phospholipid Fatty Acid (PLFA) Analysis

*Methylomicrobium album* BG8 and *Methylocystis* sp. Rockwell were selected for PLFA analysis. Cultures were grown as detailed above, with either 2.5 mmol methane or 1 mmol methanol provided as carbon source as these conditions were most favourable for biomass accumulation. Cultures were also grown with either ammonium or nitrate as N source for comparison. Samples for analysis were collected upon reaching maximum OD540 but prior to the

onset of stationary phase. Cells were collected by vacuum filtration onto a 0.22 μm filter, which was washed with sterile medium, at which time the cells were transferred into a microcentrifuge tube and pelleted before being frozen at -80°C. Cell pellets (n=6 for each condition) were analyzed for PLFA content at the National Renewable Energy Laboratory (NREL) in Golden, CO.

Whole biomass lipid content was measured through fatty acid methyl esters (FAMEs) analysis as described previously (Henard, Smith et al., 2016). Briefly, 10 mg of lyophyilized biomass (dried overnight at 40 °C under vacuum) were homogenized with 0.2 mL of chloroform:methanol (2:1, v/v), and the resulting solubilized lipids were transesterified *in situ* with 0.3 mL of HCl:methanol (5%, v/v) for 1 h at 85 °C in the presence of a known amount of tridecanoic acid (C13) methyl ester as an internal standard. FAMEs were extracted with hexane (1 mL) at room temperature for 1 h and analyzed by gas chromatography: flame ionization detection (GC:FID) on a DB-WAX column (30 m × 0.25 mm i.d. and 0.25 μm film thickness).

### 3.3.4. Metabolite Analysis

Supernatant (1 mL) from the same cultures used for PLFA analysis were collected via sterile syringe and passed through a 0.22 μm syringe filter to remove cells, with replicates grown for each condition (n=3). Culture supernatants (n=3), were analyzed for metabolites at the National Renewable Energy Laboratory (NREL) in Golden, CO. High pressure liquid chromatography (HPLC) was used to detect lactate, formate, acetate, and methanol in culture supernatants, as described previously (Henard et al., 2016). Briefly, culture supernatant was filtered using a 0.2 μm syringe filter or 0.5 mL 10 K MWCO centrifuge tube (Life Technologies) and then separated using a model 1260 HPLC (Agilent, Santa Clara, CA) and a cation H HPx-87H column (Bio-Rad). A 0.1-mL injection volume was used in 0.01 N sulfuric acid with a 0.6 mL/min flow rate at 55 °C. DAD detection was measured at 220 nm and referenced at 360 nm, and organic acid concentrations were calculated by regression analysis compared to known standards. For analysis of comparisons between

conditions, significance was determined by standard t-test, with $\alpha<0.05$; all differences denoted as significant met this standard.

### 3.3.5. RNA Extraction

Total RNA was extracted from *Methylocystis* sp. Rockwell and *M. album* BG8 cells grown in either AMS or NMS, with methanol (1 mmol) or methane (2.5 mmol) provided as carbon source, at late log phase, using the MasterPure RNA purification kit (Epicentre). Briefly, cells were inactivated with phenol-stop solution (5% phenol, 95% ethanol) and pelleted through centrifugation. Nucleic acid from *Methylocystis* sp. Rockwell and *M. album BG8* were purified according to manufacturer's instructions, with the following modifications: 1 mg total Proteinase K was added for *Methylocystis* sp. Rockwell, and 0.35 mg total Proteinase K was added for *M. album* BG8. In addition, samples of *Methylocystis* sp. Rockwell grown on methanol were processed with organic solvent extraction in place of MPC precipitation as follows: extract sequentially with equal volume of phenol (acetate-buffered, pH 4.2), equal volume of 1:1 phenol:chloroform, and equal volume of 24:1 chloroform:isoamyl alcohol, before resuming MasterPure total nucleic acid precipitation protocol at the isopropanol addition step. RNA quantity and quality were assessed using a BioAnalyzer (Agilent Technologies).

### 3.3.6. RNA Sequencing and Assembly

RNA-Seq was performed by the Department of Energy Joint Genome Institute (DOE, JGI), using Illumina HiSeq-2000 technology. Raw reads, JGI transcriptomic analysis, and additional supporting information were made available through the JGI Genome Portal, under proposal ID 1114. Raw reads were trimmed and quality checked using CLC Genomics Workbench with quality scores (limit 0.05) and length filter (>30 bp). CLC RNASeq Assembler was then used to map reads to genome using default settings. Gene expression and differential expression were calculated using CLC Genomics Workbench, using reads per kilobase of transcript per million mapped reads (RPKM)

as normalized gene expression levels. Due to its prevalence in literature, nitrate-methane was selected as the reference condition to serve as a standard of comparison, and all other expression levels were judged relative to expression under this condition. Significance in differential expression was considered at an *n*-fold change of > | 1.25 | and false-discovery rate (FDR) adjusted *p*-value of < 0.05, calculated by CLC Genomics Workbench. All *Methylocystis* sp. Rockwell conditions were completed with n=3 replicates, as was *M. album* BG8 NMS/$CH_3OH$, while the remaining three samples were n=2 replicates.

## 3.4. Results

### 3.4.1. Effect of Carbon and Nitrogen Sources on Growth Rates and Yields of Methanotrophs

The effects of two carbon (methane and methanol) and two nitrogen (ammonium and nitrate) sources on the growth rates and yields of three alpha-MOB and two gamma-MOB were compared. The range of carbon amounts added to the 100-mL cultures was chosen from a point of limitation to excess as follows. At 0.5 mmol methane, the cultures were found to be carbon-limited, as demonstrated by complete depletion of methane coinciding with the onset of stationary phase (Supp. Figure A-1). At 2.5 mmol methane, the cultures were found to be oxygen-limited as the onset of stationary phase coincided with the depletion of oxygen, while methane remained in the gas headspace (Supp. Figure A-2). Therefore, the comparison of growth between 0.5 and 2.5 mmol carbon were selected to include growth conditions that ranged between carbon limitation and oxygen limitation.

Figure 3.1 shows the maximum $OD_{540}$ obtained for all strains and conditions tested (varying amounts of C source, with 10mM ammonium or nitrate in 100-mL cultures). The time points at which maximum optical densities were achieved, from the average of replicates, are given in Supp. Table A-1. Due to the mass transfer limitation of methane into the liquid medium, the apparent

carbon availability to the culture is mediated by the surface area of the liquid-gas interface, whereas methanol is immediately available to the culture. This could lead to faster growth rates in methanol-grown cultures, as a much higher proportion of substrate is readily available for use from the time of inoculation. In some cases, the toxicity of methanol could actually result in the opposite effect, with growth inhibition occurring at higher concentrations of methanol in batch culture.

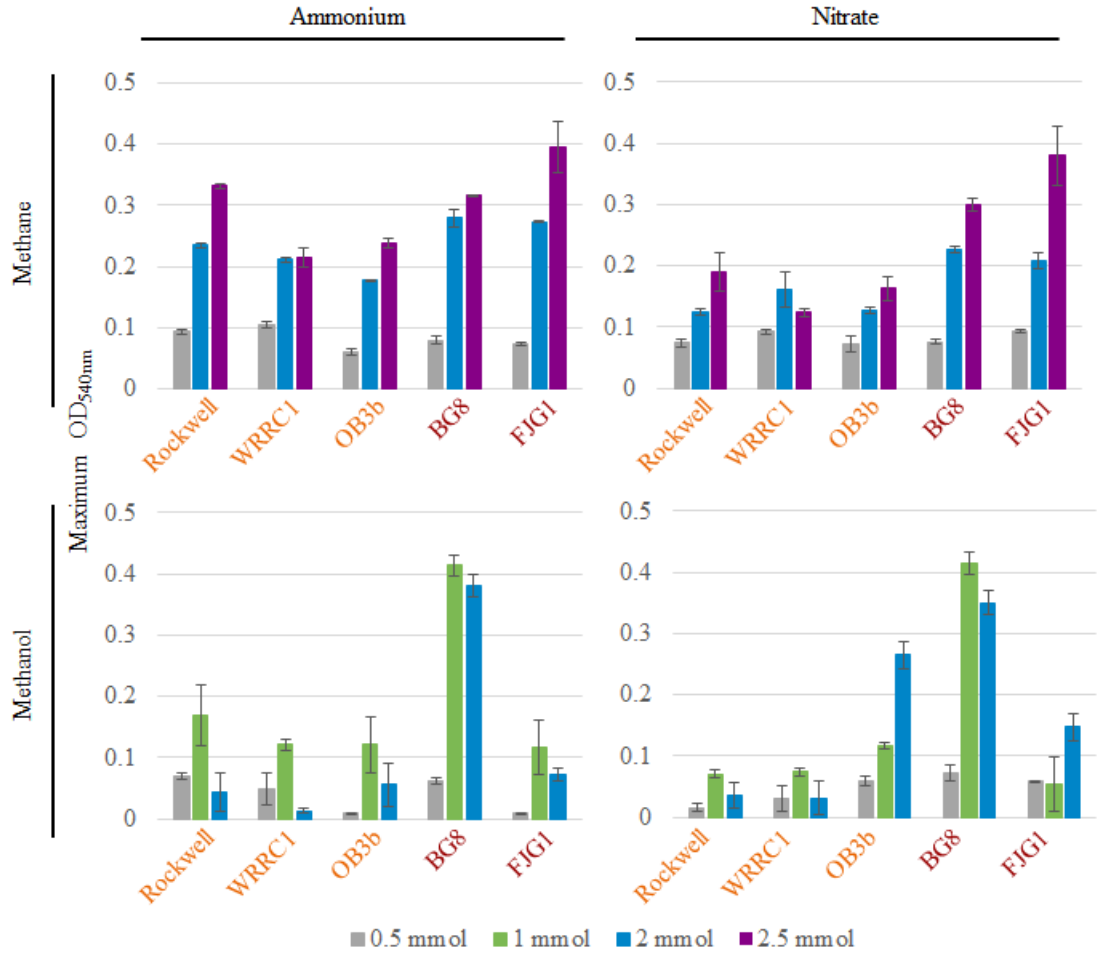


**Figure 3.1**. Maximum OD540 of 100-mL cultures of methanotrophic bacteria provided with 10 mM ammonium or nitrate and varying amounts of methane or methanol. Error bars represent standard deviations for n = 3 technical replicates per condition. Alpha-MOB strain name are indicated in orange and Gamma-MOB names are indicated in red.

For methane-grown cultures, ammonium as the N source resulted in overall higher biomass ($OD_{540}$) than with nitrate, particularly for the three alpha-MOB (Figure 3.1). At the highest methane amount tested (2.5 mmol), the two gamma-MOB showed little difference in $OD_{540}$ between

ammonium and nitrate. In all strains, the 0.5 mmol methane condition showed low $OD_{540}$ in agreement with the carbon limitation that this condition imposes. With nitrate, the *Methylocystis* sp. WRRC1 achieved lower $OD_{540}$ when grown with 2.5 mmol compared to 2 mmol methane, unlike the other strains. Methanol-grown cultures generally reached a lower maximum $OD_{540}$ than methane-grown cultures, which is apparent in both the 0.5 and 2 mmol carbon amended cultures. A notable exception to this trend was with *M. album* BG8, which showed a maximum $OD_{540}$ when grown in 1 or 2 mmol methanol, in either ammonium or nitrate (Figure 3.1; Supp. Figure A-3).

**Table 3.1**. Growth yields ($OD_{540nm}$/mol-C source) of methanotrophic bacteria grown in combinations of different carbon and nitrogen sources.

| Strain | Carbon (mmol) | Methane | | Methanol | |
|---|---|---|---|---|---|
| | | $NH_4^+$ | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| Rockwell | 0.5 | **188 (±7.98)** | 149 (±13.2) | 138 (±9.44) | 29.7 (±13.8) |
| | 1 | – | – | 169 (±50) | 70.5 (±6.75) |
| | 2 | 118 (±1.78) | 62.2 (±2.48) | 21.7 (±15.9) | 17.6 (±10.7) |
| | 2.5 | 133 (±1.88) | 75.8 (±12.7) | – | – |
| WRRC1 | 0.5 | **210 (±9.51)** | 185 (±7.5) | 98.3 (±51.8) | 60.1 (±39.6) |
| | 1 | – | – | 121 (±8.44) | 73.7 (±6.4) |
| | 2 | 106 (±1.69) | 80.4 (±14.9) | 6.17 (±1.93) | 15.6 (±13.3) |
| | 2.5 | 86.0 (±6.42) | 49.3 (±2.89) | – | – |
| OB3b | 0.5 | 123 (±10.2) | **144 (±26.8)** | 19.5 (±1.71) | 120 (±15.7) |
| | 1 | – | – | 121 (±45.9) | 116 (±5.14) |
| | 2 | 89.1 (±0.671) | 63.7 (±2.09) | 27.9 (±17.5) | 132 (±10.7) |
| | 2.5 | 95.5 (±3.12) | 65.1 (±7.68) | – | – |
| BG8 | 0.5 | 159 (±12.7) | 151 (±7.56) | 123 (±10.2) | 144 (±26.8) |
| | 1 | – | – | 414 (±17) | **415 (±17.3)** |
| | 2 | 140 (±7.59) | 113 (±2.79) | 190 (±9) | 175 (±9.67) |
| | 2.5 | 127 (±0.327) | 120 (±4.01) | – | – |
| FJG1 | 0.5 | 147 (±4.63) | **187 (±3.57)** | 19.9 (±1.15) | 117 (±2.27) |
| | 1 | – | – | 116 (±43.3) | 54.9 (±44.5) |
| | 2 | 137 (±0.283) | 104 (±6.65) | 36.2 (±5.1) | 73.5 (±11.3) |
| | 2.5 | 158 (±16.5) | 152 (±19.3) | – | – |

*Standard deviations of three technical replicates are reported in parentheses. Bold values are the maximum yields for each strain. Dashes indicate conditions that were not examined.*

Methane-grown cultures were generally more replicable in terms of growth yields ($OD_{540}$/mol-C source) (Table 3.1) and length of lag phase (Supp. Table A-2) than methanol-grown cultures. Extremely low, or even absence of growth was observed among replicate cultures grown on methanol. However, higher growth yields were still achieved with 1 mmol versus 2 mmol methanol for all strains, suggesting toxicity for 2 mmol methanol (representing a concentration of 0.2 mM). As all of the carbon was consumed in the 0.5 to 1 mmol carbon-amended cultures, the

calculated growth yields were highest under these conditions, and were higher with methane than with methanol except for *M. album* BG8 (Table 3.1).

**Table 3.2**. Growth rates of methanotrophic bacteria in different combinations of carbon and nitrogen sources, reported as change in optical density (540 nm) per hour.

| Strain | Carbon (mmol) | Methane | | Methanol | |
|---|---|---|---|---|---|
| | | $NH_4^+$ | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| Rockwell | 0.5 | 0.112 (±0.002) | **0.116 (±0.007)** | 0.0246 (±0.002) | 0.0186 (±0.001) |
| | 1 | – | – | 0.0389 (±0.004) | 0.0144 (±0.006) |
| | 2 | 0.0995 (±0.004) | 0.0614 (±0.007) | 0.0402 (±0.006) | 0.0579 (±0.032) |
| | 2.5 | 0.113 (±0.004) | 0.0491 (±0.009) | – | – |
| WRRC1 | 0.5 | 0.111 (±0.019) | **0.128 (±0.002)** | 0.0266 (±0.01) | 0.0302 (±0.004) |
| | 1 | – | – | 0.0570 (±0.003) | 0.0263 (±0.002) |
| | 2 | 0.123 (±0.004) | 0.0763 (±0.008) | 0.0201 (±0.003) | 0.0167 (±0.001) |
| | 2.5 | 0.0640 (±0.005) | 0.0572 (±0.011) | – | – |
| OB3b | 0.5 | 0.0778 (±0.011) | 0.0594 (±0.012) | 0.0146 (±0.007) | 0.0393 (±0.01) |
| | 1 | – | – | 0.0460 (±0.005) | 0.0411 (±0.011) |
| | 2 | **0.121 (±0.009)** | 0.0685 (±0.007) | 0.0547 (±0.033) | 0.0566 (±0.006) |
| | 2.5 | 0.0811 (±0.012) | 0.0497 (±0.008) | – | – |
| BG8 | 0.5 | 0.144 (±0.011) | 0.0918 (±0.006) | 0.0340 (±0.004) | 0.0383 (±0.001) |
| | 1 | – | – | **0.144 (±0.044)** | 0.131 (±0.05) |
| | 2 | 0.130 (±0.033) | 0.0978 (±0.023) | 0.0551 (±0.016) | 0.0471 (±0.009) |
| | 2.5 | 0.119 (±0.039) | 0.101 (±0.053) | – | – |
| FJG1 | 0.5 | 0.0856 (±0.016) | 0.110 (±0.007) | 0.0224 (±0.007) | 0.0596 (±0.004) |
| | 1 | – | – | 0.127 (±0.008) | 0.107 (±0.037) |
| | 2 | 0.164 (±0.005) | 0.129 (±0.008) | 0.0590 (±0.025) | 0.0648 (±0.01) |
| | 2.5 | **0.289 (±0.07)** | 0.188 (±0.034) | – | – |

*Standard deviations of three technical replicates are reported in parentheses. Bold values are the maximum growth rates during exponential phase for each strain. Dashes indicate conditions that were not examined.*

Conditions in which methane was the carbon source and ammonium was the nitrogen source resulted in generally high growth rates for all strains. Methanol led to generally slower growth than methane, with the exception of *M. album* BG8 (Table 3.2). Lag phases also tended to be much longer for growth on methanol than methane (Supp. Fig A-3; Supp. Table A-2), although the duration of lag phases for methanol-grown cultures was generally shorter for the gamma-MOB than for the alpha-MOB. This may be related to poorer growth in the inoculum culture or periods of adaptation to the condition and it is important to note that continuous bioprocessing operation may mitigate these impacts. Some of the strains were not able to achieve exponential growth on methanol (0.5 mmol methanol: *Methylocystis* sp. Rockwell with nitrate, *M. trichosporium* OB3b with ammonium, *M. denitrificans* FJG1 with nitrate. 2 mmol methanol: *Methylocystis* sp. WRRC1 with ammonium, *M. denitrificans* FJG1 with ammonium). Notably, an exponential phase could be

measured for all strains grown in either ammonium or nitrate when provided with 1 mmol methanol, suggesting that this intermediate methanol amount (representing a concentration of 0.1 mM) was neither carbon limiting nor toxic to the cells and was the optimal concentration among the conditions tested in this study.

While clearly distinct growth outcomes can be noted, multivariate ANOVA analysis was completed to distinguish how strain type, carbon amount, carbon source, and nitrogen source alone and in combination contributed to maximum OD, growth rate, and growth yield for each strain (Table 3.3). All factors and combinations had statistically significant effects on maximum OD. Growth rate was also significantly impacted by each individual major factor, as well as by a variety of combinatorial factors. Growth yield was least affected by the analyzed factors though strain, carbon amount and carbon type all had significant effects.

**Table 3.3**. Multifactorial analysis of variance (ANOVA) on measurements of maximum optical density, growth rate, and yield for each condition tested.

| | Maximum OD | Growth rate | Yield |
|---|---|---|---|
| Strain | **$<2.00 \times 10^{-16}$** | **$<2.00 \times 10^{-16}$** | **$7.83 \times 10^{-5}$** |
| CAmt | **$<2.00 \times 10^{-16}$** | **$9.87 \times 10^{-12}$** | **$6.91 \times 10^{-13}$** |
| Carbon | **$<2.00 \times 10^{-16}$** | **$<2.00 \times 10^{-16}$** | **$8.40 \times 10^{-13}$** |
| Nitrogen | **$3.10 \times 10^{-7}$** | **$3.97 \times 10^{-5}$** | $3.02 \times 10^{-1}$ |
| Strain:CAmt | **$<2.00 \times 10^{-16}$** | **$<2.00 \times 10^{-16}$** | **$5.67 \times 10^{-3}$** |
| Strain:carbon | **$<2.00 \times 10^{-16}$** | **$2.57 \times 10^{-2}$** | $1.36 \times 10^{-1}$ |
| CAmt:carbon | **$7.47 \times 10^{-3}$** | $1.48 \times 10^{-1}$ | **$5.52 \times 10^{-6}$** |
| Strain:nitrogen | **$5.67 \times 10^{-12}$** | $9.45 \times 10^{-1}$ | $4.28 \times 10^{-1}$ |
| CAmt:nitrogen | **$9.76 \times 10^{-10}$** | **$3.17 \times 10^{-4}$** | $3.58 \times 10^{-1}$ |
| Carbon:nitrogen | **$1.36 \times 10^{-10}$** | **$7.49 \times 10^{-4}$** | $1.67 \times 10^{-1}$ |
| Strain:CAmt:carbon | **$3.84 \times 10^{-15}$** | **$3.95 \times 10^{-2}$** | $7.64 \times 10^{-1}$ |
| Strain:CAmt:nitrogen | **$2.06 \times 10^{-4}$** | **$3.01 \times 10^{-2}$** | $9.57 \times 10^{-1}$ |
| Strain:carbon:nitrogen | **$3.21 \times 10^{-5}$** | $7.55 \times 10^{-1}$ | $6.30 \times 10^{-1}$ |
| CAmt:carbon:nitrogen | **$1.27 \times 10^{-8}$** | $1.50 \times 10^{-1}$ | $7.26 \times 10^{-1}$ |
| Strain:CAmt:carbon:nitrogen | **$7.43 \times 10^{-3}$** | $4.35 \times 10^{-1}$ | $7.74 \times 10^{-1}$ |

*Values represent calculated p-values from F-tests. Bolded values represent those factors and combinations of factors (interactions) showing statistically significant, measureable effects on the outcome assessed at $\alpha = 0.05$.*

Analysis of gene expression of the central methane oxidation pathway showed no notable difference in expression of methane monooxygenase genes for *Methylocystis* sp. Rockwell grown on

methane with either nitrate or ammonium despite the observed differences in growth (Figure 3.1 and Supp. Table A-3). However, significant decreases in *pmo* gene expression was observed for growth of *Methylocystis* sp. Rockwell on methanol when compared to methane-nitrate. Minor decreases in expression of methanol dehydrogenase genes was observed for cells grown in ammonium when compared to the nitrate-methane standard. Formate and formaldehyde oxidation gene expression were unchanged, except for a significant down-regulation of a gene for formaldehyde activating protein in ammonium-methanol (Supp. Table A-3). While this may point to a potential growth bottleneck, related to formaldehyde toxicity, this effect was not observed in the nitrate-methanol grown cells. In *M. album* BG8, expression of *pmo* genes increased only in the ammonium-methanol condition relative to the nitrate-methane control (Supp. Table A-4). This is in contrast to the downregulation of *pmo* genes in Rockwell grown in methanol, and may point to a possible cause of the noticeably different growth profiles noted in these strains in methanol-grown cells. When grown in nitrate-methanol, methanol dehydrogenase, formaldehyde oxidation, and formate oxidation genes showed increased expression relative to the methane-nitrate control, while cells grown on ammonium-methane and ammonium-methanol showed no differences in expression of these genes.

### 3.4.2. Effect of Carbon and Nitrogen Sources on Small Metabolites

To expand the analysis of carbon and nitrogen effects on methanotrophs, two strains, the alpha-MOB *Methylocystis* sp. Rockwell and the gamma-MOB *M. album* BG8, were selected for analysis of excreted metabolites, representing different types of methanotrophs as well as distinct substrate-based growth effects as measured by optical density. Cultures were grown with either 1 mmol methanol or 2.5 mmol methane with either ammonium or nitrate at 10 mM. For all conditions tested – either strain with all carbon-nitrogen combinations – a significant amount of glycerol was measured (Table 3.4). Lactic acid was measurable for *Methylocystis* sp. Rockwell grown in methane-ammonium and methanol-nitrate. *Methylocystis* sp. Rockwell, but not *M. album*

BG8, excreted formic acid in all cultures except when grown on methane-ammonium, with more detected in the methanol-grown cultures. Interestingly, *M. album* BG8 grown in methanol and nitrate produced small amounts of xylitol. While the origins of this sugar alcohol were not further investigated, its source could potentially be X5P-derived xylulose, which could implicate a pentose-phosphate pathway or phosphoketolase (PKT) bottleneck with implications for bioindustrial potential. RNA-Seq analysis identified no change in gene expression of PKT in this condition relative to nitrate-methane in *M. album* BG8. This condition did however show significant upregulation of formaldehyde-activating protein genes and down-regulation of formate dehydrogenase genes, which is not observed in either ammonium-methane or ammonium-methanol. (Supp. Table A-4).

**Table 3.4**. Concentrations of metabolites excreted to supernatant by *Methylocystis* sp. Rockwell and *M. album* BG8 grown with different carbon and nitrogen sources reported in g/L.

| Strain | Metabolite (g/L) | Methane | | Methanol | |
|---|---|---|---|---|---|
| | | $NH_4^+$ | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| Rockwell | Glycerol | 0.311 (±0.027) | 0.290 (±0.026) | 0.338 (±0.053) | 0.396 (±0.048) |
| | Lactic acid | 0.039 (±0.055) | – | – | 0.019 (±0.027) |
| | Formic acid | – | 0.009 (±0.013) | 0.138 (±0.005) | 0.106 (±0.023) |
| | Xylitol | – | – | – | – |
| BG8 | Glycerol | 0.381 (±0.054) | 0.371 (±0.037) | 0.279 (±0.020) | 0.370 (±0.110) |
| | Lactic acid | – | – | – | – |
| | Formic acid | – | – | – | – |
| | Xylitol | – | – | – | 0.052 (±0.074) |

*Methane was supplied at 2.5 mmol while methanol was supplied at 1 mmol per 100 mL of culture; respective nitrogen sources were supplied at 10 mM. Standard deviations of three technical replicates are reported in parentheses. Dashes indicate metabolites that were under the limit of detection.*

### 3.4.3. Effect of Carbon and Nitrogen Sources on PLFA Composition and Abundance

In order to determine if the combinations of carbon and nitrogen sources were significantly altering membrane structure, FAMEs analysis was conducted on *Methylocystis* sp. Rockwell and *M. album* BG8. All measured fatty acids were between C10 and C18, with no measurable C8 or C20-24 (which were included in the analysis standards). Overall abundance of percent biomass was determined for each strain and growth condition (Figure 3.2), and ANOVA analysis was completed to determine whether strain type, carbon type, and nitrogen type contributed to overall measured

FAMEs (Supp. Tables A-5 & A-6). Total fatty acid abundance was significantly lower in methanol-grown cultures of *Methylocystis* sp. Rockwell. Furthermore, cultures of *Methylocystis* sp. Rockwell grown with ammonium had lower abundance of fatty acids than cultures grown with nitrate. In contrast, there was no significant difference in total fatty acid abundance across conditions for *M. album* BG8. Overall, strain-type, carbon and nitrogen sources and their interactions were determined to have significant impact on abundance of FAMEs. (Supp. Table A-5).

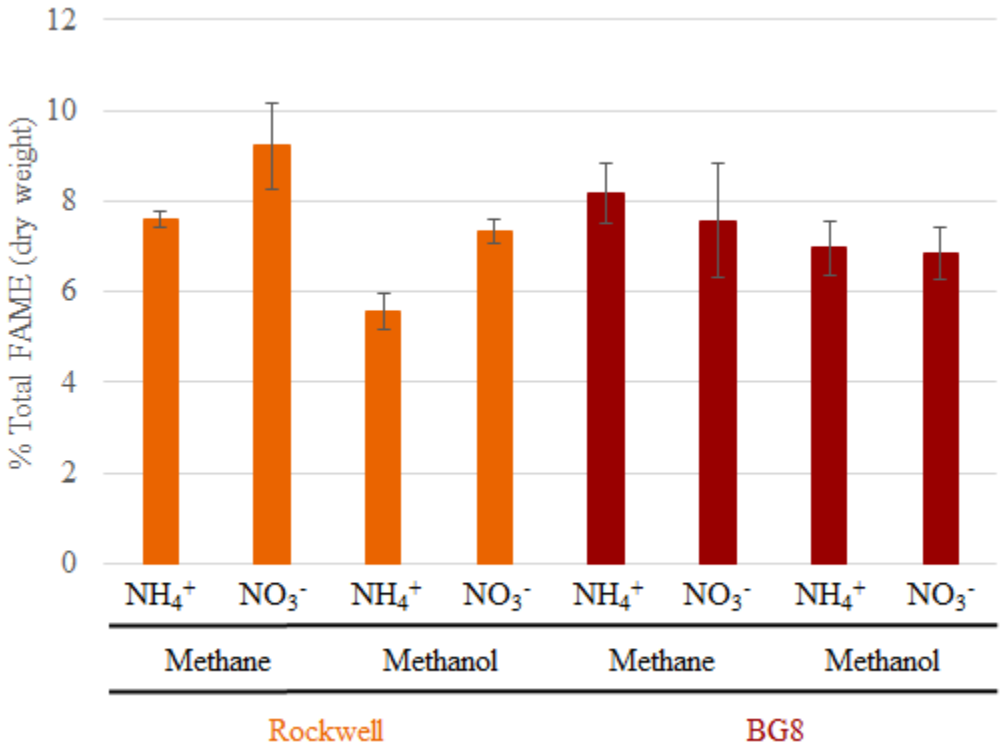


**Figure 3.2**. Total FAMEs measured in each sample as a percentage of total cell dry weight. Error bars represent standard deviations (n = 6). Results from Alpha-MOB strains are indicated in orange and from Gamma-MOB are indicated in red.

In all conditions tested, over 93% of the fatty acid content in *Methylocystis.* sp. Rockwell was composed of only two species: C18:1n9, accounting for approximately 70-75% of the measured FAMEs, and C18:1n7, accounting for approximately 18-25% of the total FAMEs (Supp. Table A-7). All other fatty acids measured individually contributed less than 1.55% of the measured FAMEs. In contrast, the profile of *M. album* BG8 showed four different fatty acids contributing a substantial

portion (12% or higher) of the total FAMEs measured. In descending order of prominence, these fatty acids were: C16:1n6 (36-38%), C16:1n9 (23-27%), C16:1n7 (15-20%), and C16:0 (12-15%) (Supp. Table A-7).

While the general profiles held true in all cultures conditions, the relative abundance of each fatty acid varied (Figure 3.3). In *M. album* BG8, the abundance of fatty acid C16:1n6 in cells grown in methane compared to methanol was ca. 0.95:1 for both nitrogen sources. Conversely, higher proportions of the fatty acid C16:1n7 can be found in methane-fed compared to methanol-fed cultures, with differences in the abundance of this fatty acid measured at values of 1.13:1 in cells grown on ammonium and 1.33:1 in nitrate-grown cells. Both fatty acid proportions changed significantly in their response to carbon source (Supp. Table A-6).

Other effects of nitrogen source were noted in the C16:0 proportions, with nitrate-grown cells containing approximately 1.13 times the proportion found in ammonium-grown cells (Supp. Table A-6). Interestingly, the proportion of C16:1n9 was 1.12-1.15× more abundant in the methanol plus nitrate condition relative to all the other conditions, though neither carbon nor nitrogen source was judged to have a significant effect.

In *Methylocystis* sp. Rockwell, a significantly lower proportion of C18:1n7 was measured as a component of total FAMEs in methanol-grown cells, with methane-grown cells possessing approximately 1.32× more C18:1n7, proportionally, regardless of nitrogen source. Carbon source likewise appeared to affect C18:1n9 composition, although conversely: methane-grown cells contained proportionally less of this fatty acid compared to methanol-grown cells, approximately 0.95:1. Both major fatty acids, C18:1n7 and C18:1n9, were significantly affected by carbon but not nitrogen source (Supp. Table. A-6). RNA-Seq analysis of the fatty acid biosynthesis pathway in both *Methylocystis* sp. Rockwell and *M. album* BG8 showed no difference in gene expression across growth conditions (Supp. Table A-3 and A-4).

a) **C16:0**

| | | Methane | | Methanol | |
|---|---|---|---|---|---|
| | | $NH_4^+$ | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| Methane | $NH_4^+$ | 1.00 | | | |
| | $NO_3^-$ | **1.13** | 1.00 | | |
| Methanol | $NH_4^+$ | 0.98 | **0.86** | 1.00 | |
| | $NO_3^-$ | **1.21** | 1.07 | **1.23** | 1.00 |

b) **C16:1n6**

| | | Methane | | Methanol | |
|---|---|---|---|---|---|
| | | $NH_4^+$ | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| Methane | $NH_4^+$ | 1.00 | | | |
| | $NO_3^-$ | 0.99 | 1.00 | | |
| Methanol | $NH_4^+$ | **0.96** | 0.96 | 1.00 | |
| | $NO_3^-$ | **0.95** | **0.96** | 0.99 | 1.00 |

c) **C16:1n7**

| | | Methane | | Methanol | |
|---|---|---|---|---|---|
| | | $NH_4^+$ | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| Methane | $NH_4^+$ | 1.00 | | | |
| | $NO_3^-$ | 0.92 | 1.00 | | |
| Methanol | $NH_4^+$ | **1.13** | **1.23** | 1.00 | |
| | $NO_3^-$ | **1.22** | **1.33** | 1.08 | 1.00 |

d) **C16:1n9**

| | | Methane | | Methanol | |
|---|---|---|---|---|---|
| | | $NH_4^+$ | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| Methane | $NH_4^+$ | 1.00 | | | |
| | $NO_3^-$ | 1.03 | 1.00 | | |
| Methanol | $NH_4^+$ | 1.01 | 0.98 | 1.00 | |
| | $NO_3^-$ | **0.88** | **0.85** | **0.87** | 1.00 |

e) **C18:1n7**

| | | Methane | | Methanol | |
|---|---|---|---|---|---|
| | | $NH_4^+$ | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| Methane | $NH_4^+$ | 1.00 | | | |
| | $NO_3^-$ | 0.98 | 1.00 | | |
| Methanol | $NH_4^+$ | **1.33** | **1.36** | 1.00 | |
| | $NO_3^-$ | **1.29** | **1.31** | 0.97 | 1.00 |

f) **C18:1n9**

| | | Methane | | Methanol | |
|---|---|---|---|---|---|
| | | $NH_4^+$ | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| Methane | $NH_4^+$ | 1.00 | | | |
| | $NO_3^-$ | **1.03** | 1.00 | | |
| Methanol | $NH_4^+$ | **0.96** | 0.93 | 1.00 | |
| | $NO_3^-$ | **0.97** | **0.94** | 1.01 | 1.00 |

| | ***M. album* BG8** | | | | ***M.* sp. Rockwell** | |
|---|---|---|---|---|---|---|
| | **C16:0** | **C16:1n6** | **C16:1n7** | **C16:1n9** | **C18:1n7** | **C18:1n9** |
| Carbon | 6.90E-01 | 2.32E-03 | 1.49E-03 | *9.59E-02* | 3.00E-07 | 5.77E-03 |
| Nitrogen | 1.06E-03 | 6.13E-01 | 7.97E-01 | 2.22E-01 | 5.20E-01 | 2.79E-01 |
| Carbon:Nitrogen | 3.61E-01 | 9.75E-01 | 1.58E-01 | *5.83E-02* | 9.31E-01 | 5.22E-01 |

**Figure 3.3**. Relative changes in the abundances of primary FAMEs for cells grown with various combinations of carbon and nitrogen sources in *M. album* BG8 (A–D) and *Methylocystis* sp. Rockwell (E,F). Bold values signify statistically different by unpaired t-test ($\alpha < 0.05$).

## 3.5. Discussion

### 3.5.1. Optimal Carbon–Nitrogen Combinations for Growth of Methanotrophic Strains

Optimization of growth is generally approached in one of two ways, either from a maximum biomass or a fastest growth rate perspective. In an industrial context, both of these parameters have value and should be accounted for in a multi-objective optimization approach. By evaluating growth yields (Table 3.1) and growth rates (Table 3.2) together, we can determine for each strain an optimal combination of carbon-nitrogen sources, and to a lesser extent, carbon amount, leading to the best growth outcomes (Figure 3.4). The biggest limitations to these analyses are: 1) lag phase was not accounted for since the use of pre-cultures and continuous cultures can overcome this limitation, 2) there is incomplete methane oxidation at higher concentrations due to $O_2$ limitation (representative data in Supp. Figure A-2), and A-3) methanol toxicity was observed at high concentrations. However, the analysis did reveal preferred combinations of carbon-nitrogen sources for each strain tested that can be further optimized to achieve the best outcomes in industrial applications.

For *Methylocystis* sp. Rockwell, methane-ammonium was the preferred carbon-nitrogen combination enabling greater yield and high growth rates, particularly for the 0.5 mmol methane amount where the carbon was completely oxidized (Figure 3.4). This condition is also most favourable for *M. trichosporium* OB3b which, while achieving slightly greater yield in methane-nitrate, experienced its fastest growth rate in methane-ammonium (Table 3.1 and 3.2). The optimal condition for *Methylocystis* sp. WRRC1, however, was found to be methane-nitrate at 0.5 mmol carbon source, though the weighted difference with growth rate and yield in methane-ammonium was small. In terms of industrial application, this could impact strain selection, especially when considering alternative products to biomass, fatty acids, and organic acids, as described here; previous work has found, for instance, that ammonium is a preferred nitrogen source for PHB

production in *Methylocystis parvus* OBBP, but nitrate was more productive for *Methylosinus trichosporium* OB3b (Rostkowski et al., 2013). Combinatorial factors must also be considered however, as different carbon sources may be preferred given certain nitrogen sources, or vice versa. A novel modeling-based approach has been applied to *M. trichosporium* OB3b examining such effects and demonstrates that optimal growth conditions do not match optimal PHB production conditions, and that the source of carbon, methane or methanol, changes nitrogen source preference for both metrics (Zaldívar Carrillo, Stein, & Sauvageau, 2018).

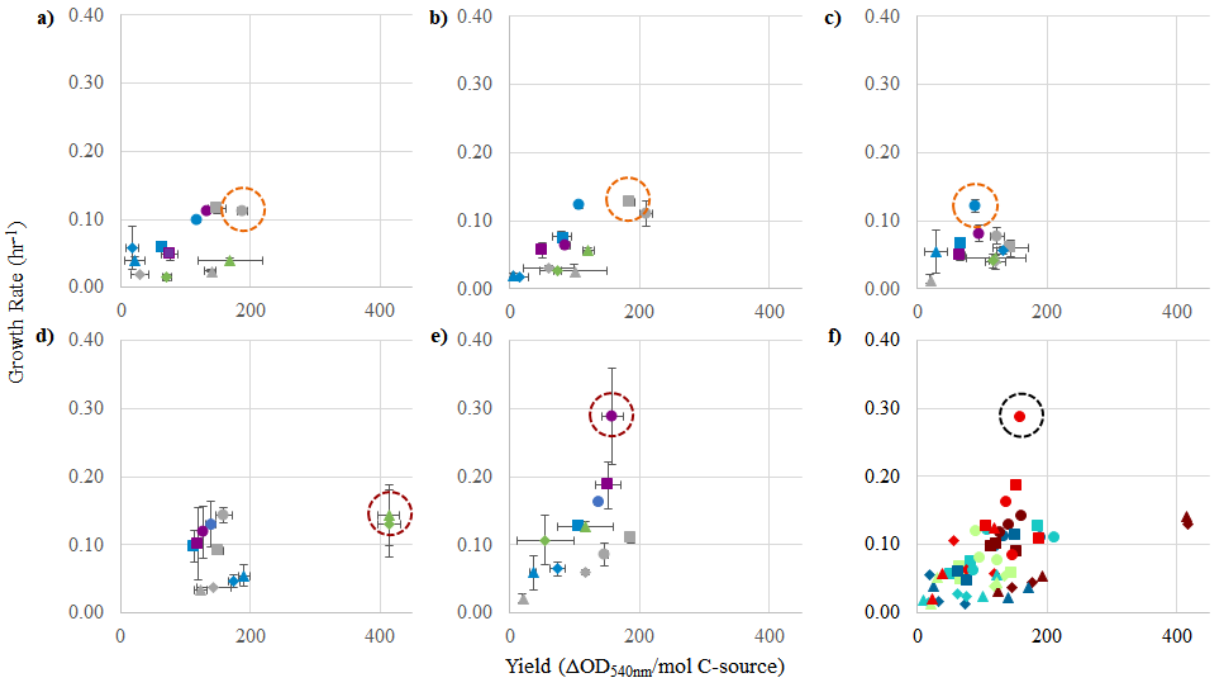


**Figure 3.4**. Comparison of yield (OD540 nm/mol C) and growth rate (h-1) for each strain, and in each condition tested. For panels (A–E): (A) Methylocystis sp. Rockwell, (B) Methylocystis sp. WRRC1, (C) M. trichosporium OB3b, (D) M. album BG8, and (E) M. denitrific ans FJG1. Carbon source amounts are: 0.5 mmol (gray), 1 mmol (green), 2 mmol (blue), and 2.5 mmol (purple). Carbon/nitrogen conditions are represented by: methane/NH+4 (circles), methane/NO−3 (squares), methanol/NH+4 (triangles), and methanol/NO−3 (diamonds). Panel (F) shows a combination of panels (A–E) together. Circles indicate best conditions for each strain (A–E), or overall (F).

With these results, a balance between improved growth or product yield must be considered for *M. trichosporium* OB3b, which may not be required for *Methylocystis* sp. WRRC1 or

*Methylocystis* sp. Rockwell, as PHB optimization has not yet been formally evaluated in these strains. This balance of optimization can have significant effects and must be carefully considered; use of methanol as a carbon source for production of PHB in *M. trichosporium* OB3b led to five times more PHB than methane, but also resulted in significantly longer lag phase and delayed growth (Zaldívar Carrillo et al., 2018). Even in terms of product quality, use of methanol as a carbon source can also lead to improved molecular weight of PHB (Ezhov, Doronina et al., 2017; Xin, Zhang et al., 2011), but as noted in this study, may not favour optimal biomass accumulation, significantly effecting the efficiency of the overall process.

Application in a bioprocess will also necessarily consider rate and titer of the desired product, as these might dictate which substrate condition is most favourable for the particular process, including, for instance, operational mode (*i.e.* batch vs. fed-batch, continuous etc.). Other factors than nitrogen and carbon sources must also be considered when developing an industrial process. Copper is well noted for its significance in controlling expression of pMMO and sMMO in methanotrophs (Semrau et al., 2010), and lanthanides have recently been implicated in regulating the expression of alternative methanol dehydrogenases (Farhan Ul Haque et al., 2015); neither of which were examined in this study. Nevertheless, beyond biomass, these findings may have widespread implications for diverse products, and specifically the optimized conditions for processes developed to generate these bioproducts.

While *M. album* BG8 grew favourably in most conditions tested, the 1 mmol methanol conditions proved most preferable for *M. album* BG8, with a slight preference for the methanol-ammonium combination over methanol-nitrate, largely due to the high yield resulting from these conditions. Of the five strains tested, *M. album* BG8 showed the least inhibition by substrate condition, with relatively high values resulting from analysis of weighted growth rates and yield in every experimental group. This outcome could lend well to potential future process development with this strain, given its inherent adaptability. Likely, the growth condition chosen for

bioindustrial operation will need to reflect the product and process being developed; ultimately, incorporation of oxygen usage will be required to define key cost drivers and optimal process configurations. Regardless, a related industrially-relevant strain, *Methylomicrobium buryatense* 5GB1, was previously found to grow faster in methane, not methanol (Gilman et al., 2015); so this finding could point to a specialized use of *M. album* BG8 in certain industrial effluents, wherein higher concentrations of methanol can serve as a challenge for many methanotrophs.

In contrast, the best combined growth yield and rate for *M. denitrificans* FJG1 was observed with 2.5 mmol methane with either N source suggesting efficient use of methane by this strain even under $O_2$ limitation. This is interesting as this strain has an active metabolism under hypoxia, allowing for continued methane oxidation even under exceedingly low $O_2$ tensions (Kits, Klotz, et al., 2015), but only in nitrate, not ammonium. The growth benefit of ammonium is therefore, in this strain, unexpected. Although the lag phases for these cultures could be quite long, especially under methanol growth (Supp. Table A-2), the shortest lag times were observed with higher methane amounts (2.5 mmol). In an industrial process context, these data suggest that initial growth of methanotrophs could be augmented by using a higher initial methane condition before altering the carbon loading rate to achieve optimal growth yields and rates.

The excretion of particular metabolites lends clues to the efficiency of metabolism and growth of the two strains examined in more details. The accumulation of formate during growth of *Methylocystis* sp. Rockwell, particularly when grown on methanol, could imply sub-optimal conditions, and specifically an imbalance in intracellular redox potential or assimilatory bottlenecks (Table 3.3). Excretion of excess formate suggests that the C1 assimilatory pathway is not going to completion; which could explain the noticeably poorer growth outcomes, especially when growing on methanol. As no differential expression was observed in the transcription of formaldehyde and formate oxidation genes, this may be due to a lack of long-term adaptation to the growth conditions. *M. album* BG8 grew robustly on methanol and did not excrete formate (Table 3.3), suggesting the

presence of this metabolite could be used as a good indicator of unbalanced growth conditions in process monitoring and control. Formate has been observed as an excreted metabolite during growth of other gamma-MOB, and its concentration increases as a function of unbalanced growth, for instance, under oxygen limitation (Kalyuzhnaya et al., 2013) or during growth on methanol (Gilman et al., 2015). Production of lactate by *Methylocystis* sp. Rockwell suggests anaerobic metabolism, although this product has not been reported for other alphaproteobacterial methanotrophic strains. However, *Methylocystis parvis* has been reported to produce other fermentation products like succinate and acetate during anaerobic metabolism (Vecherskaya, Dijkema et al., 2009). *M. album* BG8 did not excrete measurable formate into the medium under any condition, suggesting complete oxidation of methane/methanol to $CO_2$ under all tested conditions.

### 3.5.2. Carbon and Nitrogen Effects on Lipid Composition in Alpha- and Gamma-MOB

Analysis of PLFA compositions and abundances in *Methylocystis sp.* Rockwell confirmed prior studies of other *Methylocystis* sp.s in which relative PLFA abundances, but not compositions, changed for cells grown in methane or methanol or in methane plus methanol (Bodelier et al., 2009). Overall, analysis of total fatty acids as a percentage of cell dry weight showed greater change in abundance with variation in carbon and nitrogen source in *Methylocystis* sp. Rockwell compared to *M. album* BG8 (Figure 3.2). However, both strains showed specific PLFA changes in response to different carbon and nitrogen sources (Figure 3.3). *Methylocystis* sp. Rockwell generally grew more robustly with ammonium, yet it produced significantly less PLFA than when growing with nitrate in either methane or methanol. Furthermore, methanol growth decreased the abundance of PLFA even further when compared to growth on methane. This is in agreement with previous work on *M. buryatense* 5GB1, which similarly showed a decrease in total FAMEs when grown in methanol compared to methane (Gilman et al., 2015). Overall, the FAMEs profile of *Methylocystis* sp. Rockwell, 93% composed of only 2 separate fatty acid types and over 75% C18:1n9, may point to suitability

for use in biodiesel production, as high abundance, heavily-synthesized fatty acid. The relationship between PLFA abundance and growth characteristics remains to be defined and points to an interesting area for future investigation. The PLFA abundance changes in response to nitrogen and carbon source by *Methylocystis* sp. Rockwell is in stark contrast with the relative lack of change in *M. album* BG8.

Only minor differential gene expression was noted in the analysis of the fatty acid biosynthesis pathway in either strain under the examined growth conditions. Upregulation of ACP synthase in methanol grown cells can be noted in *M. album* BG8 and not in *Methylocystis* sp. Rockwell (Supp. Tables A-3 and A-4), which may explain some differences in relative fatty acid abundance in these conditions, as these genes are very likely involved in upstream intermediate supply. Overall, however, expression profiles, particularly in *Methylocystis* sp. Rockwell do not change with condition, despite the changes in FAMEs profile measured. This implies that the difference in FAMEs profiles was not caused by regulation of the fatty acid biosynthesis pathway. Contrary to our results, growth on methanol was previously shown to repress fatty acid biosynthesis genes in *Methylomicrobium buryatense* 5G(B1) (Demidenko et al., 2017). These results indicate that though these fatty acid biosynthesis pathways may be well-characterized, there is more complexity to their regulation. While transcriptomic analysis remains a powerful and versatile tool for informing process and culturing decisions, it also must be paired with other strategies to achieve concrete insights into microbial behaviour.

## 3.6. Conclusions

The results of this study clearly show that nutrient combinations greatly impact growth yields and rates in alpha- and gamma-MOB, and must be carefully considered on a strain-by-strain basis when developing bioprocessing strategies. In all cases, a multi-objective optimization

approach, even rudimentary, should be considered to assess advantageous conditions for both growth yields and rates.

While a single medium may support growth of most methanotrophs (*i.e.* NMS and AMS), some formulations are obviously better suited to some strains rather than others. Though pathways and enzymes in these organisms may be well understood, we do not yet possess the ability to necessarily predict these optimal conditions based purely on theoretical understanding (*i.e.* which is calculated to be most efficient). Further work will need to be completed to address this aspect of the work, if bioindustrial optimization is to be streamlined.

These results also highlight the benefit of using certain key metabolites to evaluate nutrient effects on growth, as accumulation may point to unbalanced growth or challenging growth conditions. This has implications in understanding carbon flux, an important consideration in optimizing bioindustrial processes. These growth conditions also lead to variable FAMEs synthesis, helpful if the industrial process could benefit from a higher accumulation of lipids in the cell. Overall, notable differences in FAMEs response across strains are expected, which further points to strain-specific optimization (although preliminary evidence suggest that total PLFA abundance in alpha-MOB may not be as sensitive to C- and N sources).

While this work provides a survey of different strains growing on various combinations of carbon and nitrogen sources, many other aspects of culture optimization – including copper concentrations, phosphorous and other trace elements, and lanthanides – should also be addressed in a similar fashion. The application of these optimized conditions to common bioindustrial processes, *e.g.* bioreactors operating in continuous or semi-continuous modes, would also provide an interesting avenue of further study, examining efficiency through scale up and industrial applications.

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# 4. Comparative transcriptomics of *Methylomicrobium album* BG8 in different carbon and nitrogen growth conditions

## 4.1. Abstract

### 4.1.1. Abstract

Methanotrophs, bacteria whose sole source of carbon and energy is methane, have long been of interest for industrial use, given their low-cost feedstock and wide inventory of natural bioproducts. Their implementation in industrial-scale processes has been hampered by limited understanding of how these species grow and regulate their metabolism in response to varied growth conditions. This is problematic since many bioprocesses are highly pathway-specific, relying on the high expression of certain desired metabolic pathways in the cell. This study aimed to address this problem by conducting whole-genome transcriptomics on an industrially-relevant, methanol-favouring, gammaproteobacterial methanotroph strain, *Methylomicrobium album* BG8, growing on either methane or methanol coupled with either ammonium or nitrate, which are the most common carbon and nitrogen conditions used for growth of these bacteria. Overall differential gene expression was classified by clusters of orthologous groups category. Our results demonstrate significant differential expression amongst the four tested growth conditions, showing diverse regulation despite similar growth seen in batch culture. Most notable was a very pronounced transcript profile shift from growth in methanol, particularly when paired with nitrate. This is seen most notably in the nitrogen assimilatory pathway, as well as stabilizing and fluidity components of the cell membrane. These findings point to the importance of lipid biosynthesis and membrane regulation as being key components of this bacterium's adaptation to methanol-growth, and point to its suitability for development as a bio-industrial asset.

### 4.1.2. Importance

Methane, a potent greenhouse gas, is a common by-product of many modern industrial activities, which often results in wasteful flaring or harmful release. Diversion of waste methane emissions to use in methanotroph-based biorefineries could instead result in production of highly valuable, environmentally-friendly, commodity chemicals or consumer products. This process is only possible however with a highly detailed understanding of the physiology of the organisms and a comprehensive model of bacterial behavior under varied conditions, as might be encountered in industrial settings. The interactive effects of carbon source and nitrogen source are likely to play a huge role in any future success of these processes, but they remain largely under-studied. This work addressed this current deficit in the research by providing a multi-variable, comprehensive evaluation of growth behaviours and their implications thereof in future works.

## 4.2. Introduction

In nature, one of the most important methane sinks is through the action of a specialized class of bacteria known as the methane-oxidizing bacteria (MOB), or methanotrophs. There are many different families of methanotrophs, but some of the most well-characterized are the aerobic proteobacterial members, including the gammaproteobacterial methanotrophs (gamma-MOB) (Hanson & Hanson, 1996). The gamma-MOB are notably dominant in most mixed communities over alphaproteobacterial methanotrophs, and tend to win in competition studies, including under carbon limitation, likely owing to their higher efficiency carbon assimilation (Graham et al., 1993; Hanson & Hanson, 1996; Hu & Lu, 2015; Shrestha et al., 2010). This prevalence points to the importance of the gamma-MOB in regulating the global methane cycle. However, while these bacteria have been studied physiologically for nearly a century, much still remains to be elucidated about their regulation, their responses to various environments, and how to capitalize on their noted potential for biotechnological applications (Cantera et al., 2019; Hanson & Hanson, 1996; Murrell, 2010).

The *Methylomicrobium album* BG8 strain of methanotroph is notable for its long status as a model organism of the gamma-MOB and the many physiological studies conducted to explore its behaviour (Brantner, Buchholz et al., 1997; Caceres, Gentina, & Aroca, 2014; Campbell et al., 2011; Kits, Campbell, et al., 2015; Nyerges et al., 2010; Tays, Guarnieri et al., 2018). Relevant to this study is an investigation on its growth in ammonium aimed at exploring co-metabolism and toxicity response, which found that ammonium significantly slowed methane oxidation rates in this strain (Nyerges & Stein, 2009). Examination of growth in nitrite was also explored, which found that this strain is particularly resistant to nitrite toxicity, particularly when competing with the strain *Methylocystis* sp. Rockwell (Nyerges et al., 2010). Recently, the growth characteristics of *M. album* BG8 was assayed in various concentrations of methane and methanol, paired with ammonium or nitrate (Tays et al., 2018). This work found that methanol is a favoured growth substrate for this strain over methane, unlike the four other methanotrophs tested.

Due to solvent effects on bacterial cells, methanol usually leads to reduced growth rate, yield, or both when used as a substrate for cultures of methanotrophs (Gilman et al., 2015; Whittenbury et al., 1970). Likewise, ammonium can be co-metabolised to toxic hydroxylamine by methane monooxygenase (He et al., 2017; Murrell & Smith, 2010). Both methanol and ammonium, however, would be theoretically less energetically demanding to incorporate into cell biomass when compared to methane and nitrate (Anthony, 1978; He et al., 2017; Nyerges & Stein, 2009; van Dijken & Harder, 1975). Examination of the regulation of *M. album* BG8, which demonstrates preference towards methanol and nitrate, may well allow for insight into how this or other methanotrophic strains of interest may be adapted to increase growth efficiencies and improve the economics of bioprocess applications.

One of the current limitations in developing these bioproduction processes however lies in the uncertainty surrounding optimal culturing conditions. Though Whittenbury et al. (1970) outlined a robust standard growth medium for methanotrophs, alterations to this medium have

proven beneficial both for straightforward growth assays as well as for specialized applications such as inducing increased lipid yield (Fei et al., 2014; Hoefman et al., 2014). Some studies examining the effects of nitrogen sources have been conducted. In *M. album* BG8, nitrate was shown to upregulate the denitrification pathway but only under hypoxia, while ammonium induced increased expression of hydroxylamine oxidoreductase as a response to ammonia oxidation (Campbell et al., 2011; Kits, Klotz, et al., 2015). However, in a study of methanotroph mixed communities from rice field soils dominated by gamma-MOB, amendment with nitrate- and ammonium-fertilizers led to differential assimilation and growth, favouring ammonium (Hu & Lu, 2015). On the carbon side, investigation into methane- and methanol-based growth in another gamma-MOB, *Methylomicrobium buryatense* 5GB1, did show that the carbon source affects core metabolism (Fu et al., 2019), while another study of yet another gamma-MOB, *Methylomonas* sp. DH-1, found that methane and methanol also lead to distinct transcriptome profiles (Nguyen et al., 2019). Clearly, both nitrogen and carbon play important roles in determining transcription.

Indeed, the nitrogen and carbon sources are two of the most important components to defining methanotroph growth media and show many crosslinks in regulatory effect (Commichau et al., 2006; Nyerges & Stein, 2009). Previous studies of gamma-MOB have generally focused on the effect of only one variable at time, *e.g.* either carbon or nitrogen. These studies provide valuable insight into the mechanisms of metabolic regulation in these bacteria but they do not account for the possibility of cross-regulation by accounting for both C source and N source effects. This aspect of regulation in methanotrophs therefore remains ill-defined.

In this work, the interactive effects of carbon and nitrogen sources will be examined, aimed at shedding light on how changing one or both of these components can affect growth behaviour and regulation. Specifically, this will be achieved via transcriptomic analysis, to provide a regulatory-based analysis of *M. album* BG8 and its response to growth conditions that might be scientifically, industrially, and agriculturally relevant. Evaluation and comprehension in this area

may be instrumental in the future understanding and optimization of bioprocesses and natural processes alike.

## 4.3. Methods

### 4.3.1. Growth and Maintenance

*M. album* BG8 was selected to provide an assessment of growth characteristics on different carbon/nitrogen source combinations due to it's unusual growth on methanol compared to other strains of methanotroph (Tays et al., 2018). Cultures were grown as previously reported (Tays et al., 2018), using either ammonium mineral salts (AMS) or nitrate mineral salts (NMS) medium (Whittenbury et al., 1970), containing either 10 mM ammonium chloride (AMS) or 10 mM potassium nitrate (NMS) as N source.

For growth experiments, Wheaton media bottles (250 mL) closed with butyl-rubber septa caps were filled with 100 mL medium. The copper ($CuSO_4$) concentration in the final medium was 5 µM for all media formulations. The media were buffered to pH 6.8 through addition of 1.5 mL phosphate buffer (26 g/L $KH_2PO_4$, 33 g/L $Na_2HPO_4$) and inoculated with 1 mL (1%) of previously grown cultures that had been passaged once in identical conditions to each of the experimental conditions; as such, initial biomass at inoculation ranged in $OD_{540nm}$ from 0.097 to 0.102, reflecting the growth result of the inoculum culture.

In methane-grown cultures, 2.5 mmol methane was provided via injection through a 0.22-µm filter-fitted syringe, following initial removal of gas headspace to ensure pressure at the beginning of incubations was ~1.05 atm. In methanol-grown cultures, initial pressure was 1 atm and 1 mmol of pure HPLC-grade methanol was added. All cultures were incubated at 30°C with shaking at 150 rpm. Experiments were performed with replication (n=3) for all conditions.

### 4.3.2. Analysis of Growth

To monitor growth, 500-µL samples were extracted from cultures via sterile syringe at regular intervals over lag, exponential, and stationary phases. Three replicates were grown and assayed for each condition such that standard deviations could be calculated. Growth was assessed using optical density (OD) measurements at 540 nm in a 48-well microplate (Multiskan Spectrum, Thermo Scientific). Representative growth curves, demonstrating when RNA extraction was accomplished, are represented in Supp. Figure B-1. Culture purity was assured through phase contrast microscopy and plating of culture on TSA/nutrient agar plates, where lack of growth supported lack of contamination.

### 4.3.3. RNA Extraction

Total RNA was extracted at late log phase from all strains grown in either AMS or NMS with methanol (1 mmol) or methane (2.5 mmol) provided as carbon source using the MasterPure RNA purification kit (Epicentre). Briefly, cells were inactivated with phenol-stop solution (5% phenol, 95% ethanol) and pelleted through centrifugation. Nucleic acids were purified according to manufacturer's instructions, except for the modification of Proteinase K addition to 0.35 mg total. RNA quantity and quality were assessed using a BioAnalyzer (Agilent Technologies).

### 4.3.4. RNA Sequencing and Assembly

RNA-Seq was performed by the Department of Energy Joint Genome Institute (DOE, JGI), using Illumina HiSeq-2000 technology. In both methane-ammonium and methane-nitrate conditions, one replicate sample was sequenced via Illumina MiSeq. Raw reads, JGI transcriptomic analysis, and additional supporting information were made available through the JGI Genome Portal, under proposal ID 1114. Geneious 11.0.2 (https://www.geneious.com) was then used to map reads to the *M. album* BG8 genome (GCA_000214275.3 ASM21427v3) with Bowtie2, using default settings (high sensitivity) and local-use alignment method (Langmead & Salzberg, 2012;

Langmead, Wilks et al., 2018). Gene expression and differential expression were calculated using DESeq2 (Love et al., 2014).

In all tested conditions except methanol-ammonium (for which two replicates were completed), three replicates were sequenced and mapped to the reference genome. After mapping, principal component analysis (PCA) via Geneious was used to determine suitability of replicates for further analysis, using the distance equation: $a^2 + b^2 = c^2$. Notably distant – and therefore divergent (un-grouping) – replicates were determined to be outliers and removed from further analysis. Both of the replicates sequenced with MiSeq were cut from further analysis due to lack of coherence with the other replicates for those conditions (Supp. Figure B-2). Therefore, methane-nitrate, ammonium-nitrate, and methanol-ammonium data sets were analyzed with n=2 replicates, while the methanol-nitrate data set was analyzed with n=3 replicates. In the literature, methane-nitrate is considered the standard growth condition for methanotrophic bacteria; therefore, this condition was selected as the reference for comparison with the other three conditions. Significance in differential expression of transcripts was considered at an *n*-fold change of $> | 1 |$ and false-discovery rate (FDR) adjusted *p*-value of $< 0.01$, calculated by DESeq2 plugin in Geneious.

### 4.3.5. Differential Gene Expression (DGE) Classification and Analysis

Significant differential gene expression in each of the test conditions compared to expression in the reference condition (methane-nitrate) were further analysed by gene orthology using the Kyoto Encyclopedia of Genes and Genomes (KEGG) program, BLAST Koala (Kanehisa, Sato, & Morishima, 2016), and the European Molecular Biology Laboratory (EMBL) program, EggNOG (Huerta-Cepas, Szklarczyk et al., 2015). Genes unclassified by the EggNOG analysis, or classified only as Function Unknown, were further assessed via the National Centre for Biotechnology Information Basic Local Alignment Search Tool (NCBI-BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine if any uncategorized or hypothetical genes could be assigned preliminarily to a function or functional group.

## 4.4. Results

### 4.4.1. Gene Orthology of Highly Differentially Expressed Genes

To provide a snapshot of how carbon and nitrogen conditions might differentially affect gene expression, all significant differential gene expression (DGE) for each comparison was identified (Table 4.1). Compared to the reference condition of methane-nitrate, a change in nitrogen source, *i.e.* methane-ammonium, resulted in 39 incidents of significant DGE. A different carbon source, *i.e.* methanol-nitrate, resulted in a much higher DGE count of 432. When both carbon and nitrogen source were different however, *i.e.* methanol-ammonium, only 14 DGE counts were found (Table 4.1).

**Table 4.1**. Number of differential gene expressions (DGE) in each tested comparison of growth conditions in *M. album* BG8. Significance was determined by log-fold change > 1, and adjusted p-value < 0.01. Count of total genes includes both genes showing increased and decreased transcription in the test condition, compared to the reference condition.

| **Test Condition** | *Methane-Ammonium* | *Methanol-Nitrate-* | *Methanol-Ammonium* | *Methanol-Ammonium* | *Methanol-Ammonium* |
|---|---|---|---|---|---|
| **Reference Condition** | *Methane-Nitrate* | *Methane-Nitrate* | *Methane-Nitrate* | *Methane-Ammonium* | *Methanol-Nitrate* |
| **Upregulated** | 34 | 290 | 0 | 0 | 182 |
| **Downregulated** | 5 | 142 | 14 | 19 | 272 |
| **Total DGE** | 39 | 432 | 14 | 19 | 454 |

However, when nitrogen sources were compared in methanol-grown cultures, rather than methane-grown, 454 DGE were observed. Likewise, when carbon sources were compared in ammonium-grown cultures, only 19 DGE were found. Based on these data, the methanol-nitrate condition induces the most highly divergent pattern of transcription when compared to the methane-nitrate reference.

After being identified as significant in differential expression, genes were then each classified according to their orthology through the use of both BLAST-Koala and EggNOG. The BLAST-Koala pipeline led to a generally low number of classified genes, often less than a third of the total genes submitted, and results were thus not informative (data not shown). In comparison, EggNOG was able to reliably categorize between 56-79% of the genes, with the uncategorized or function unknown comprising solely hypothetical genes (Figure 4.1).

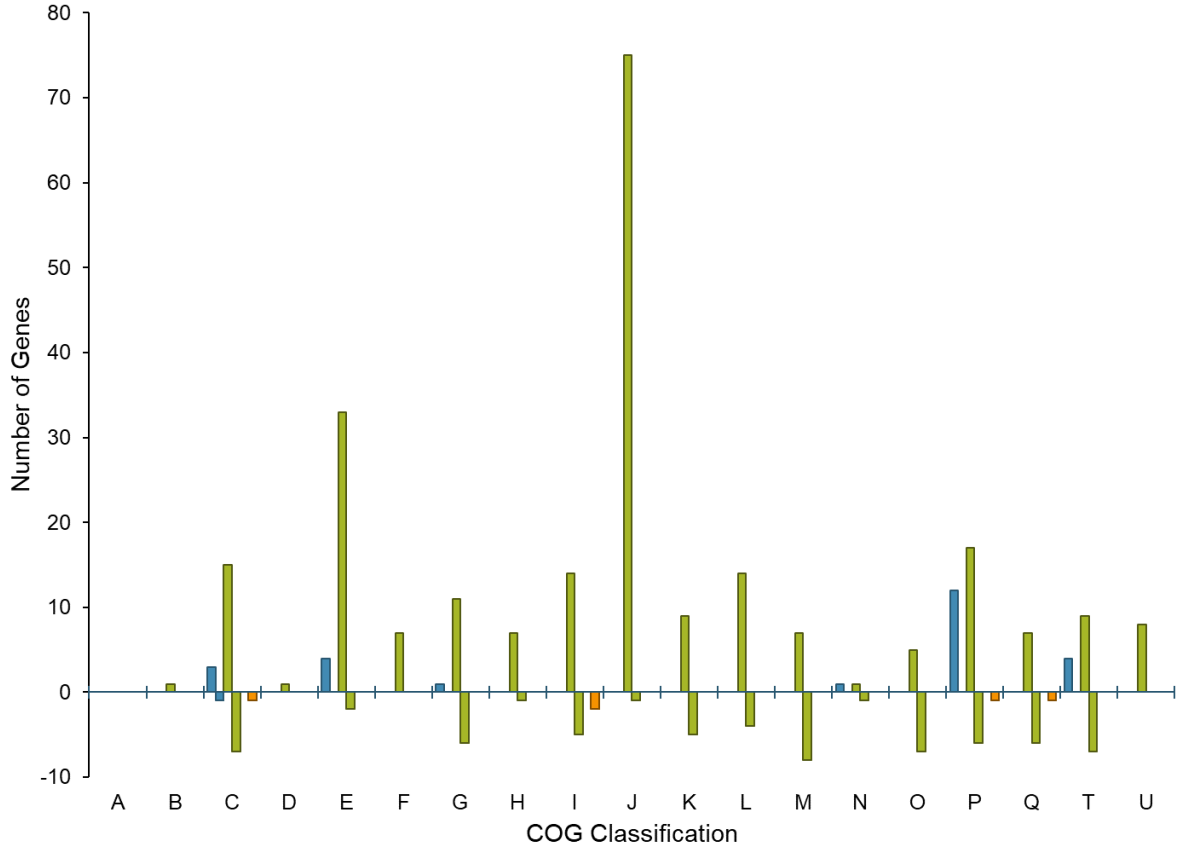


**Figure 4.1**. Classification of significant differential gene expression (DGE) in *M. album* BG8, based on COG classification according to the EggNOG database. Blue represents methane-ammonium vs. methane-nitrate, green represents methanol-nitrate vs. methane-nitrate, and orange represents methanol-ammonium vs. methane-nitrate. A = RNA processing and modification; B = Chromatin structure and dynamics; C = Energy production and conversion; D = Cell cycle control and mitosis; E = Amino acid metabolism and transport; F = Nucleotide metabolism and transport; G = Carbohydrate metabolism and transport; H = Coenzyme metabolism; I = Lipid metabolism; J = Translation; K = Transcription; L = Replication and repair; M = Cell wall/membrane/envelop biogenesis; N = Cell motility; O = Post-translational modification, protein turnover, chaperone functions; P = Inorganic ion transport and metabolism; Q = Secondary structure; T = Signal transduction; U = Intracellular trafficking and secretion.

It is notable that there were no genes showing significantly higher expression levels in methanol-ammonium compared to methane-nitrate, nor in methanol-ammonium compared to methane-ammonium. While in general few instances of DGE were noted in comparing methane-ammonium to methane-nitrate, a high proportion of these genes were classed as relating to inorganic ion transport and metabolism. This included nitrate, nitrite, and ammonium transporter genes, as well as genes only identified as ABC transporter-related, and genes coding for receptors and a urea carboxylase-associated protein. Only one gene showing lower expression in this comparison was classified, a cytochrome c gene. This was categorized by EggNOG as energy production and conversion, a category which also saw three genes with higher expression in methane-ammonium: nitrite reductase genes *nirB* and *nirD*, and a gene coding for an FAD binding domain.

Genes showing increased expression in the methanol-nitrate condition were primarily in the category of translation. Genes with decreased transcription were spread between the categories of: energy production and conversion; carbohydrate metabolism and transport; cell wall/membrane/envelope; post-translational modification, protein turnover, chaperone functions; inorganic ion transport and metabolism; and signal transduction. In the other methanol-grown case (methanol-ammonium), despite the greatest variety in growth condition, only five genes with DGE could be categorized compared to methane-nitrate; all with decreased expression, including a formate dehydrogenase gene (*fdh*), two genes for squalene phytoene synthase (*fdfT*) and squalene-hopene cyclase (*sqhC*), a methyltransferase gene (*yafE*), and cyanase gene (*cynS*).

Finally, in comparing methanol-based growth between ammonium and nitrate, genes related to energy production and conversion, post-translational modification, protein turnover, chaperone functions, and signal transduction were more highly expressed. Also showing increased expression were cell motility genes, with genes for flagella, Type IV pili and twitching motility upregulated. 67 genes related to translation showed lower levels of expression, as did 23 amino

acid metabolism and transport genes and 19 energy production and conversion genes, among others.

Following classification of gene orthology, the most highly differentially expressed genes were analysed, these are presented in Supp. Table B-1. In the methane-ammonium vs. methane-nitrate comparison, nitrogen-related genes dominated the list. This included nitrite and nitrate reductases, transporters, and channel proteins. Chemotaxis-related genes were also more highly expressed. Methanol grown cells with either ammonium or nitrate did not show a similar pattern however, with high DGE in a wider variety of genes including a chaperone protein, a competence protein, chemotaxis proteins, and 30S and 50S ribosomal proteins.

In the methanol-nitrate vs. methane-nitrate comparison, highly expressed genes were more varied, and included hemerythrin, TonB components, electron carriers, and a formyltransferase. Methanol-ammonium vs. methane-nitrate showed increased expression of a number of cell membrane fluidity influencers, squalene/phytoene synthase and squalene-hopene cyclase, as well as some degradation enzymes like cyanase and an EthD family reductase. The latter genes were likewise less expressed in methanol-ammonium compared to methane-ammonium, as were certain Nir, Nar, PII, and N transport genes.

### 4.4.2. Carbon and Nitrogen Assimilation Pathways

To determine if carbon and nitrogen sources both have effects on carbon assimilation and regulation, the expression of genes involved in methane oxidation and the RuMP pathway were analyzed. Overall, methane induced very similar expression of methane oxidation genes and RuMP genes, regardless of nitrogen source (Figure 4.2a). Transcriptional response to growth in methanol is, however, affected by nitrogen source; growth on ammonium decreased expression of a number of RuMP genes when compared to growth on nitrate, and upregulated *pmoCAB* (Supp. Figure B-4b).

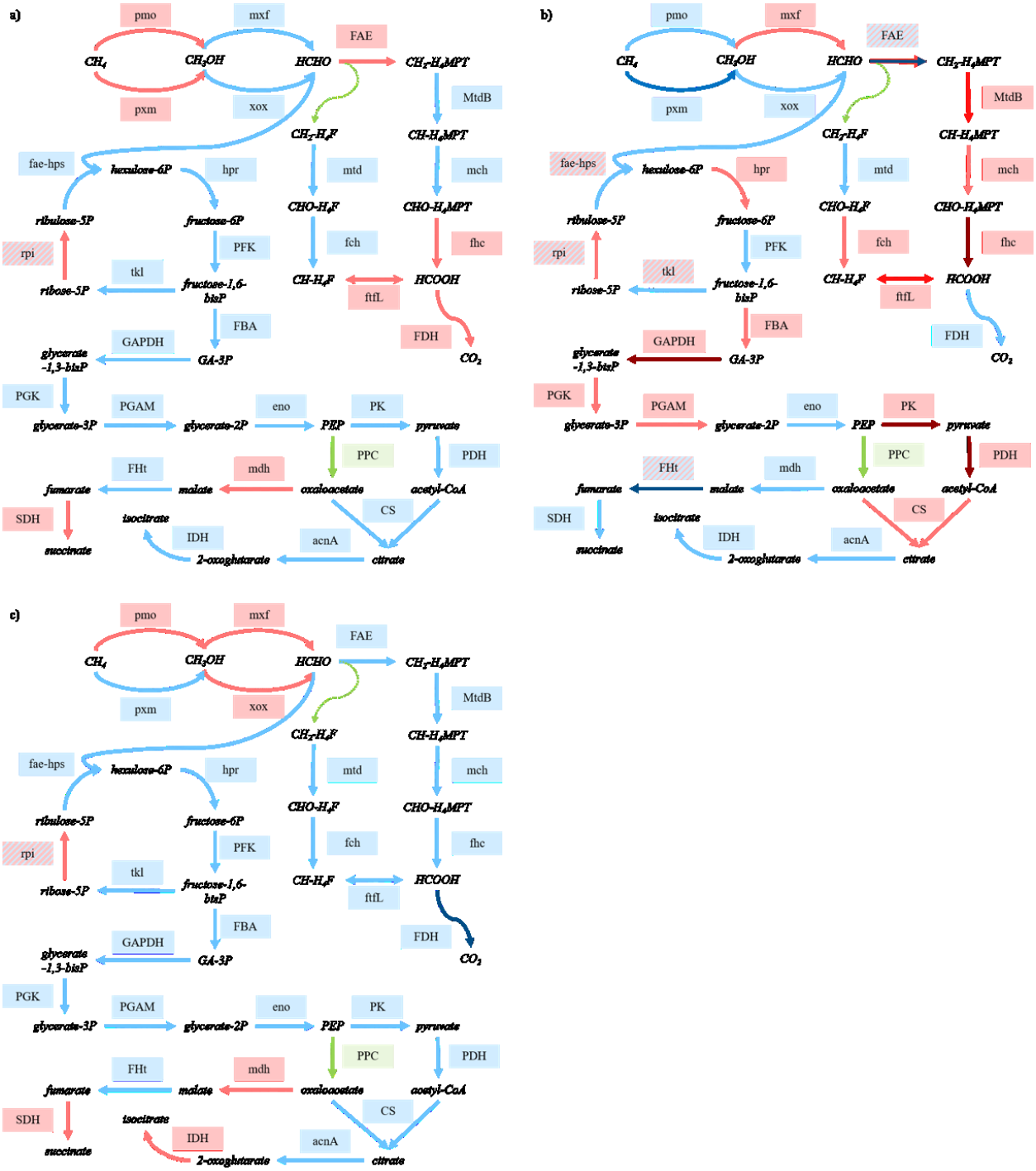


**Figure 4.2**. Differential regulation of methane oxidation and carbon fixation *via* the RuMP pathway in *M. album* BG8. Methane-ammonium vs. methane-nitrate (a), methanol-nitrate vs. methane-nitrate (b), methanol-ammonium vs. methane-nitrate (c). Genes shown in red are comparatively upregulated in the experimental conditions, blue denotes downregulation. Dark arrows represent significant differential regulation (log-fold change > 1, adj. p-value < 0.01), medium-coloured represent differential regulation under adj-p-value < 0.05. Where striped, multiple genes showed both up- and down-regulation in test condition and the overall expression difference was used to determine the colour of the arrow.

Compared to the reference of growth on methane-nitrate, however, methanol-nitrate showed the most DGE overall. This is noted in significant up- and down-regulation in different formaldehyde activating enzymes, as well as increased expression of genes in the tetrahydromethanopterin and tetrahydrofolate pathways, and some decreased expression of the *pxmABC* operon, which encodes a second type of methane described by Tavormina et al. (2011). Growth on methane-ammonium and methanol-ammonium in comparison were generally associated with downregulation of carbon pathways, despite a few instances of upregulation, and most differences in expression did not reach significance. Likewise for comparison of methanol-ammonium vs. methane-ammonium, the other C source comparison (Figure 4.2, Supp. Figure 4.4).

Compared to growth with nitrate, expression of particulate methane monooxygenase was higher when cells were grown in ammonium regardless of carbon source (Figure 4.2a,b,c), Expression of *pmoCAB* genes were highest in methanol-ammonium grown cells, whereas expression of *pxmABC* were highest in methane-ammonium grown cells (Supp. Figure 4.4a, 4.4b). Also of note is the expression of the methanol dehydrogenases, mxaF-type and xoxF-type. Compared to methane-nitrate, methane-ammonium induced less expression of both *mxaF* and *xoxF* genes (Figure 4.2a), methanol-nitrate increased expression of *mxaF* and decreased expression of *xoxF* (Figure 4.2b), while methanol-ammonium increased expression of both (Figure 4.2c).

In the RuMP pathway, little distinction was noted between methane-grown cells with nitrogen source (Figure 4.2a). In comparison, the methanol-nitrate condition showed higher expression of a number of pathway components (Figure 4.2b). Growth on methanol-ammonium did not prompt a similar differential response, however (Figure 4.2c). Methanol-ammonium vs. methane-ammonium growth likewise showed similar expression levels of RuMP genes (Supp. Figure B-3a).

Differences in the pathways and genes associated with nitrogen uptake and assimilation were analysed. Compared to the reference condition of methane-nitrate, ammonium grown cells

showed higher expression in a number of these genes (Figure 4.3a), including nitrogen regulatory protein PII gene *glnB*, ammonium transporters (*amt*), nitrate transporters (*nrt*), nitrate reductase (*nar*) and nitrite reductase (*nir*).

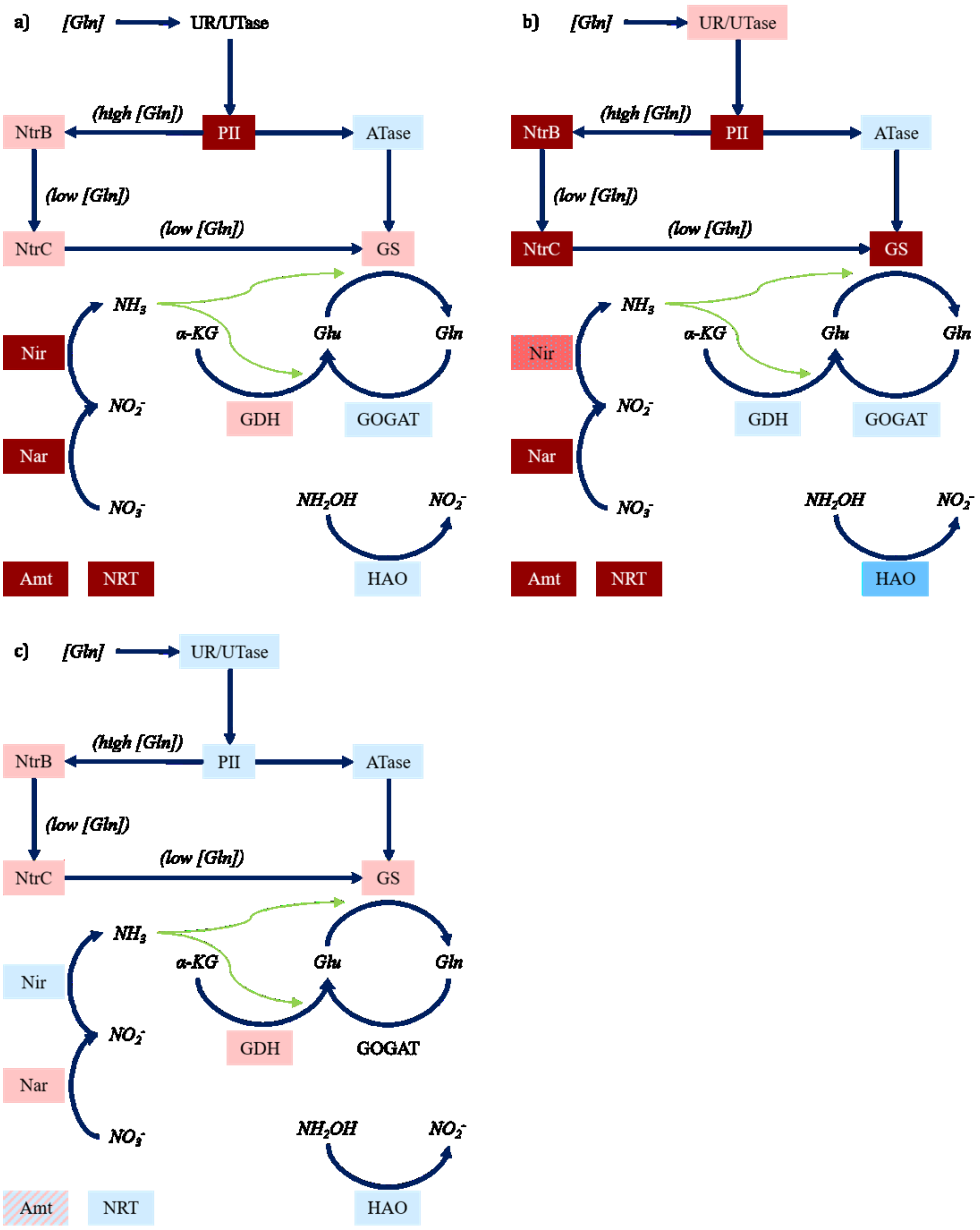


**Figure 4.3**. Differential regulation of nitrogen uptake and assimilation in *M. album* BG8. Methane-ammonium vs. methane-nitrate (a), methanol-nitrate vs. methane-nitrate (b), methanol-ammonium vs. methane-nitrate (c). Genes shown in red are comparatively upregulated in the experimental conditions, blue denotes downregulation. Dark arrows represent significant differential regulation (log-fold change > 1, adj. p-value < 0.01), medium-coloured represent differential regulation under adj-p-value < 0.05. Where striped, multiple genes showed both up- and down-regulation in test condition and the overall expression difference was used to determine the colour of the arrow.

Growth on methanol-nitrate compared to methane-nitrate showed higher expression in a number of the same genes: *glnB*, *amt*, *nrt*, *nar*, and *nir*, as well as glutamine synthetase (GS) gene *glnA* (Figure 4.3b). Hydroxylamine oxidoreductase was significantly down-regulated in methanol- compared to methane-grown cells. Growth on methanol-ammonium showed little effect on DGE compared to growth on methane-nitrate (Figure 4.3c), but significant decreases in expression of in *glnB*, *nrt*, *nar*, *nir*, and *glnA* were found.

## 4.5. Discussion

### 4.5.1. Overall Image of Differential Gene Expression

Carbon and nitrogen serve as two of the most important elements in a cell, and their availability and form can have dramatic effects on cellular function (Dam et al., 2014; Fu et al., 2019; Kalyuzhnaya et al., 2009; Kits, Campbell, et al., 2015; Kits, Klotz, et al., 2015; Nguyen et al., 2019; Vorobev et al., 2014). In this study, the effects of carbon and nitrogen sources on the growth of *M. album* BG8 were examined through the lens of transcriptomic analysis. Previous studies support the position that *M. album* BG8 is a promising candidate for development as an industrial microbe, with potential for rapid growth in a wide variety of conditions, and the ability to overcome challenges including hypoxia, nitrite, and methanol stress (Kits et al., 2013; Nyerges et al., 2010; Tays et al., 2018).

This study addresses how these growth behaviours, and ability to adapt to potentially challenging conditions, relate to intracellular regulation in terms of transcription. This data may indicate which pathways and genes are affected, explaining observed growth behaviours and physiological responses of *M. album* BG8, and providing more functional background for process development and even further applications including refinement of metabolic mapping (de la Torre, Metivier et al., 2015). Overall, this work showed that gene expression profiles were notably affected by the choice of carbon and nitrogen sources, alone and in combination.

To provide a basis for comparison, methane-nitrate was selected as reference condition, as this carbon-nitrogen combination is used as the most common growth medium in the literature (He et al., 2017). When comparing transcriptional responses during growth on methane with ammonium or nitrate, ammonium resulted in increased expression of 34 genes and decreased expression of 5 genes. As growth between these two conditions is largely indistinguishable in terms of rate, yield, and final growth density (Tays et al., 2018), this low DGE is not unexpected.

In contrast, the change in carbon source from methane-nitrate to methanol-nitrate resulted in 290 genes with increased expression and 142 with decreased expression. In a survey of five tested alpha- and gamma-MOB, only *M. album* BG8 was able to grow robustly in methanol-nitrate (Tays et al., 2018), perhaps due to its ability to better regulate gene expression towards survival, unlike that in the alphaproteobacterial methanotroph, *Methylocystis* sp. Rockwell (Tays et al., 2019, *Chapter 5)*. Of the genes with decreased expression, 8 could be classified as cell wall/membrane/envelope biogenesis genes, including three outer-membrane lipoprotein genes, two glycosyl transferase genes, and one transglycosylase gene.

This is of note, as a recurring category of genes showing increased expression during growth on methanol were those responsible for synthesis of hopanoids, squalenes, and porins. The former are structures that affect the rigidity and fluidity of the cell membrane (Ourisson, Rohmer, & Poralla, 1987), while porins serve as entrance and exit to the cell – often specific in function and synthesized directly in response to environmental cues and the needs of the cell (Achouak, Heulin, & Pagès, 2001). This points to the importance of cell wall regulation as a mechanism for acclimating to methanol, whose toxicity as an alcohol leads to a challenging situation when present in high concentration (Whittenbury et al., 1970). *M. album* BG8 is quite capable of handling much higher methanol concentrations than those used in this study (data not shown), and it is reasonable to assume that mechanisms affecting cell wall characteristics play a large role.

When comparing transcriptional responses to a change in both carbon and nitrogen sources, i.e. from methane-nitrate to methanol-ammonium, only 14 genes were differentially regulated. This response in *M. album* BG8 is notably different from the response of the alphaproteobacterial methanotroph, *Methylocystis* sp. Rockwell, which showed the largest changes in gene expression when both carbon and nitrogen sources were changed (Tays et al., 2019, *Chapter 5*). This difference in regulation is notable, and as of yet not fully explored.

Further, while growth between methanol-ammonium and methanol-nitrate was quite similar in *M. album* BG8 (Tays et al., 2018), the transcriptional response was not, with 454 genes differentially regulated. This implies that a great deal of transcriptional response is key to good growth on methanol, especially when also considering different N source. Of note is the upregulation in methanol-ammonium of 16 cell motility genes, which suggest less desirable growth conditions (Zhao, Liu, & Burgess, 2007). On the other hand, when comparing ammonium to nitrate in methane, only 1 gene of 34 total upregulated genes is motility-related, a chemotaxis sensory transducer, implying that this motility may be methanol-dependent. As methane- vs. methanol-dependent growth behaviours have been noted in methanotrophs before (Kalyuzhnaya et al., 2009), this is of interest for future works.

Finally, it should be noted that methanol-ammonium vs. methane-ammonium does not demonstrate the same pattern of DGE as methanol-nitrate vs. methane-nitrate. Only 19 genes are significantly differentially regulated in the ammonium-based comparison, compared to 432 in nitrate. This vast difference in transcriptional regulation is unexpected, given that the overall culture growth, as seen in Supp. Figure B-1, does not seem to be noticeably differentiated by N source (Tays et al., 2018). Again, this may point to the importance of carbon-nitrogen co-regulation in methanotrophs and remains a point to be examined more deeply.

### 4.5.2. Regulation of Carbon and Nitrogen

Given the undeniable importance of both nitrogen and carbon to regular cell functioning and growth, analysis of the uptake, regulation, and assimilation pathways of both these nutrients is vital. With the carbon source, though methanol is an intermediate of the same pathway as methane, implications exist in the sense of energetic balance of the cell (Fu et al., 2019). For example, previous work has found that methane vs. methanol does directly affect transcription profiles in gammaproteobacterial methylotrophs. A recent work on *Methylomicrobium buryatense* 5GB1 noted that methanol affects flux through the core carbon pathways, in line with decreased NADH requirements, but that this is not always seen at the level of transcriptomic profile (Fu et al., 2019).

The results of the study of *M. buryatense* 5GB1 may aid in explaining the results seen herein (Figure 4.2, Supp. Figure B-4) – though metabolite pools may change depending on carbon source, it is possible that the majority of the regulation of these processes in gamma-MOB is through post-translational, not transcriptional, mechanisms. Certainly, posttranslational mechanisms may contribute to the low degree of DGE observed between transcriptional profiles observed between growth on methane-ammonium and methanol-ammonium (Supp. Figure B-3). However, posttranslational modification events alone do not account for the high degree of DGE between growth on methanol versus methane with nitrate as N source. Therefore, the interplay between transcriptional, translational, and posttranslational mechanisms of modulating cellular activity is an important area of future study.

Incidences of high DGE between cells grown on methane versus methanol have been reported in another gamma-MOB, *Methylomonas* sp. DH-1, where expression of the central carbon pathway genes and those of secondary metabolites, including decreased expression of stress-response transcriptional regulators indicative of stress, were shifted as a result of methanol-growth (Nguyen et al., 2019). Conversely, a strong stress response to methanol did not appear in the transcriptome profile of *M. album* BG8 in the current study (Figure 4.1 and Figure 4.2b).

Despite the use of methanol as a variable, no methanol dehydrogenase gene was differentially regulated under any condition in this study, as shown in Figure 4.2. While XoxF has been shown to be the dominant methanol dehydrogenase in *M. buryatense* and very strongly upregulated at the transcriptional level in the presence of lanthanides (Chu & Lidstrom, 2016), its regulation was not strongly affected in *M. album* BG8 with methanol, nor was its more classical counterpart, *mxaF*-type methanol dehydrogenase (Figure 4.2). Genes encoding the classical form of particulate methane monooxygenase, *pmoCAB*, also did not have a strong transcriptional response to carbon or nitrogen source in general, only upregulated significantly in the comparison of methanol-ammonium vs. methanol-nitrate (Supp. Figure B-5b). Previous work in a related strain, *M. buryatense* 5GB1 did predict increased metabolic activity of MMO on methane but this was not explored with transcriptomic analysis (Fu et al., 2019).

*M. album* BG8 also encodes a separate CuMMO, *pxmABC*, with evolutionary relatedness to pMMO (Tavormina et al., 2011). This operon was previously found to be significantly upregulated in situations of nitrite availability and hypoxia, but not hypoxia alone, and therefore responsive to denitrifying conditions (Kits, Campbell, et al., 2015). Indeed, few DGE of this operon were noted in this study, with only marginal downregulation noted in methanol-nitrate compared to methane-nitrate (Figure 4.2). The methane oxidation and central carbon pathways overall showed little significant DGE between conditions, implying that other mechanisms, likely posttranslational, are more significant in shifting metabolic responses to methane vs. methanol, or ammonium vs. nitrate.

In considering nitrogen, separate uptake and, in the case of nitrate, reduction processes must take place prior to assimilation. Perhaps expectedly, nitrogen source did have a strong transcriptional effect on nitrogen uptake and assimilation pathways and genes when cells were grown on methane (Figure 4.3a) and methanol (Supp. Figure B-5b). Interestingly, these same genes showed a strong response to change in carbon source as well. Growth on methanol-nitrate vs. methane-nitrate showed increased expression of many genes (Figure 4.3b), growth on methanol-

ammonium vs. methane-ammonium resulted in more decreases in expression of many genes (Supp. Figure B-5a). This may imply that the difference in cellular energy balance of methanol oxidation vs. methane oxidation (Fu et al., 2019) also affects nitrogen assimilation, which itself is connected to carbon cycling via amino acid metabolism (Commichau et al., 2006). Further investigation into the levels of specific amino acid metabolites under the different growth conditions may address this mechanism.

## 4.6. Conclusions

Transcriptomic analysis allows for insight into the regulation of the cell and can be a valuable accompaniment to traditional culturing and enzymatic assays to assess cellular states, mechanisms, and other areas of interest. This is doubly true for industrially-focused research, which must tackle media refinement, process development, growth optimization, etc. RNASeq analysis demonstrated the varied responses of one strain, *M. album* BG8, to only two variables with two levels each, resulting in a large degree of differential gene expression across growth conditions. It should be noted that transcriptomics does not always directly align with metabolome or proteome data (Fu et al., 2019; Vogel & Marcotte, 2012), and further follow-up work is recommended to be confident in overall cellular response to the conditions tested, not just response via regulation of transcription.

However, the response of *M. album* BG8 to methanol in this study demonstrates that membrane regulation may be a key acclimatization mechanism to methanol stress. As well, the results strongly suggest that both carbon and nitrogen sources should be carefully tested when determining appropriate growth strategies, and that these variables should be examined together as one clearly affects the other in both pure culture and in the environment (Bodelier & Laanbroek, 2004; Commichau et al., 2006). Further work on additional growth variables including copper, feeding strategies, and reactor-based growth will aid in future refinements of growth conditions, whether for industry or discovery-based science.

## 4.7. Acknowledgements

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# 5. Comparative transcriptomics of *Methylocystis* sp. Rockwell in different carbon and nitrogen growth conditions

## 5.1. Abstract

### 5.1.1. Abstract

Methanotrophs are bacteria that can use methane as their sole source of carbon and energy. They play an important role in many environments and have garnered interest for the low-cost production of a variety of bioproducts. Currently, limitations in understanding of certain aspects of growth and metabolic regulation in response to culture conditions hamper implementation in large-scale industrial processes. In bioprocesses, this deficiency in understanding of how these bacteria regulate their metabolism in response to varied growth conditions can be critically problematic, as many processes inherently require a certain amount of pathway-specificity towards the desired bioproduct. This study aimed to address this problem by conducting whole-genome transcriptomics on an industrially-relevant, ammonium-favouring alphaproteobacterial methanotroph strain, *Methylocystis* sp. Rockwell, growing on either methane or methanol coupled with either ammonium or nitrate. Differential gene expression in cross-comparisons between growth conditions was classified by clusters of orthologous groups category. Our results demonstrate the nitrate assimilation pathway is highly affected by differences in nitrogen source. As well, significant differential gene regulation is observed when the bacteria are grown in methanol compared to methane, but only when provided with ammonium rather than nitrate as N source. Despite highly different growth outcomes, methane-nitrate and methanol-nitrite were found to induce very similar gene expression. These results provide a strong basis for the explanation of the wide variations in growth rates and yields observed when *Methylocystis* sp. Rockwell grows on the various combinations of carbon and nitrogen sources tested, and why growth is most robust with methane-ammonium over the other three combinations.

### 5.1.2. Importance

Methane, a potent greenhouse gas, is a common by-product of many modern industrial activities, which often results in wasteful flaring or harmful release. Diversion of waste methane emissions to use in methanotroph-based biorefineries could instead result in production of highly valuable, environmentally-friendly consumer products. This process is only possible, however, with a highly detailed understanding of the physiology of the organisms and a comprehensive model of bacterial behavior under varied conditions as might be encountered in industrial settings. The interactive effects of carbon source and nitrogen source are likely to play a major role in any future process' success, but the effects of these nutrients on bacterial metabolism and gene regulation remain largely under-studied. This work addressed this current deficit in the research by providing a comprehensive evaluation of transcriptional responses during bacterial growth on four combinations of carbon-nitrogen source.

## 5.2. Introduction

Much of the biotic control of methane oxidation in the environment occurs through the action of methane-oxidizing bacteria (MOB), also known as methanotrophs. Of the many types of MOB, the alphaproteobacterial methanotrophs (alpha-MOB) have garnered particular interest towards use in bioindustry (Hanson & Hanson, 1996; Murrell, 2010). These bacteria are of note for their ability to grow in less favourable or eutrophic conditions, including lower oxygen concentration and nitrogen limitation, compared to the gammaproteobacterial methanotrophs (Graham et al., 1993; Pfluger, Wu et al., 2011), and may therefore be of value in potentially challenging biotechnology processes. Alpha-MOB utilize the serine cycle for carbon assimilation, and from that pathway can produce a number of value-added products, including the biopolyester polyhydroxybutyrate (PHB), which can be used as biodegradable bioplastic (Hanson & Hanson, 1996; Murrell, 2010; Pieja, Rostkowski, & Criddle, 2011).

In this study, the effects of carbon and nitrogen sources on the growth and regulation of an industrially-promising alpha-MOB, *Methylocystis* sp. Rockwell, was investigated using transcriptomic analysis. This strain is notable for its affinity for ammonium, and has previously been explored in relation to high rates of ammonia-oxidation activity and resistance to high ammonium concentrations in batch culture (Nyerges & Stein, 2009). Indeed, in a growth competition experiment with the gammaproteobacterial methanotroph *Methylomicrobium album* BG8, *Methylocystis* sp. Rockwell dominated in cultures grown with ammonium, but not with nitrate as N source (Nyerges et al., 2010). Recently, the growth behaviour of *Methylocystis* sp. Rockwell was assayed under a range of methane and methanol concentrations, paired with ammonium or nitrate (Tays et al., 2018), which confirmed its preference for ammonium as N source in contrast to other methanotrophs that preferred nitrate.

This preferred growth with ammonium is notable in that it can be co-metabolised to toxic hydroxylamine by methane monooxygenase due to the evolutionary relatedness of this enzyme to ammonia monooxygenase (He et al., 2017; Murrell & Smith, 2010). As an alcohol and a solvent, methanol often leads to reduced growth rate, yield, or both when used as a sole carbon/energy source for growing methanotrophs (Gilman et al., 2015; Whittenbury et al., 1970). Both methanol and ammonium, however, theoretically require less energy for their incorporation into cell biomass compared to methane and nitrate, respectively (Anthony, 1978; He et al., 2017; Nyerges & Stein, 2009; van Dijken & Harder, 1975). Elucidating gene regulation in *Methylocystis* sp. Rockwell growing under various conditions, can lay the foundation towards the development of strategies and processes enabling improved growth efficiencies and/or advantageous economics for bioproduction at industrial scales.

Most of the literature investigating methanotrophic bacteria rely on the standard growth medium established almost 50 years ago by Whittenbury et al. (1970). Alterations to this medium have proven beneficial both for straightforward growth assays as well as specialized applications

like inducing increased PHB accumulation (Hoefman et al., 2014; Sundstrom & Criddle, 2015). Two of the most important components to defining methanotroph growth media are the carbon and nitrogen sources, which show many crosslinks in regulatory effect (Commichau et al., 2006; Nyerges & Stein, 2009). Specifically for alpha-MOB, nitrogen species, namely ammonium and nitrate, have a long history of inducing significant effects in growth behaviour and physiology, which were highlighted in recent transcriptomic analyses (Dam et al., 2014; He et al., 2017). Carbon source, either methane or methanol, too is demonstrably critical to determining growth rates, yields, and other aspects of growth in this MOB type (Zaldívar Carrillo et al., 2018). What is less explored is the combined effects of both carbon and nitrogen sources on growth, and deconvoluting the complex web of regulation on the various C and N species.

Previous studies of alpha-MOB have provided valuable insight into the mechanisms of regulation in these bacteria. *Methylosinus trichosporium* OB3b was profiled for global gene expression when grown on methane-nitrate, providing insight into carbon flux regulation (Matsen et al., 2013), but this work did not comparatively analyze the results to global gene expression in other methanotrophs or to cultures grown in different carbon-nitrogen combinations. *M. trichosporium* OB3b was also previously investigated for transcriptional response to copper, and it was found that the pMMO operon, encoding particulate methane monooxygenase, was reciprocally regulated with the sMMO operon, encoding soluble methane monooxygenase, and the methanobactin operon, encoding a copper-chelating chalkophore; copper induced expression of the former and repression of the latter (Kenney et al., 2016).

Other RNASeq studies have examined the effects of ammonium vs. nitrate on expression, finding that the resultant transcriptomic profile is distinct in both the methane-oxidizing and nitrogen-related genes in *Methylocystis* sp. SC2 (Dam et al., 2014). The effects of different carbon source too have begun to be examined through transcriptomics, Vorobev et al. finding distinctly separate regulation as a result of *Methylocystis* sp. SB2 growing in either methane or ethanol

(2014). As mentioned above carbon and nitrogen sources combine to provide various regulatory effects, an aspect of regulation in methanotrophs that remains ill-defined.

In this work, the transcriptomic profiles of *Methylocystis* sp. Rockwell undergoing growth on different combinations of carbon and nitrogen sources will be examined. This regulatory-based analysis will be combined with analysis of growth, providing valuable information for the understanding and the implementation of this organism in both industrial and natural bioprocesses.

## 5.3. Methods

### 5.3.1. Growth and Maintenance

*Methylocystis* sp. Rockwell was selected due to its variation of growth characteristics on different combinations of carbon and nitrogen sources (Tays et al., 2018). Cultures were grown as previously reported (Tays et al., 2018), using either ammonium mineral salts (AMS) or nitrate mineral salts (NMS) medium (Whittenbury et al., 1970), containing either 10 mM ammonium chloride (AMS) or 10 mM potassium nitrate (NMS) as N source.

For growth experiments, Wheaton media bottles (250 mL) closed with butyl-rubber septa caps were filled with 100 mL medium. The copper ($CuSO_4$) concentration in the final medium was 5 µM for all media formulations. The media were buffered to pH 6.8 through addition of 1.5 mL phosphate buffer (26 g/L $KH_2PO_4$, 33 g/L $Na_2HPO_4$) and inoculated with 1 mL (1%) of previously grown cultures that had been passaged once in identical conditions to each of the experimental conditions; as such, initial biomass at inoculation ranged in $OD_{540nm}$ from 0.087 to 0.096, reflecting the growth result of the inoculum culture.

In methane-grown cultures, 2.5 mmol methane was provided via injection through a 0.22 µm filter-fitted syringe, following initial removal of gas headspace to ensure pressure at the beginning of incubations was approximately 1.05 atm. In methanol-grown cultures initial pressure was 1 atm and 1 mmol of pure HPLC-grade methanol was added. All cultures were incubated at

30°C with shaking at 150 rpm. Experiments were performed with replication (n=3) for all conditions.

### 5.3.2. Analysis of Growth

To monitor growth, 500-µL samples were extracted from cultures via sterile syringe at regular intervals over lag, exponential, and stationary phases. Three replicates were grown and assayed for each condition such that standard deviations could be calculated. Growth was assessed using optical density (OD) measurements at 540 nm in a 48-well microplate (Multiskan Spectrum, Thermo Scientific). Representative growth curves, demonstrating when RNA extraction was accomplished, are represented in Supp. Fig. 1. Culture purity was assured through phase contrast microscopy and plating of culture on TSA/nutrient agar plates, where lack of growth demonstrated lack of contamination.

### 5.3.3. RNA Extraction

Total RNA was extracted from all strains grown in either AMS or NMS with methanol (1 mmol) or methane (2.5 mmol) provided as carbon source at late log phase using the MasterPure RNA purification kit (Epicentre). Briefly, cells were inactivated with phenol-stop solution (5% phenol, 95% ethanol) and pelleted through centrifugation. Nucleic acids were purified according to manufacturer's instructions, excepting modification of Proteinase K addition to 1 mg total. In addition, samples grown on methanol were processed with organic solvent extraction in place of MPC precipitation as follows: extract sequentially with equal volume of phenol (acetate-buffered, pH 4.2), equal volume of 1:1 phenol:chloroform, and equal volume of 24:1 chloroform:isoamyl alcohol, before resuming MasterPure total nucleic acid precipitation protocol at the isopropanol addition step. RNA quantity and quality were assessed using a BioAnalyzer (Agilent Technologies).

### 5.3.4. RNA Sequencing and Assembly

RNA-Seq was performed by the Department of Energy Joint Genome Institute (DOE, JGI), using Illumina HiSeq-2000 technology. Raw reads, JGI transcriptomic analysis, and additional supporting information were made available through the JGI Genome Portal, under proposal ID 1114. Geneious 11.0.2 (https://www.geneious.com) was then used to map reads to *Methylocystis* sp. Rockwell reference genome (ASM18815v3, GCA_000188155.3) with Bowtie2, using default settings (high sensitivity) and local-use alignment method (Langmead & Salzberg, 2012; Langmead et al., 2018). Gene expression and differential expression were calculated using DESeq2 (Love et al., 2014).

In all tested conditions, 3 replicates were sequenced and mapped to the reference genome. After mapping, principal component analysis (PCA) via DESeq2 application in Geneious was used to determine suitability of replicates for further analysis, using the distance equation: $a^2 + b^2 = c^2$. Notably distant, and therefore divergent (un-grouping), replicates were determined to be outliers and removed from further analysis. Two replicates, one each in methane-ammonium and methanol-nitrate, were cut from further analysis at this stage, due to lack of coherence with the other replicates of those conditions (Supp. Fig. 2). Therefore, methane-nitrate and methanol-ammonium data sets were completed with n=3 replicates, while methane-ammonium and methanol-nitrate growth conditions were completed with n=2 conditions. In the literature, methane-nitrate is considered the standard growth condition for methanotrophic bacteria; therefore, this condition was selected as the reference for comparison with the other three conditions. Significance in differential expression was considered at an log-fold change of $> | 1 |$ and false-discovery rate (FDR) adjusted *p*-value of $< 0.01$, calculated by DESeq2 plugin in Geneious.

### 5.3.5. Differential Gene Expression (DGE) Classification and Analysis

Significant differential gene expression in each of the test conditions compared to expression in the reference condition were further analysed by gene orthology using the Kyoto

Encyclopedia of Genes and Genomes (KEGG) program, BLAST Koala (Kanehisa et al., 2016), and the European Molecular Biology Laboratory (EMBL) program, EggNOG (Huerta-Cepas et al., 2015). Genes unclassified by the EggNOG analysis, or classified only as Function Unknown, were further assessed via the National Centre for Biotechnology Information Basic Local Alignment Search Tool (NCBI-BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine if any uncategorized or hypothetical genes could be assigned preliminarily to a function or functional group.

## 5.4. Results

### 5.4.1. Gene Orthology of Highly Differentially Expressed Genes

The transcriptomes obtained from growth on each combination of carbon and nitrogen sources were compared to identify genes with significant differential gene expression (DGE) between conditions and obtain profiles of the differential global gene expression (Table 5.1).

**Table 5.1**. Number of differential gene expressions (DGE) in each tested comparison of growth conditions in *Methylocystis* sp. Rockwell. Significance was determined by log-fold change > 1, and adjusted p-value < 0.01. Count of total genes includes both genes showing increased and decreased transcription in the test condition, compared to the base condition.

| **Test Condition** | *Methane-Ammonium* | *Methanol-Nitrate-* | *Methanol-Ammonium* | *Methanol-Ammonium* | *Methanol-Ammonium* |
|---|---|---|---|---|---|
| **Base Condition** | *Methane-Nitrate* | *Methane-Nitrate* | *Methane-Nitrate* | *Methane-Ammonium* | *Methanol-Nitrate* |
| **Upregulated** | 226 | 52 | 649 | 371 | 198 |
| **Downregulated** | 65 | 9 | 496 | 409 | 190 |
| **Total DGE** | 291 | 61 | 1145 | 780 | 388 |

The DGE as a result of nitrogen source was directly related to the carbon source, i.e. methane-grown cultures in ammonium compared to nitrate showed 291 total DGE, while the same comparison of nitrogen source in methanol-grown cultures resulted in 388 total DGE. In the inverse case, comparing the two carbon sources in ammonium-grown cultures showed 780 total DGE, while

comparing the two carbon sources in nitrate-grown cultures led to a DGE of 61 genes. In comparing methane-nitrate to methanol-ammonium, the DGE was 1145 genes.

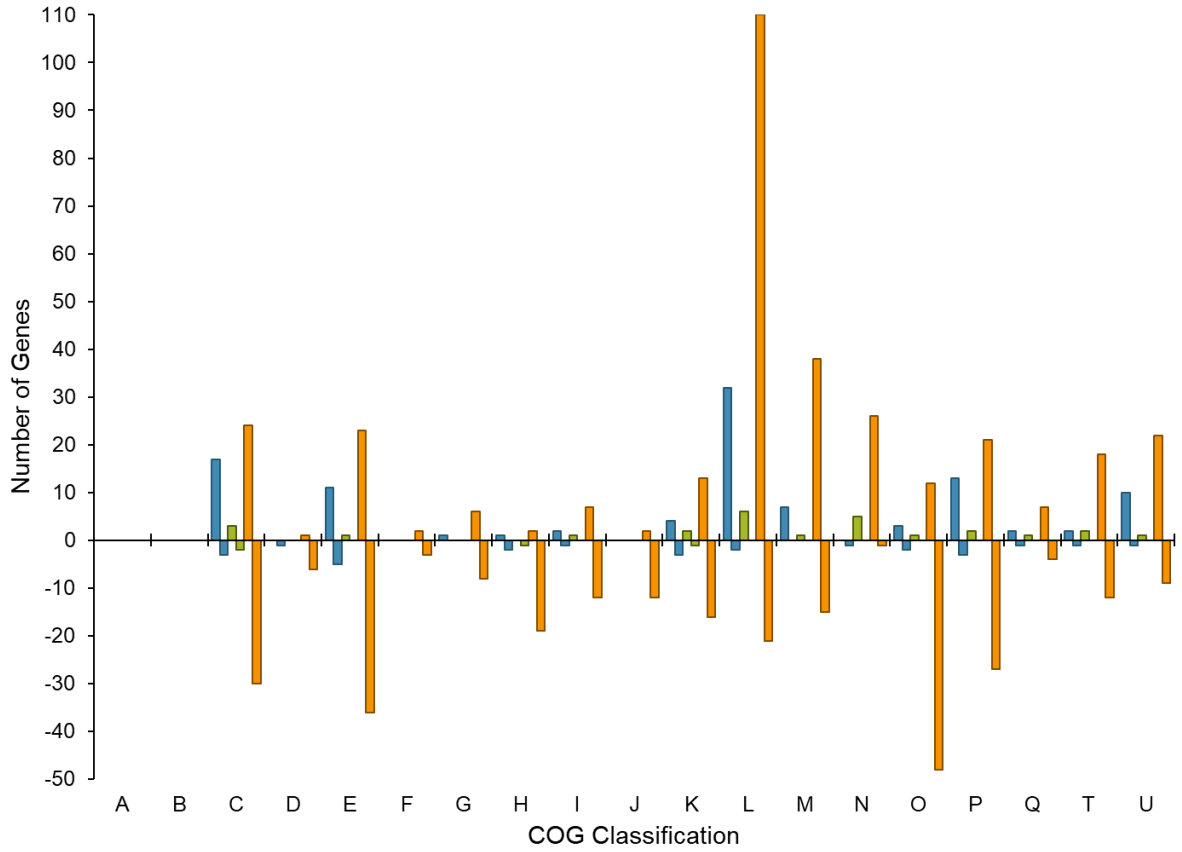


**Figure 5.1**. Classification of significant differential gene expression (DGE) in *Methylocystis* sp. strain Rockwell, based on categories of orthologous gene classification according to EggNOG database. Blue represents methane-ammonium vs. methane-nitrate, green represents methanol-nitrate vs. methane-nitrate, and orange represents methanol-ammonium vs. methane-nitrate. A = RNA processing and modification; B = Chromatin structure and dynamics; C = Energy production and conversion; D = Cell cycle control and mitosis; E = Amino acid metabolism and transport; F = Nucleotide metabolism and transport; G = Carbohydrate metabolism and transport; H = Coenzyme metabolism; I = Lipid metabolism; J = Translation; K = Transcription; L = Replication and repair; M = Cell wall/membrane/envelop biogenesis; N = Cell motility; O = Post-translational modification, protein turnover, chaperone functions; P = Inorganic ion transport and metabolism; Q = Secondary structure; T = Signal transduction; U = Intracellular trafficking and secretion.

Genes that displayed a significant DGE were then classified according to their orthology using both BLAST-Koala and EggNOG. As was demonstrated in a previous study (Tays et al., 2019, *Chapter 4*), few genes were classified using the BLAST-Koala tool – often fewer than a third of total

genes submitted – which rendered the results not informative (data not shown). The EggNOG platform, on the other hand, was able to reliably categorize 46-62% of the genes submitted, with genes falling in the uncategorized or function unknown group only including hypothetical genes. The results of this categorization of DGE are presented in Figure 5.1.

Across all transcriptome comparisons, replication and repair genes showed high amounts of DGE, with significant proportions being up- and down-regulated. Overall, however, there were more replication and repair genes with increased expression as a result of growth in methanol-ammonium, which is consistent with methanol being a toxic growth substrate for this bacterium (Tays et al., 2018). Energy production and conversion was also a highly differentially regulated category across growth conditions, as was inorganic ion transport, cell wall/membrane/envelope, and amino acid metabolism and transport.

Overall, comparison of methane-ammonium to methane-nitrate highlighted increased expression in genes associated with replication and repair, energy production and conversion, and inorganic ion transport and metabolism, which makes sense owing to the fact that growth of *Methylocystis sp.* Rockwell is less robust on methane-nitrate than methane-ammonium (Tays et al., 2018), and these gene categories are associated with stress responses. Amino acid metabolism and transport genes were the most prevalent genes with lower expression in ammonium as the bacteria had ample supply of readily assimilable nitrogen. In the case of methanol-ammonium vs. methanol-nitrate, genes related to cell wall/membrane/envelope displayed significantly higher expression due to membrane stress caused by solvent effects. Decreased expression levels were found in a high number of genes categorised as: energy production and conversion; lipid metabolism; cell wall/membrane/envelope; and inorganic ion transport and growth was considerable weaker in methanol versus methane as carbon source (Figure 5.1).

Comparison of methanol-nitrate to methane-nitrate resulted in only 30 genes with DGE categorized. Of note, a number of cell motility genes were up-regulated in methanol. In the case of

methanol-ammonium vs. methane-ammonium, a high number of genes with increased expression were categorized within replication and repair, and cell wall/membrane/envelope; all indications of solvent stress due to methanol as carbon source. A high number of genes in energy production and conversion, inorganic ion transport, amino acid metabolism and transport, post-translational modification, and amino acid metabolism showed lower expression levels in this comparison, which matches the poor outcome of growth on methanol as carbon source when compared to methane (Tays et al., 2018).

Finally, comparison of methanol-ammonium to methane-nitrate highlights a large number of genes with significantly lower expression in the class of post-translational modification, protein turnover, and chaperone functions, as well as amino acid metabolism and transport, energy production and conversion, and inorganic ion transport. Higher expression was seen in many genes with replication and repair functions, as well as cell wall/membrane/envelop functions. This comparison is between the two growth conditions that were not optimal for *Methylocystis* sp. Rockwell but indicates that both the preferred carbon (methane) and nitrogen (ammonium) sources for this bacterium are required for expression of vital pathways involved in biosynthesis and energy conversion.

Following classification of gene orthology of DGE, the most highly expressed genes were assessed for each comparison between growth conditions (Supp. Table C-1). Nitrogen uptake, nitrogen regulation, and nitrogen assimilation genes were prominently expressed when ammonium was the N source regardless of the carbon source. A number of transposases, as well as genes for flagellar biosynthesis and chemotaxis, were significantly more expressed in methanol-nitrate growth compared to methane-nitrate. A hemerythrin gene was the most significant positive DGE in the comparison of methanol-ammonium vs. methane-ammonium, while general categories of transcriptional regulators were not as highly expressed.

### 5.4.2. Carbon and Nitrogen Assimilation Pathways

To determine if various carbon-nitrogen combinations had effects on gene expression related to methane oxidation or formaldehyde assimilation, in this case the serine cycle, DGE in these pathways was compared across all growth conditions (Figure 5.2). Growth in methane-ammonium compared to methane-nitrate showed decreased expression of both methane oxidation and the serine cycle pathways, but to a low degree, not reaching significance cut-offs (Figure 5.2a). Interestingly, in both N sources, growth in methanol was associated with increased transcription of the methane monooxygenase genes compared to methane (Figure 5.2c, Supp. Figure C-4a). Comparison of the methanol-grown conditions, in ammonium compared to in nitrate, demonstrated more significant DGE compared to the same N source comparison of growth in methane. For instance, growth in methanol-ammonium compared to methanol-nitrate, resulted in significantly increased transcription of genes encoding methanol dehydrogenase genes (*mxf*), as well as some increased transcription of genes encoding particulate methane monooxygenase and formaldehyde activating enzyme (FAE) (Supp. Figure C-4b).

Compared to the reference methane-nitrate, growth on methanol with either nitrogen source resulted in increased expression of genes encoding the methanol dehydrogenases, MxaF-type, but decreased the expression of XoxF (Figure 5.2b an 5.2c) In general, growth on methane-nitrate showed the highest expression of genes encoding formate dehydrogenase and genes encoding many enzymes of the serine cycle. *glyA* was a notable exception, with decreased expression in methane-nitrate relative to the other three carbon-nitrogen combinations. Together, these results were surprising, as robust expression of genes for methane oxidation and formaldehyde assimilation pathways were expected in the methane-ammonium growth condition since this is the favoured combination for *Methylocystis sp*. Rockwell.

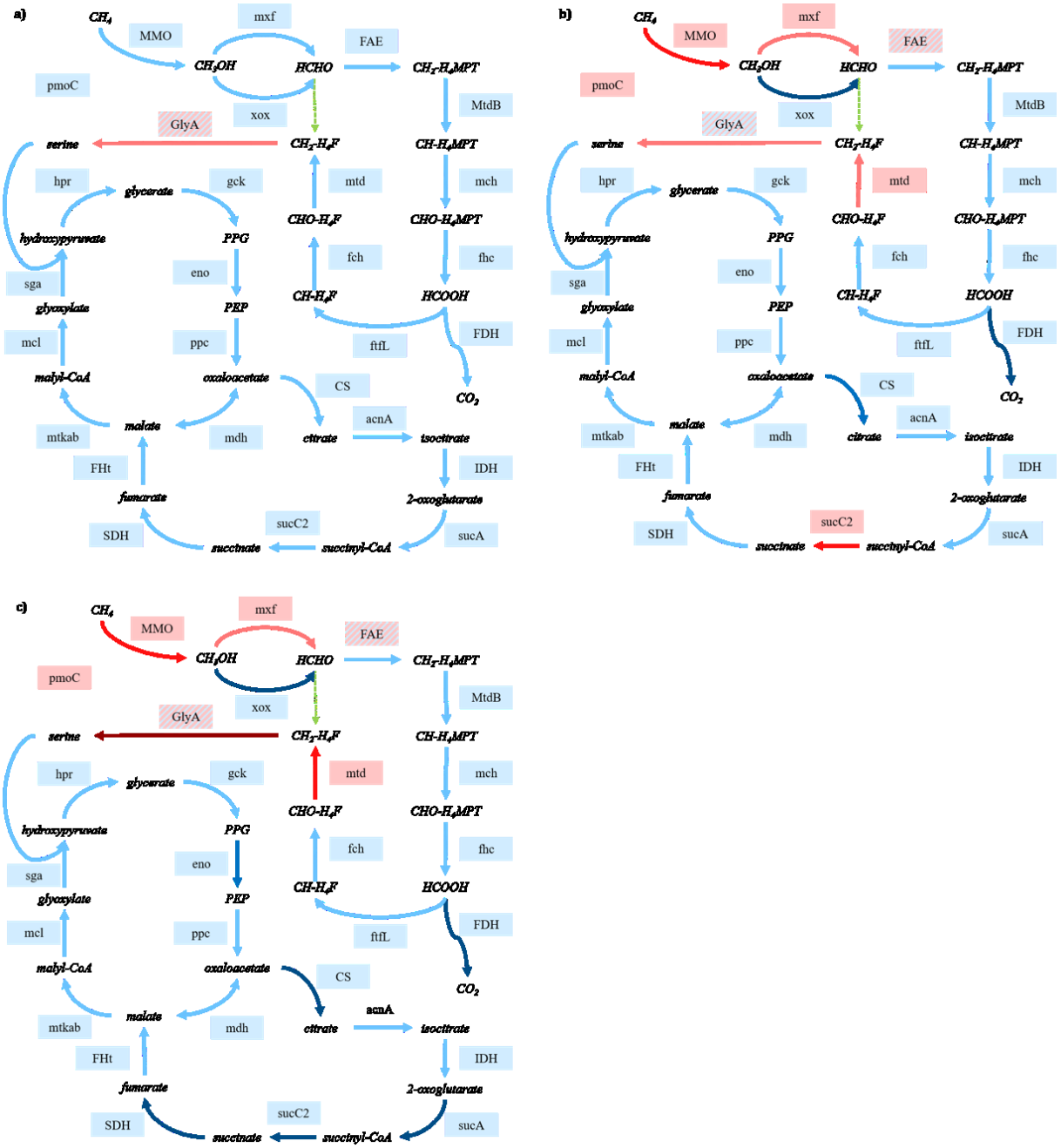


**Figure 5.2**. Differential regulation of methane oxidation and carbon assimilation *via* the serine cycle in *Methylocystis* sp. Rockwell. Methane-ammonium vs. methane-nitrate (a), methanol-nitrate vs. methane-nitrate (b), methanol-ammonium vs. methane-nitrate (c), methanol-ammonium vs. methane-ammonium (d), and methanol-ammonium vs. methanol-nitrate (e). Genes shown in red are comparatively upregulated in the experimental conditions, blue denotes downregulation. Dark arrows represent significant differential regulation (log-fold change > 1, adj. p-value < 0.01), medium-coloured represent differential regulation under adj-p-value < 0.05. Where striped, multiple genes showed both up- and down-regulation in test condition and the overall expression difference was used to determine arrow colour.

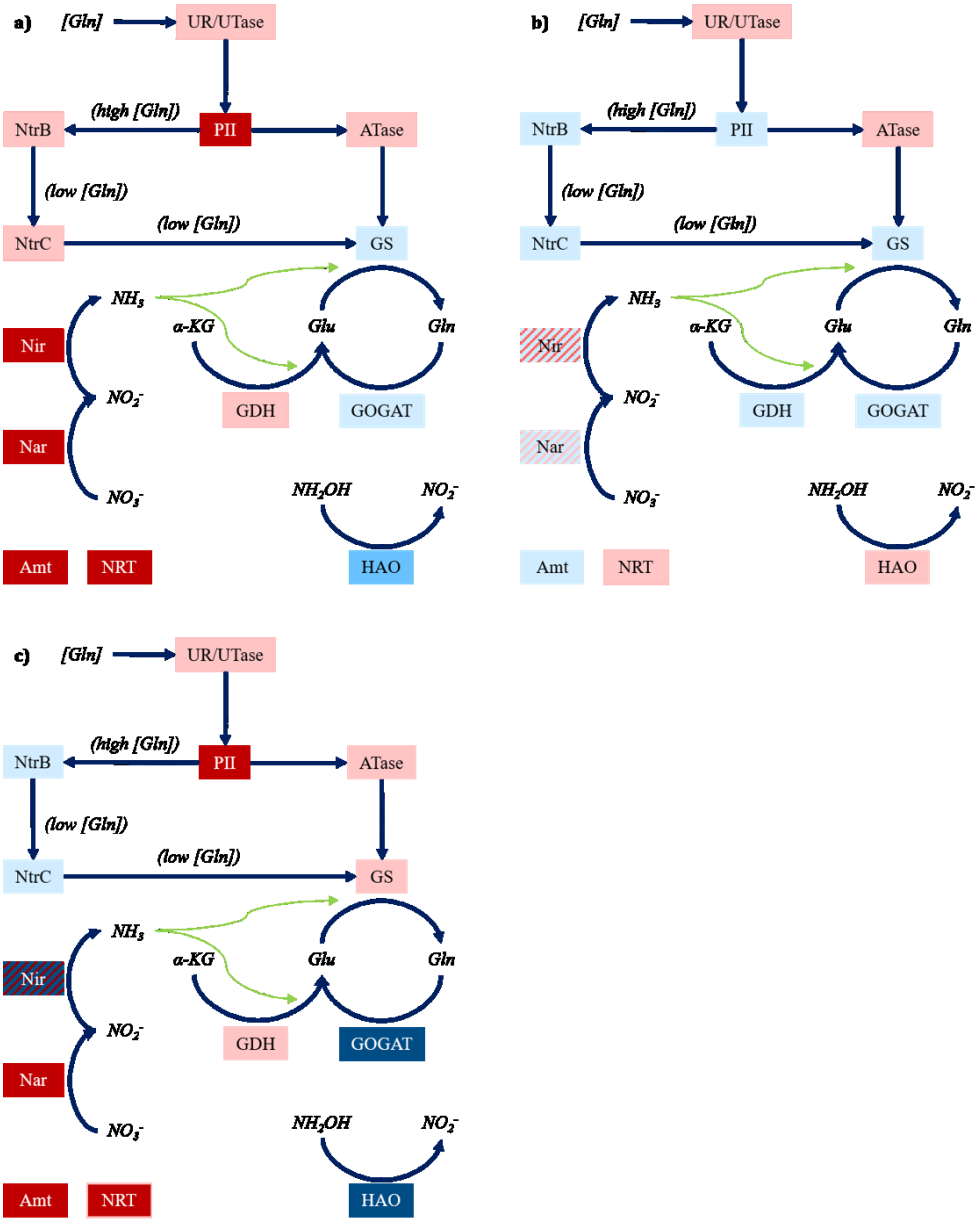


**Figure 5.3**. Differential regulation of nitrogen uptake and assimilation in *Methylocystis* sp. Rockwell. Methane-ammonium vs. methane-nitrate (a), methanol-nitrate vs. methane-nitrate (b), methanol-ammonium vs. methane-nitrate (c). Genes shown in red are comparatively upregulated in the experimental conditions, blue denotes downregulation. Dark arrows represent significant differential regulation (log-fold change > 1, adj. p-value < 0.01), medium-coloured represent differential regulation under adj-p-value < 0.05. Where striped, multiple genes showed both up- and down-regulation in test condition and the overall expression difference was used to determine arrow colour.

Expression of genes involved in nitrogen uptake and assimilation pathways were also analysed for DGE between growth conditions. First, in comparing methane-ammonium to methane-

nitrate, a pattern of increased expression was noted (Figure 5.3a) including genes for ammonium transport (*amt*), nitrate reductase (*nar*), and nitrogen regulatory protein PII. In the case of methanol-nitrate compared to methane-nitrate, little significant DGE can be noted (Figure 5.3b).

Finally, the methanol-ammonium to methane-nitrate comparison showed the highest level of DGE across all the comparisons (Figure 5.3c). This includes higher expression of genes encoding PII, Nar, and Amt as well as ATase. Various genes for assimilatory nitrite reductase (Nir) were not consistently up- or down-regulated. More expression of glutamine synthetase (GS) was observed, while lower expression of glutamate synthase/glutamine oxoglutarate aminotransferase (GOGAT) corresponded. Finally, in growth conditions with ammonium as N source (Figure 5.3a and 5.3c), genes encoding hydroxylamine dehydrogenase (HAO) were less highly expressed than in the nitrate-grown cells, which is the opposite case observed in gamma-MOB (Campbell et al., 2011).

**Table 5.2**. Differential regulation of polyhydroxybutyrate (PHB) biosynthesis cycle in *Methylocystis* sp. Rockwell. All comparisons to standard condition of methane-nitrate: methane-ammonium, methanol-nitrate, and methanol-ammonium. Genes shown in red are comparatively upregulated in the experimental conditions, blue denotes downregulation. Bold values represent significant differential regulation (log-fold change > 1, adj. p-value < 0.01), italicized are significant under adj. p-value < 0.05.

| | | Test Condition | *Ammonium-Methane* | *Nitrate-Methanol* | *Ammonium-Methanol* |
|---|---|---|---|---|---|
| Locus | Gene | Reference Condition | *Nitrate-Methane* | *Nitrate-Methane* | *Nitrate-Methane* |
| MET49242_RS21865 | phbA | acetyl-CoA C-acetyltransferase | -0.10 | -0.58 | **-2.42** |
| MET49242_RS20855 | phbB | 3-hydroxyacyl-CoA dehydrogenase | -0.35 | -0.12 | *-1.40* |
| MET49242_RS21780 | phbC | poly-beta-hydroxybutyrate polymerase | 0.52 | 1.65 | -0.51 |
| MET49242_RS13165 | phaZ | poly-hydroxyalkanoate depolymerase | -0.34 | -1.84 | **-3.14** |
| MET49242_RS16490 | bdhA | 3-hydroxybutyryl-CoA dehydrogenase | -0.30 | -1.41 | **-2.38** |
| MET49242_RS16295 | acsA2 | CoA-transferase | -0.92 | -0.14 | -0.36 |
| MET49242_RS21870 | phaR | poly-hydroxyalkanoate synthesis repressor PhaR | -0.74 | -0.33 | 0.00 |

As methanotrophs have long been noted for their biotechnological potential, and the alphaproteobacterial group has been scrutinized for their potential in biosynthesis of polyhydroxybutyrate (PHB), effects of carbon-nitrogen combinations on expression of genes in this pathway was examined. Only growth on methanol-ammonium resulted in significant decreases in expression of PHB biosynthetic genes, suggesting that this is the least favourable carbon-nitrogen combination for generating this polymer during growth (Table 5.2).

## 5.5. Discussion

### 5.5.1. Overall Image of Differential Gene Expression

Carbon and nitrogen are the two most important elements for cellular growth and metabolism, and their availability and form can have dramatic effects on cellular function (Dam et al., 2014; Fu et al., 2019; Kalyuzhnaya et al., 2009; Kits, Campbell, et al., 2015; Kits, Klotz, et al., 2015; Nguyen et al., 2019; Vorobev et al., 2014). Previous studies point to optimal growth of *Methylocystis* sp. Rockwell in the carbon-nitrogen combination of methane-ammonium over methanol or nitrate (Nyerges et al., 2010; Nyerges & Stein, 2009; Tays et al., 2018). This study addressed how differences in growth based on carbon-nitrogen combination affect transcriptional regulation, *i.e.* which pathways and genes are affected, resulting in differential growth behaviours. Patterns of gene expression can serve as helpful insight into cell regulation, highlighting key or yet unestablished processes (Matsen et al., 2013). In fact, striking differences in gene expression profiles were noted in *Methylocystis sp.* Rockwell as a result of both carbon and nitrogen sources.

Methane-nitrate was selected to serve as a reference condition based on the fact it is often regarded as a standard for the cultivation of methanotrophs – primarily in order to avoid potential inhibitory effects from ammonium co-metabolism (He et al., 2017). While this preference has proven untrue in the case of *Methylocystis* sp. Rockwell (Tays et al., 2018), the prevalence of its use and ease of comparison to other studies (Tays et al., 2019, Chapter 4) makes it a useful reference for comparisons, despite not being the most favourable combination for growth. Transcription

profiles in ammonium-methane, methanol-nitrate, and methanol-ammonium were thus compared to this case. Overall, ammonium-methanol lead to an increase in transcription, with 226 genes significantly upregulated, and only 65 genes downregulated (Table 5.1). This may be linked to the faster and more abundant growth observed in ammonium (Supp. Figure C-1), leading to more activity in the cells, or perhaps connected to overcoming the effects of ammonium co-metabolism.

Comparing ammonium to nitrate as N source, energy production and conversion genes showed increased expression with either methane or methanol as carbon source (Figure 5.1, Supp. Figure C-3). Expression of genes related to amino acid metabolism and transport, and inorganic ion transport and metabolism genes also increased with ammonium as N source, regardless of carbon source. With methane as carbon source, ammonium also resulted in high expression for numerous genes related to intracellular trafficking and secretion. All these findings could support the theory of more favourable, active metabolism in the methane-ammonium condition versus all other carbon-nitrogen combinations.

When the comparison moves to carbon source, however, very little differences in transcription were found when nitrate was the N source with only 52 genes upregulated and 9 genes downregulated when comparing growth on methane or methanol. This finding is notable as the growth of *Methylocystis* sp. Rockwell in methanol-nitrate was the weakest of the four conditions tested (Supp. Figure C-1) (Tays et al., 2018). In this case, less DGE may point to an inability of this strain to adjust to methanol and nitrate together, thus nearly preventing growth. A matching study of *Methylomicrobium album* BG8 shows, by comparison, a large level of DGE between growth in methane-nitrate and methanol-nitrate that is supported by optimal growth on methanol-nitrate compared to the other carbon-nitrogen combinations (Tays et al., 2019, *Chapter 4*). Most notable in the comparison between growth on methane-nitrate and methanol-nitrate is the activation of several cell motility genes, including regulator-associated genes *flaF* and *flbT*, flagellum component genes *flg*B and *flg*K, and flagellar biosynthesis gene *flh*A. It has been previously observed that

methanotroph motility is influenced by growth condition, with transcriptional response matching a preference for different modes of motility in different media: flagella in methylamine-supplemented media or pili associated with twitching motility and adherence without nutrient supplementation (Kalyuzhnaya et al., 2009). Further, in non-methanotrophic *Escherichia coli*, motility has been linked to survival in nutrient-poor conditions, as synthesis of this machinery is energy-intensive, such that less-desirable carbon sources are linked to increased expression of motility genes and behaviour (Zhao et al., 2007). This result therefore points to methanol-nitrate being a stress-inducing growth condition for *Methylocystis sp.* Rockwell, which again correlates well with the poor growth observed in batch culture (Supp. Figure C-1).

Finally, the third comparison between methanol-ammonium and methane-nitrate, led to the highest number of DGE, with 649 genes significantly upregulated and 496 downregulated. This result is particularly interesting given the relatively equivalent growth outcome of these two conditions (Supp. Figure C-1) (Tays et al., 2018). Currently, the fact such a high number of DGE is observed between these conditions (much higher than the previous two comparisons) cannot be explained further but may point to significant interactions of carbon- and nitrogen-induced regulatory behaviour.

The toxic solvent effects of methanol were most pronounced with increased expression of cell wall/membrane/envelop genes (24) when *Methylocystis sp.* Rockwell was grown in methanol-ammonium versus methane-ammonium. As methanol is a solvent, and toxic to cells, even for methanotrophs that tightly control its intracellular concentration as a critical metabolite (Best & Higgins, 1981; Hanson & Hanson, 1996), it is sensible that these genes be upregulated. This observation also brings to light the absence of this response in the methanol-nitrate condition, supporting the theory of non-acclimatization leading to negligible growth (Tays et al., 2018). While it is difficult to comment on the methanol-nitrate case, given its stunted growth (Tays et al., 2018), genes from the same COG category of cell wall/membrane/envelop, are also differentially

expressed in the comparison of methanol-grown cultures (Supp. Table C-1). When growing in ammonium, a significant proportion of cell wall, membrane, and envelope genes were either upregulated (27 genes) or downregulated (14 genes), paired with a cadre of lipid metabolism genes that were also down-regulated (15 genes). As methanol-ammonium resulted in much stronger growth than methanol-nitrate (Tays et al., 2018), this significant DGE may point again to membrane adaptation for survival in methanol

### 5.5.2. Regulation of Carbon, Nitrogen, and PHB Pathways

Analysis of carbon and nitrogen effects on the bacterium's regulation would not be complete with paying extra attention to the genes that effect uptake, regulation, and assimilation of these vital nutrients. Nitrate is reduced and assimilated differently than ammonium, with separate pathways and enzymes involved (Stein & Klotz, 2011). Energetics of redox balance become important when considering the value of methanol or methane as a carbon source, though the former is the first step of the oxidation of the latter (Fu et al., 2019).

In examining the analysis of methane oxidation and formaldehyde assimilation genes, methane-grown cells showed little by way of significant DGE between the two nitrogen sources (Figure 5.2a). Similarly, little significant change in DGE was observed between the carbon sources, *i.e.* methanol vs. methane, in nitrate-grown cells, though this showed a somewhat stronger effect than the methane-fed ammonium-nitrate comparison (Figure 5.2b). Interactive effects can be noted however, between the carbon and nitrogen effects; unlike with methane, methanol lead to more DGE between ammonium-grown and nitrate-grown cells (Table 5.1). As well, ammonium-grown cells showed more differential regulation between methanol and methane (Supp. Table C-1) than did the nitrate-grown cultures. Vorobev et al. (2014) have reported relatively low levels of DGE in the alpha-MOB *Methylocystis* sp. SB2 growing on methane or ethanol (rather than methanol), particularly in nitrate. Like in this report, some significant DGE was noted in the methane oxidation

and formaldehyde assimilation pathways, though scales of expression were modest with decreased expression of the methane oxidation and serine pathways.

In considering nitrogen sources, separate uptake and, in the case of nitrate, reduction processes must take place to incorporate either N-species (Stein & Klotz, 2011). Compared to the reference of methane-nitrate, genes related to nitrogen regulation and assimilation were among the most strongly differentially regulated genes in cells grown in the methane-ammonium and methanol-ammonium conditions. This included strong ammonium-induced expression of assimilatory nitrite and nitrate reductases, ammonium transporters, and nitrogen fixation proteins (Figure 5.3, Supp. Table C-1). This is to be expected, given the extensive nitrogen pathways methanotrophs possess, as well as the fine control they exhibit over different forms of nitrogen species (Campbell et al., 2011; Stein & Klotz, 2011). Interestingly, in this study hydroxylamine oxidoreductase (HaoAB) was not found to be upregulated in the presence of ammonium as previously noted (Campbell et al., 2011), but rather downregulated. This finding may imply that there are other triggers or controls to hydroxylamine oxidation that have yet to be defined, that the timing of its regulation is either earlier in the cell cycle, or that the function of Hao can be post-translationally controlled.

Other work examining regulation of nitrogen in methanotrophs has been pursued. A recent study of a related strain, *Methylocystis* strain SC2, found that ammonium similarly affected regulation of cell transcription as was found here, though that study did not examine the combined effects of methanol (Dam et al., 2014). Specifically, the authors noted that even high (30 mM) concentrations of ammonium affected expression of relatively few genes, compared to growth in nitrate, and that ammonium vs. nitrate did not significantly affect expression of particulate methane monooxygenase, methanol dehydrogenase, or formate dehydrogenase.

As industrial biotechnology becomes more prevalent, and methane emissions a more pressing concern, methanotrophs are gaining attention as bioconversion vectors for methane. This

is most apparent in the development of process strategies to promote accumulation of a PHB, which can be used as biodegradable bioplastic (Pieja et al., 2017). Through genome-scale modelling, alpha-MOB in the genus *Methylocystis* have been identified as promising candidates for industrial production strains, capable of high carbon flux towards acetoacetate, a precursor to PHB (Bordel, Rodríguez et al., 2019). This study found only one differential effect of carbon-nitrogen source on transcription of PHB pathway genes; methanol-ammonium growth led to a decrease in pathway expression, which points to use of methane as a future potential feedstock in industrial bioproduction rather than methanol (Table 5.2). This result is favourable for process optimization, as it aligns with optimal growth conditions (Tays et al., 2018), though PHB measurement and determination should be completed to support these findings.

## 5.6. Conclusions

Despite certain stark differences in growth of *Methylocystis sp.* Rockwell under the four tested carbon-nitrogen combinations, transcriptomic analysis is not straightforward – complicated by the many regulatory interactions between carbon and nitrogen in cells (Commichau et al., 2006). The two growth conditions with the most similar growth outcome, methane-nitrate and methanol-ammonium, were in fact the most distinct in transcriptome profiles. On the other hand, conditions with largely different growth profiles, methane-nitrate and methanol-nitrate, were very similar in levels of specific gene expression. It is clear however, that regulation, response, and adjustment to growth medium is a complex topic, and transcriptomics provides only a part of the picture. Certainly, it has been noted that transcript pool and metabolite pool do not always coincide in the most obvious way (Fu et al., 2019; Vogel & Marcotte, 2012).

Regardless, there are valuable lessons to be gleaned from global transcriptional analysis and evaluation. This method provides insight into regulation, which can help inform process development by bringing to light responses and concerns as varied as stress, bioproduction of valuable intermediates, and pure physiological understanding of an organism. This study

demonstrates that membrane regulation may be key to adapting certain methanotroph strains to methanol stress, and that both carbon and nitrogen sources should be carefully weighed by determining appropriate growth strategies. Studies such as these are useful in producing metabolic maps, which help inform bioindustrial processes and contribute hugely to optimized growth strategies (Bordel et al., 2019). While more work should be completed to relate these results to metabolite or protein pools, this study lays the groundwork for future studies to follow, examining yet more media refinements or combinations. This global transcriptome profiling helps generate a heightened understanding of the organism and demonstrates how drastically and sometimes unexpectedly growth mediums can impact cellular function.

## 5.7. Acknowledgements

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# 6. Summary, Conclusion, and Future Directions

## 6.1. Summary and Conclusions

The study of methanotrophy is a complex field, even within the relative constraints of examination only of its aerobic, proteobacterial members. The breadth of diversity inherent in its diverse taxonomic members is well reflected in the associated physiology and characteristics of each class, let alone each individual strain. The works presented in this thesis show that methanotrophy should be recognized for this extensive range of possibilities and that many interesting physiologies likely remain yet uncharacterized. This is especially true in the fields of carbon and nitrogen metabolisms, which even now are areas with many questions yet remaining unanswered. The response of the five strains examined in this thesis to different carbon-nitrogen growth conditions begins to address some of these gaps, and adds to the pool of critical, elementary knowledge that is required to understand, predict, and optimize the growth of these biotechnologically-relevant organisms.

The focus of this thesis concerned the effects of four different carbon and nitrogen culturing conditions on the growth and regulatory response in five biologically and industrially interesting MOB strains. Two carbon sources, methane and methanol, and two nitrogen sources, ammonium and nitrate, were assessed throughout. Importantly, experiments were designed to allow for comparison both within and between strains, in essence a survey of potential that so far remains rare in the field. Of the five strains chosen, two are representative, model strains of the two main categories of proteobacterial MOB: an alpha-MOB, *Methylosinus trichosporium* OB3b, and a gamma-MOB, *Methylomicrobium album* BG8. Of the three remaining strains, all represented industrially promising, and physiologically interesting models: an alpha-MOB with unusually good growth in ammonium, *Methylocystis* sp. Rockwell, another alpha-MOB industrially-employed for its strong

PHB biosynthesis, *Methylocystis* sp. WRRC1, and a gamma-MOB with preference for growth in nitrate and showing denitrification capacity, *Methylomonas denitrificans* FJG1.

Arguably the most vital aspect to truly understanding a microbe is in determining its general physiology, and specifically how environmental or culture conditions affect its behaviour and growth. As carbon and nitrogen are two of the most vital nutrients in a cell, this is especially true for them, and even moreso in MOB, which have demonstrated extremely variable response to different conditions. In Chapter 3, this was approached by examination of all five test strains grown in combinations of methane or methanol with either ammonium or nitrate. Further, various levels of carbon source were used, from very limiting amounts to excess (based on oxygen availability). From these experiments, optimal growth conditions were identified, as selected by growth rate and yield per mol carbon supplied. Interestingly, methane-ammonium was found to best support growth of a number of strains, including *Methylocystis* sp. Rockwell, against commonly accepted knowledge that ammonium is too much a detriment to growth compared to nitrate. Equally of note, one strain, *Methylomicrobium album* BG8, grew best in methanol, quite unlike any other strain assayed.

Based on these results, further examination of two strains – *M. album* BG8 and *Methylocystis* sp. Rockwell, followed, aimed at further determining how these carbon-nitrogen conditions affected growth beyond macro-level culture characteristics. This was primarily accomplished by fatty acid methyl esters (FAME) analysis, to identify lipid effects, and analysis of small organic acids in the medium, to identify which metabolites might be produced in excess or excreted. For the former, total FAME was significantly affected by growth condition, including a reduction in total FAME in methanol. Overall, the major lipid components in each species was constant in type but varied in total proportion, according to both C source, N source, and combined C-N conditions. For metabolite analysis, interestingly, formate was found to be excreted by *Methylocystis* sp. Rockwell in all conditions except the favoured condition of methane-ammonium, and was only excreted by *M.*

*album* BG8 growing on nitrate-methanol, implying that excess production of this metabolite might be a signal of challenging conditions or perhaps redox imbalance in the cell.

It is clear that these growth conditions did significantly influence MOB culture outcomes, and certainly affected certain specific characteristics and processes within the cells. With these phenotypes noted, it became clear that further insight into cellular regulation would be needed to expand the view into how MOB respond to growth conditions. This was addressed in Chapters 4 and 5, sister studies into the global gene regulation of two MOB strains with particularly unique physiologies, *M. album* BG8 (in Chapter 4) and *Methylocystis* sp. Rockwell (in Chapter 5). These chapters once again tested both methane and methanol with either ammonium or nitrate, and examined how these various growth outcomes are supported by differential transcriptional regulation. Specifically, the overall pattern of differential gene expression (DGE) was examined, as well as further evaluation of the modulation of expression of the genes and pathways involved with methane oxidation, carbon fixation, and nitrogen metabolism.

In Chapter 4, the gene regulation of *M. album* BG8 was investigated, and uncovered some surprising results. While relatively good growth was observed for all growth conditions selected for analysis, with little effect of N source in particular, the amount of DGE varied immensely in the comparisons. Methane-ammonium vs. methane-nitrate, a change of N source, resulted in very few DGE. In contrast, methanol-nitrate vs. methane-nitrate, a change of C source, was associated with a very high proportion of DGE. This is unlike growth outcomes (*i.e.* some noticeable preference for methanol-growth, little effect of N source), implying that methanol in nitrate medium requires vastly different cellular regulation than methane in order for good growth to be maintained. Interestingly, this methanol effect was not seen when the nitrogen source was also changed in the comparison of methanol-ammonium vs. methane-nitrate growth. In fact, this comparison resulted in very low DGE, despite the change in both nutrient sources, which would likely be hypothesized to lead to even more differential regulation than simply changing one. This supports the hypothesis

that both nitrogen and carbon should be considered together when determining optimal conditions and C source or N source effects.

Likewise in Chapter 5, gene regulation in *Methylocystis* sp. Rockwell was examined for C and N effects. Perhaps even more so than in Chapter 4, comparison of all three other C-N combinations to a reference condition of methane-nitrate, it became clear that a strict dichotomy of methane vs. methanol cannot be considered definitive without also considering the dichotomy of ammonium vs. nitrate. Methane-ammonium vs. methane-nitrate, a change of N source, showed a high amount of DGE, and methanol-nitrate vs. methane-nitrate, a change of C source, lead to only a few scarce DGE. Methanol-ammonium vs. methane-nitrate, a change in both C and N sources, showed a very high proportion of DGE, much greater than the sum of the other two comparisons. Notably, this is unlike the result in *M. album* BG8 in the preceding chapter. However, this chapter also discussed the DGE of methanol-ammonium compared to either methanol-nitrate or methane-ammonium, providing another angle into examining C and N source effects. The results of these comparisons are quite unlike the previously described C source and N source comparisons that used methane-nitrate as a reference. Methanol-ammonium vs. methanol-nitrate showed very distinct patterns of expression compared to methane-ammonium vs. methane-nitrate, and methanol-ammonium vs. methane-ammonium had more than ten times the DGE observed in methanol-nitrate vs. methane nitrate.

In both cases, nitrogen metabolism genes were strongly affected by nitrogen source, as might be expected. Interestingly, carbon source also appeared to have a noticeable effect, demonstrating once again the tight co-regulation of these two major nutrient sources and their assimilation pathways. Methane oxidation was also differentially regulated, at least in part, in a majority of the comparisons tested and in both strains. Carbon fixation, *i.e.* the serine and RuMP cycles tended towards lower levels of DGE in the transcriptomics comparisons, implying that other mechanisms may play larger roles in acclimatizing to different growth conditions, and modulating response to nutrient source. Finally, it should be noted that both Chapter 4 and Chapter 5 found

that a major category of orthologous genes affected by these different growth conditions was cell membrane, cell wall, and envelope genes. This included hopanoids, squalenes, and other membrane components affecting both rigidity and transport. Certainly, lipids are known to be critical to methanotroph function, therefore it follows that their biosynthesis would be highly regulated by conditions effecting growth. As well, given the strain of methanol on cell membranes, this likely implicates membrane biosynthesis and control of rigidity to be a major acclimatization and survival mechanisms for MOB growing in various environments, natural or industrial.

## 6.2. Future Directions

This thesis aimed at examining MOB growth through a bioindustrial light by focusing on growth and cellular regulation as a fundamental aspect of process development. Much of the work herein described assessed effects of C source and N source on the physiology of certain industrially promising strains, evaluating the relative benefits and drawbacks inherent in the use of two relevant carbon sources and two popular nitrogen sources. This knowledge enables optimization efforts and process refinement to emerge and flourish, allowing for increased efficiencies, higher yields, and more stable processes, while also reducing risk of failure, including culture collapse or unintended pathway activity.

More work remains to be accomplished to lead to a well-reasoned, optimized bioprocess. This thesis focused mainly on optimization of growth, which is key to producing sufficient biomass in which the bioconversion of methane to bioproducts of interest can occur. Equally important, however, is the optimization of the bioproduction itself. The conditions that allow for the most efficient growth may not be the same as conditions inducing more bioproduction. For example, industrial PHB biosynthesis in MOB typically involves production of biomass, *i.e.* growth of the methanotrophs, in one step and PHB synthesis in a second step. This could be converted to physical stages with specific conditions that support growth, like *Methylocystis* sp. Rockwell in methane-

ammonium, in the first one, followed by a second stage involving the harvest of that biomass and the implementation of conditions favourable to regulation towards PHB production.

Beyond that, there are many more aspects of medium and growth condition optimization that can be considered. While C and N are perhaps most critical, copper is well known to dramatically affect MOB growth, and oxygen concentration as well. Other work investigating lanthanides and rare earth elements has brought these into focus as major determiners of growth and regulation. As well, the general method of culturing should be assessed, with batch culture, as explored in this thesis, likely leading to very distinct outcomes compared to continuous culture, fed-batch culture, or more specialized techniques like self-cycling fermentation (SCF). These could all be preferentially used depending on the product target, the feedstock conditions, and perhaps the strain being employed as bioconversion vector.

On the side of regulation, this work primarily investigated two strains of interest, but three more strains were originally assessed in the physiological survey. The remaining strains could be subjected to transcriptomic analysis as well, to broaden the knowledge available about MOB regulation and response to nutrient conditions, as well as potentially support or contrast with the current findings. As *M. album* BG8 and *Methylocystis* sp. Rockwell demonstrated such distinct profiles of cellular regulation, it would be interesting to see if more alpha-MOB and gamma-MOB follow similar patterns or if the unique phenotypes of the two studied strains are also supported by unique transcript profiles.

The optimization and regulation work in this thesis lay the foundation to better understanding many remaining curiosities about the aerobic proteobacterial methanotrophs, and it is sure that there are many informative studies to follow, in many avenues of research.

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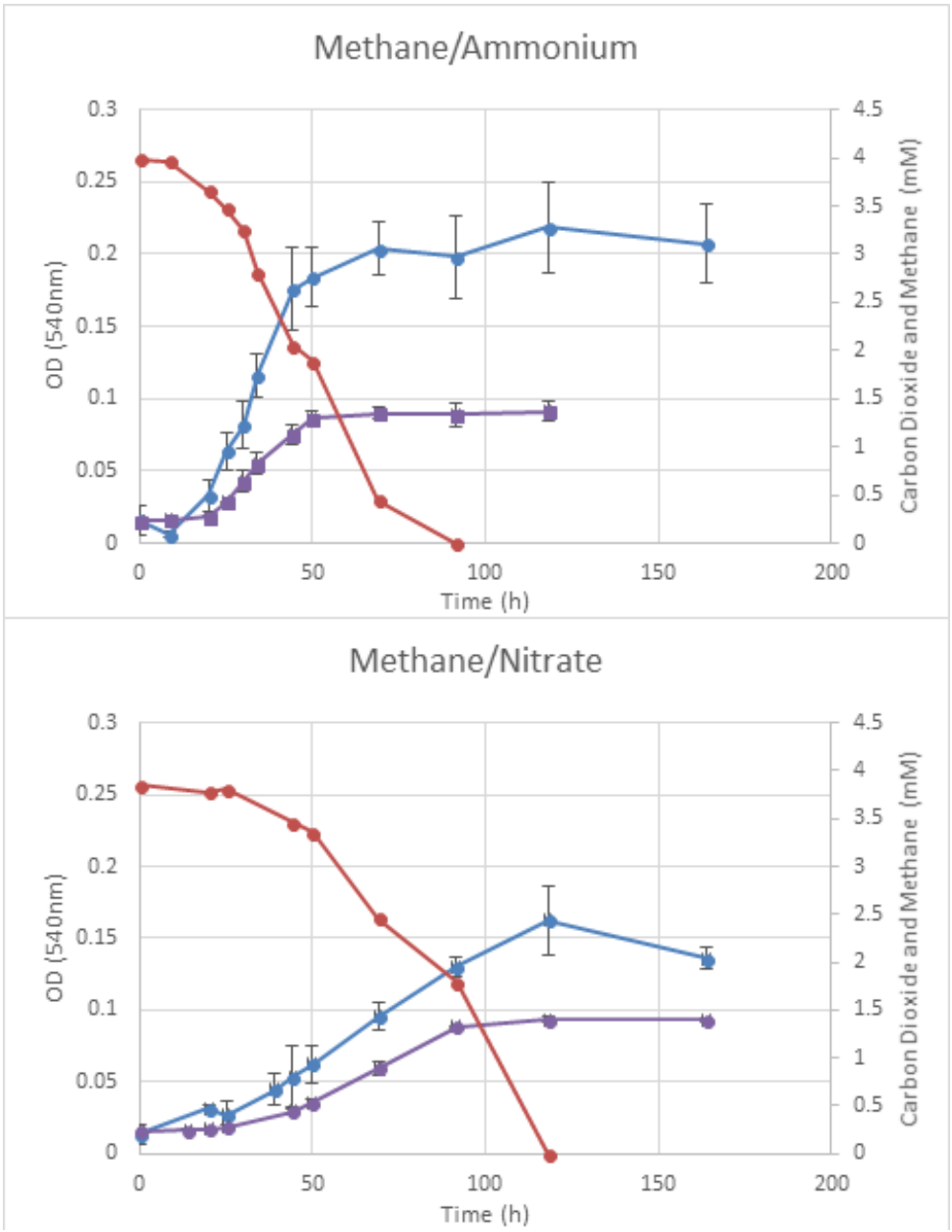
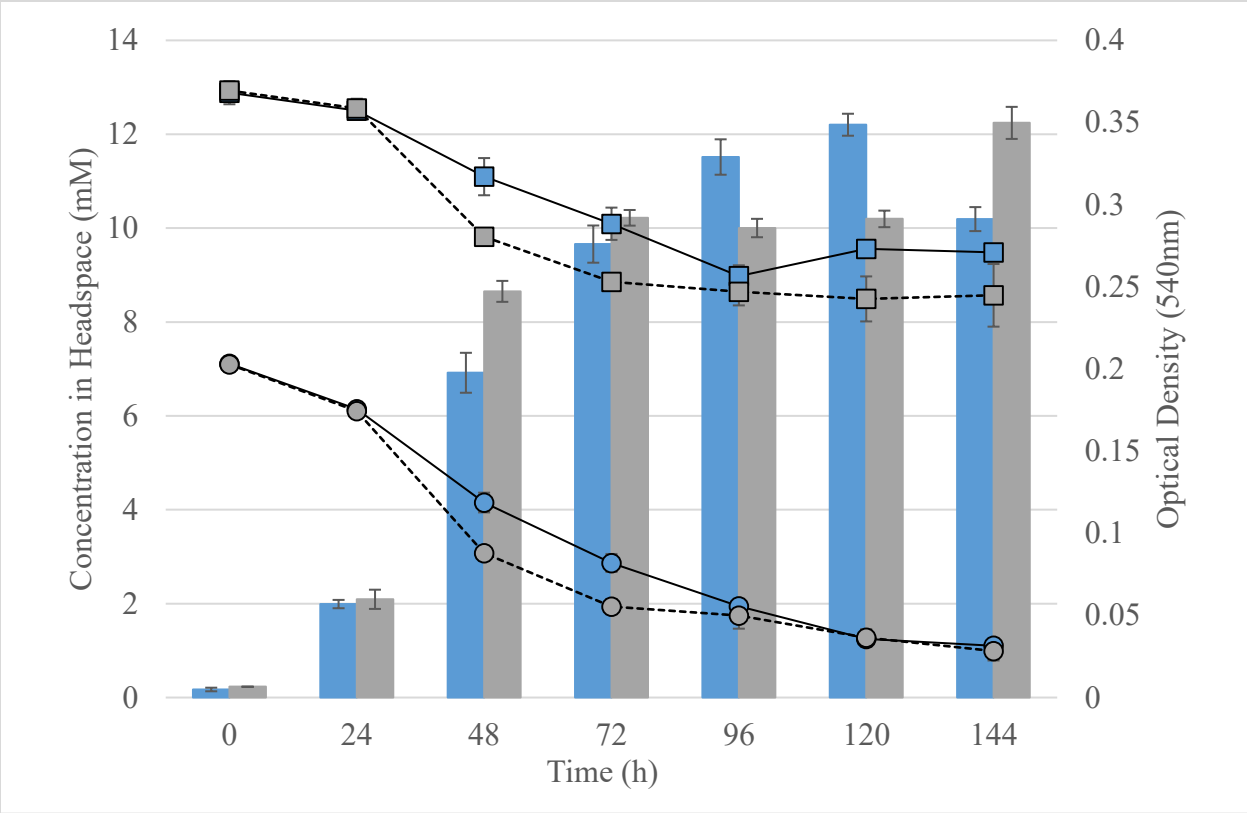
# Appendices

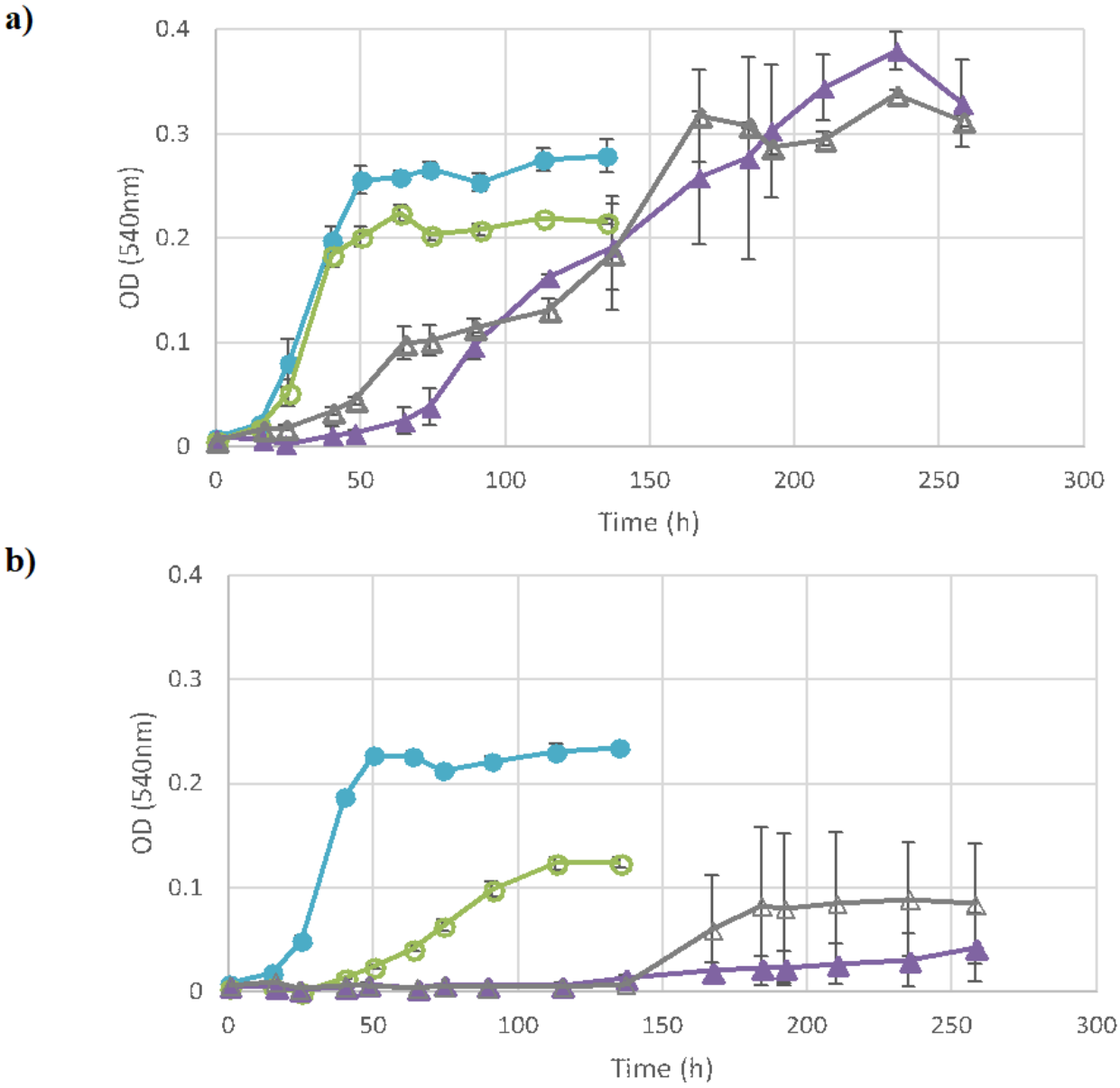
## A) Supplementary Material for Chapter 3




**Supp. Figure A-1**. Representative growth curve of with Methylocystis sp. Rockwell with 0.5 mmol methane and nitrogen sources provided at 10 mM concentrations. The complete depletion of methane in culture headspace coincided with the cessation of log phase. Cultures were grown in 50 mL of media in sealed 250-mL Wheaton bottles. OD (540nm) shown in blue, carbon dioxide in purple, and oxygen concentration in red.




**Supp. Figure A-2**. Representative growth curves of M. denitrificans FJG1. Bars represent optical density of the culture at 540nm. Methane/ammonium growth condition is represented by blue, methane/nitrate growth condition by green. Squares represent methane concentration measured in culture headspace, circles represent oxygen concentration in headspace. Nitrogen sources were provided in 10 mM concentration, and 2.5 mmol methane was provided.




**Supp. Figure A-3**. Representative growth curves of M. album BG8 (a) and Methylocystis sp. Rockwell (b). Methane/ammonium growth condition is represented by closed blue circles, methane/nitrate growth condition by open green circles, methanol/ammonium growth condition by closed purple triangles, and methanol/nitrate growth condition by open grey triangles. Nitrogen sources were provided in 10 mM concentration, while 2 mmol carbon sources were provided. f

**Supp. Table A-1**. Time at which maximum optical density (540nm) (in hours) of methanotrophic bacteria grown in combinations of carbon and nitrogen sources in batch cultures was measured. Dashes indicate conditions were not examined.

| Strain | Carbon (mmol) | Methane | | Methanol | |
|---|---|---|---|---|---|
| | | $NH_4^+$ | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| Rockwell | 0.5 | 187 | 84 | 208 | 231 |
| | 1 | - | - | 168 | 168 |
| | 2 | 135 | 135 | 258 | 235 |
| | 2.5 | 168 | 168 | - | - |
| WRRC1 | 0.5 | 138 | 113 | 231 | 231 |
| | 1 | - | - | 165 | 190 |
| | 2 | 63.5 | 113 | 235 | 258 |
| | 2.5 | 120 | 165 | - | - |
| OB3b | 0.5 | 113 | 187 | 44 | 190 |
| | 1 | - | - | 192 | 192 |
| | 2 | 63.5 | 74 | 167 | 167 |
| | 2.5 | 192 | 192 | - | - |
| BG8 | 0.5 | 68 | 113 | 208 | 208 |
| | 1 | - | - | 168 | 168 |
| | 2 | 135 | 113 | 235 | 235 |
| | 2.5 | 168 | 168 | - | - |
| FJG1 | 0.5 | 68 | 44 | 70.5 | 50 |
| | 1 | - | - | 72 | 72 |
| | 2 | 50 | 50 | 167 | 65 |
| | 2.5 | 68.5 | 68.5 | - | - |

**Supp. Table A-2**. Approximate lag phase (in hours) of methanotrophic bacteria grown in combinations of carbon and nitrogen sources in batch cultures. N.D. = not determined. Dashes indicate conditions were not examined.

| Strain | Carbon (mmol) | Methane | | Methanol | |
|---|---|---|---|---|---|
| | | $NH_4^+$ | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| Rockwell | 0.5 | 15 | 15 | 50 | N.D. |
| | 1 | - | - | 12 | 48 |
| | 2 | 16 | 24 | 137 | 137 |
| | 2.5 | 0 | 12 | - | - |
| WRRC1 | 0.5 | 15 | 22 | 50 | 114.5 |
| | 1 | - | - | 95 | 95 |
| | 2 | 25 | 40 | N.D. | 192 |
| | 2.5 | 0 | 25 | - | - |
| OB3b | 0.5 | 41 | 113 | N.D | 164 |
| | 1 | - | - | 60 | 48 |
| | 2 | 24 | 24 | 137 | 115 |
| | 2.5 | 12 | 24 | - | - |
| BG8 | 0.5 | 15 | 15 | 22 | 22 |
| | 1 | - | - | 36 | 36 |
| | 2 | 16 | 16 | 48 | 24 |
| | 2.5 | 12 | 12 | - | - |
| FJG1 | 0.5 | 22 | 15 | 22 | N.D. |
| | 1 | - | - | 40 | 40 |
| | 2 | 16 | 24 | N.D. | 40 |
| | 2.5 | 40 | 24 | - | - |

**Supp. Table A-3**. Differential gene expression in M. sp. Rockwell determined by RNA-Seq analysis, showing n-fold changes between methane-ammonium, methanol-nitrate, and methanol-ammonium growth conditions compared to the expression of genes in a methane-nitrate growth condition. Fold change was calculated from n=3 replicates for each condition. Bolded values represent those showing statistical significance (FDR-p < 0.05).

| NCBI Reference Sequence | Gene Product | $NH_4^+$ / $CH_4$ | $NO_3^-$ / $CH_3OH$ | $NH_4^+$ / $CH_3OH$ |
|---|---|---|---|---|
| WP_036279795.1 | methane, monooxygenase, subunit C | 1.08 | -1.97 | -1.69 |
| WP_036281095.1_1 | methane, monooxygenase, subunit C | -1.27 | -1.41 | -2.02 |
| WP_036281095.1_2 | methane, monooxygenase, subunit C | 1.03 | **-2.80** | **-6.32** |
| WP_036281095.1_3 | methane, monooxygenase, subunit C | 1.14 | **-2.77** | **-6.25** |
| WP_036281738.1_1 | methane, monooxygenase, subunit A | -1.22 | **-2.12** | **-7.02** |
| WP_036281738.1_2 | methane, monooxygenase, subunit A | -1.37 | **-2.24** | **-6.94** |
| WP_036287217.1 | methane, monooxygenase, subunit B | -1.19 | -1.70 | **-5.63** |
| WP_036287347.1 | methane, monooxygenase, subunit C | -1.33 | 2.78 | 1.04 |
| WP_036288357.1 | methane, monooxygenase, subunit C | -2.50 | -1.47 | -1.97 |
| WP_036284218.1 | methanol, dehydrogenase | -1.12 | 1.05 | 1.07 |
| WP_036285491.1 | methanol, dehydrogenase | -1.28 | -1.03 | **-3.40** |
| WP_036285493.1 | methanol, dehydrogenase | **-1.40** | 1.27 | **-4.54** |
| WP_036287664.1 | methanol, dehydrogenase | -1.04 | 1.63 | -1.27 |
| WP_036289203.1 | methanol, dehydrogenase 5 | -3.42 | -1.76 | -3.14 |
| WP_036282879.1 | formaldehyde-activating, protein | -1.14 | 1.02 | -1.48 |
| WP_036283168.1 | aldehyde-activating, protein | 1.04 | 1.02 | -1.09 |
| WP_036288365.1 | formaldehyde-activating, protein | -1.05 | -1.06 | -2.17 |
| WP_036288367.1 | formaldehyde-activating, protein | -1.13 | 1.16 | **-3.76** |
| WP_036282881.1 | methylenetetrahydromethanopterin, dehydrogenase | 1.24 | 1.24 | 1.36 |
| WP_036284209.1 | 5-10-methenyltetrahydromethanopterin, cyclohydrolase | 1.10 | 1.09 | -1.01 |
| WP_036280414.1 | formylmethanofuran--tetrahydromethanopterin, formyltransferase | 1.46 | -1.00 | -1.22 |
| WP_036280409.1 | formylmethanofuran, dehydrogenase | 1.37 | -1.03 | 1.28 |

| | | | | |
|---|---|---|---|---|
| WP_036280412.1 | formylmethanofuran, dehydrogenase | 1.21 | -1.04 | -1.36 |
| WP_036280416.1 | formylmethanofuran, dehydrogenase | 1.41 | -1.01 | -1.13 |
| WP_036284908.1 | methylenetetrahydrofolate, dehydrogenase | 1.47 | 1.45 | 1.44 |
| WP_036284911.1 | methenyltetrahydrofolate, cyclohydrolase | 1.47 | 1.16 | 1.23 |
| WP_036284902.1 | formate--tetrahydrofolate, ligase | 1.16 | 1.86 | 1.33 |
| WP_036282739.1 | formate, dehydrogenase | 1.42 | 1.36 | -1.12 |
| WP_036282741.1 | formate, dehydrogenase | 1.48 | 1.17 | 1.06 |
| WP_036282742.1 | formate, dehydrogenase | 1.35 | 1.49 | 1.20 |
| WP_036282743.1 | formate, dehydrogenase | 1.41 | 1.51 | 1.72 |
| WP_036287623.1 | formate, dehydrogenase | 1.49 | 1.33 | -1.07 |
| WP_036287625.1 | formate, dehydrogenase | 1.47 | 1.70 | 2.20 |
| WP_036288212.1 | formate, dehydrogenase | -2.91 | -1.44 | -3.26 |
| WP_036280890.1 | acetyl-CoA, carboxylase | -1.02 | 1.26 | -1.32 |
| WP_036282352.1 | acetyl-CoA, carboxylase | -1.03 | 1.18 | -1.60 |
| WP_036282866.1 | acetyl-CoA, carboxylase, subunit, beta | 1.27 | 1.17 | 1.27 |
| WP_036286853.1 | acetyl-CoA, carboxylase | 1.29 | 1.22 | 1.81 |
| WP_036287496.1 | biotin--acetyl-CoA-carboxylase, ligase | 1.84 | -1.10 | 1.06 |
| WP_036289314.1 | acetyl-CoA, carboxylase, subunit, alpha | 1.11 | 1.03 | -1.17 |
| WP_036284556.1 | ACP, S-malonyltransferase | 1.32 | -1.07 | -1.04 |
| WP_036280951.1 | 3-oxoacyl-ACP, synthase | 1.07 | 1.09 | 1.55 |
| WP_036284276.1 | 3-oxoacyl-ACP, synthase | 1.26 | 1.15 | -1.13 |
| WP_036284547.1 | 3-oxoacyl-ACP, synthase | 1.23 | 1.06 | -1.32 |
| WP_036284553.1 | 3-oxoacyl-ACP, synthase | 1.34 | -1.00 | 1.24 |
| WP_036286470.1 | 3-oxoacyl-ACP, synthase | 1.23 | 1.01 | 1.54 |
| WP_036286640.1 | 3-oxoacyl-ACP, reductase | -2.21 | -1.34 | -5.19 |
| WP_036280295.1 | 3-hydroxyacyl-ACP, dehydratase | 1.14 | 1.02 | -1.21 |
| WP_036284278.1 | 3-hydroxydecanoyl-ACP, dehydratase | 1.15 | -1.21 | **-1.75** |
| WP_036284272.1 | enoyl-ACP, reductase | 1.19 | 1.01 | -1.02 |
| WP_036288347.1 | enoyl-ACP, reductase | 1.11 | 1.00 | -1.24 |

**Supp. Table A-4**. Differential gene expression in *M. album* BG8 determined by RNA-Seq analysis, showing n-fold changes between methane-ammonium, methanol-nitrate, and methanol-ammonium growth conditions compared to the expression of genes in a methane-nitrate growth condition. Fold change was calculated from n=2 replicates for each condition, except $NO_3^-/CH_3OH$ (n=3). Bolded values represent those showing statistical significance (FDR-p < 0.05).

| GenBank | Locus Tag | Gene Product | $NH_4^+$ / $CH_4$ | $NO_3^-$ / $CH_3OH$ | $NH_4^+$ / $CH_3OH$ |
|---|---|---|---|---|---|
| EIC29217.1 | Metal_1432 | methane monooxygenase/ammonia monooxygenase, subunit C | -1.00 | -1.48 | 1.10 |
| EIC29218.1 | Metal_1433 | methane monooxygenase/ammonia monooxygenase, subunit B | -1.04 | -1.65 | -1.26 |
| EIC29219.1 | Metal_1434 | Ammonia monooxygenase | -1.02 | -1.98 | -1.25 |
| EIC31238.1 | Metal_3591 | methane monooxygenase/ammonia monooxygenase, subunit B | -1.00 | -1.00 | **1.40** |
| EIC31239.1 | Metal_3592 | methane monooxygenase/ammonia monooxygenase, subunit A | -1.00 | -1.00 | **1.65** |
| EIC31240.1 | Metal_3593 | methane monooxygenase/ammonia monooxygenase, subunit C | -1.00 | -1.00 | -1.00 |
| EIC29181.1 | Metal_1395 | PQQ-dependent dehydrogenase, methanol/ethanol family | -1.54 | -1.10 | 1.56 |
| EIC29717.1 | Metal_1951 | beta-propeller domain-containing protein, methanol dehydrogenase | -1.20 | -2.50 | -1.66 |
| EIC30188.1 | Metal_2469 | PQQ-dependent dehydrogenase, methanol/ethanol family | 1.02 | 1.19 | 1.27 |
| EIC30191.1 | Metal_2472 | Methanol dehydrogenase beta subunit | -1.28 | 1.06 | 1.12 |
| EIC29284.1 | Metal_1500 | formaldehyde-activating enzyme | -1.06 | -3.84 | -1.58 |
| EIC30157.1 | Metal_2435 | formaldehyde-activating enzyme | 1.12 | **1.75** | -1.40 |
| EIC31169.1 | Metal_3521 | formaldehyde-activating enzyme | -1.11 | **2.40** | -1.00 |
| EIC28889.1 | Metal_1071 | Methylene-tetrahydromethanopterin dehydrogenase | -1.03 | 1.14 | -1.07 |
| EIC31172.1 | Metal_3524 | methenyltetrahydromethanopterin cyclohydrolase | -1.43 | **1.83** | -1.54 |
| EIC28266.1 | Metal_0412 | formylmethanofuran--tetrahydromethanopterin N-formyltransferase | -1.19 | -1.03 | 1.05 |
| EIC31166.1 | Metal_3518 | putative H4MPT-linked C1 transfer pathway | -1.03 | 2.20 | 1.29 |

| | | | | | |
|---|---|---|---|---|---|
| | | protein | | | |
| EIC28267.1 | Metal_0413 | formylmethanofuran dehydrogenase subunit C | -1.09 | -1.33 | 1.12 |
| EIC27971.1 | Metal_0102 | formylmethanofuran dehydrogenase subunit A | -1.07 | -1.02 | -1.17 |
| EIC28259.1 | Metal_0404 | formylmethanofuran dehydrogenase subunit B | -1.11 | 1.06 | -1.33 |
| EIC28260.1 | Metal_0405 | formylmethanofuran dehydrogenase subunit A | -1.13 | 1.08 | -1.18 |
| EIC29032.1 | Metal_1226 | flavin-dependent oxidoreductase, F420-dependent methylene-tetrahydromethanopterin reductase | 1.37 | 1.38 | 1.16 |
| EIC29186.1 | Metal_1400 | methenyl tetrahydrofolate cyclohydrolase | -1.09 | 1.07 | -1.22 |
| EIC29736.1 | Metal_1971 | formyltetrahydrofolate synthetase | -1.35 | 2.53 | -1.23 |
| EIC29234.1 | Metal_1449 | NADH-dependent formate dehydrogenase delta subunit FdsD | 1.02 | 1.10 | 1.03 |
| EIC29235.1 | Metal_1450 | formate dehydrogenase family accessory protein FdhD | 1.07 | **-1.50** | -1.12 |
| EIC29236.1 | Metal_1451 | formate dehydrogenase, alpha subunit, archaeal-type | 1.06 | **-1.99** | -1.13 |
| EIC29203.1 | Metal_1418 | phosphoketolase | 1.09 | -1.48 | -1.48 |
| EIC28009.1 | Metal_0141 | beta-hydroxyacyl-(acyl carrier protein) dehydratase FabZ | -1.14 | 3.44 | 1.45 |
| EIC29672.1 | Metal_1906 | beta-hydroxyacyl-(acyl carrier protein) dehydratase FabA | -1.05 | -1.05 | -1.25 |
| EIC28224.1 | Metal_0368 | acyl-CoA hydrolase | -1.07 | -1.26 | -1.51 |
| EIC28378.1 | Metal_0528 | enoyl-(acyl-carrier-protein) reductase (NADH) | -1.11 | **-1.41** | -1.12 |
| EIC29396.1 | Metal_1617 | beta-ketoacyl-acyl-carrier-protein synthase II | 1.02 | 2.11 | 1.19 |
| EIC29397.1 | Metal_1618 | acyl carrier protein | 1.11 | 2.18 | 1.18 |
| EIC29398.1 | Metal_1619 | 3-oxoacyl-(acyl-carrier-protein) reductase | -1.04 | 6.91 | 1.65 |
| EIC29399.1 | Metal_1620 | malonyl CoA-acyl carrier protein transacylase | -1.07 | 6.57 | 1.55 |
| EIC29400.1 | Metal_1621 | 3-oxoacyl-(acyl-carrier-protein) synthase III | -1.15 | **5.17** | 1.30 |

| | | | | | |
|---|---|---|---|---|---|
| EIC29673.1 | Metal_1907 | 3-oxoacyl-(acyl-carrier-protein) synthase | -1.04 | **-1.68** | -1.09 |
| EIC30700.1 | Metal_3020 | 3-oxoacyl-(acyl-carrier-protein) synthase | -1.08 | -1.29 | -1.13 |
| EIC30701.1 | Metal_3021 | 3-oxoacyl-(acyl-carrier-protein) reductase, putative | -1.32 | -1.33 | -1.33 |
| EIC30702.1 | Metal_3022 | putative 3-hydroxylacyl-(acyl carrier protein) dehydratase | -1.13 | 1.24 | -1.07 |
| EIC30703.1 | Metal_3023 | 3-oxoacyl-(acyl-carrier-protein) synthase | -1.15 | 1.32 | 1.08 |
| EIC31544.1 | Metal_3907 | acyl-CoA dehydrogenase | 1.15 | -1.48 | -1.14 |
| EIC31546.1 | Metal_3909 | acyl-CoA dehydrogenase | 1.51 | -1.43 | -1.69 |
| EIC28009.1 | Metal_0141 | beta-hydroxyacyl-(acyl carrier protein) dehydratase FabZ | -1.14 | 3.44 | 1.45 |
| EIC29672.1 | Metal_1906 | beta-hydroxyacyl-(acyl carrier protein) dehydratase FabA | -1.05 | -1.05 | -1.25 |
| EIC28224.1 | Metal_0368 | acyl-CoA hydrolase | -1.07 | -1.26 | -1.51 |
| EIC28378.1 | Metal_0528 | enoyl-(acyl-carrier-protein) reductase (NADH) | -1.11 | **-1.41** | -1.12 |
| EIC29396.1 | Metal_1617 | beta-ketoacyl-acyl-carrier-protein synthase II | 1.02 | 2.11 | 1.19 |
| EIC29397.1 | Metal_1618 | acyl carrier protein | 1.11 | 2.18 | 1.18 |

**Supp. Table A-5**. Multifactorial analysis of variance (ANOVA) on total FAMEs measured as a percent of total cell weight in *M. album* BG8 and *M.* sp. Rockwell, for each condition tested. Values represent calculated F-test p-value. Bolded values represent those factors and combinations of factors (interactions) showing statistically significant, measureable effects on the outcome assessed at α=0.05.

| Factor | FAMEs (%CDW) |
|---|---|
| Strain | **>2.00E-16** |
| Carbon | **>2.00E-16** |
| Nitrogen | **>2.00E-16** |
| Strain:Carbon | **3.10E-07** |
| Strain:Nitrogen | **>2.00E-16** |
| Carbon:Nitrogen | **>2.00E-16** |
| Strain:Carbon:Nitrogen | **7.47E-03** |

**Supp. Table A-6**. Multifactorial analysis of variance (ANOVA) on specific FAMEs measured as a percent of total cell weight in *M. album* BG8 and *M.* sp. Rockwell, for each condition tested. Values represent calculated F-test p-value. Bolded values represent those factors and combinations of factors (interactions) showing statistically significant, measureable effects on the outcome assessed at α=0.05. Italized values are significant at α=0.1.

| | M. album BG8 | | | | *M.* sp. Rockwell | |
|---|---|---|---|---|---|---|
| | C16:0 | C16:1n6 | C16:1n7 | C16:1n9 | C18:1n7 | C18:1n9 |
| Carbon | 6.90E-01 | **2.32E-03** | **1.49E-03** | 9.59E-02 | **3.00E-07** | **5.77E-03** |
| Nitrogen | **1.06E-03** | 6.13E-01 | 7.97E-01 | 2.22E-01 | 5.20E-01 | 2.79E-01 |
| Carbon:Nitrogen | 3.61E-01 | 9.75E-01 | 1.58E-01 | 5.83E-02 | 9.31E-01 | 5.22E-01 |

**Supp. Table A-7**. Proportion of each FAME in different carbon and nitrogen conditions in *M. album* BG8 and *Methylocystis* sp. Rockwell, as a percent of total measured FAMEs. Standard deviations of six replicates are reported in parentheses.

| Strain | FAMEs | Methane | | Methanol | |
|---|---|---|---|---|---|
| | | $NH_4^+$ | $NO_3^-$ | $NH_4^+$ | $NO_3^-$ |
| Rockwell | C10:0 | 0.01 (±0.03) | 0.01 (±0.03) | 0 (±0) | 0.12 (±0.12) |
| | C12:0 | 0.07 (±0.01) | 0.12 (±0.09) | 0.2 (±0.05) | 0.18 (±0.06) |
| | C14:0 | 0.18 (±0.06) | 0.31 (±0.23) | 0.56 (±0.21) | 0.45 (±0.13) |
| | C15:0 | 0.05 (±0.08) | 0.13 (±0.2) | 0.2 (±0.22) | 0 (±0) |
| | C16:0 | 0.6 (±0.11) | 0.96 (±0.61) | 1.47 (±0.28) | 1.36 (±0.27) |
| | C16:1n9 | 0.24 (±0.13) | 0.59 (±0.58) | 0.23 (±0.24) | 0.65 (±0.27) |
| | C16:1n7 | 0.9 (±0.07) | 1 (±0.31) | 1.55 (±0.18) | 1.19 (±0.18) |
| | C16:1n6 | 0.07 (±0.07) | 0.08 (±0.06) | 0 (±0) | 0.04 (±0.08) |
| | C16:1n5 | 0 (±0) | 0 (±0) | 0 (±0) | 0 (±0) |
| | C16 unknown1 | 0.02 (±0.04) | 0 (±0) | 0 (±0) | 0 (±0) |
| | C16:2 | 0.41 (±0.08) | 0.65 (±0.48) | 0.74 (±0.55) | 1.03 (±0.4) |
| | C16:3 | 0 (±0) | 0.05 (±0.11) | 0.1 (±0.22) | 0.1 (±0.22) |
| | C18 | 0.59 (±0.12) | 0.87 (±0.54) | 1.28 (±0.27) | 1.17 (±0.24) |
| | C18:1n9 | 72.41 (±1.6) | 70.32 (±4.43) | 75.3 (±0.8) | 74.75 (±2.28) |
| | C18:1n7 | 24.45 (±1.87) | 24.9 (±2.23) | 18.36 (±0.47) | 18.95 (±2.03) |
| BG8 | C10:0 | 0.08 (±0.02) | 0.1 (±0.13) | 0.1 (±0.05) | 0.09 (±0.06) |
| | C12:0 | 0.1 (±0.02) | 0.33 (±0.08) | 0.16 (±0.03) | 0.24 (±0.05) |
| | C14:0 | 2.16 (±0.23) | 2.04 (±0.31) | 2.33 (±0.27) | 1.76 (±0.33) |
| | C15:0 | 0.58 (±0.05) | 0.67 (±0.07) | 0.46 (±0.04) | 0.55 (±0.07) |
| | C16:0 | 14.48 (±0.83) | 12.79 (±1.82) | 14.79 (±1.09) | 12 (±1.29) |
| | C16:1n9 | 24.12 (±1.73) | 23.33 (±3.05) | 23.84 (±1.76) | 27.31 (±2.67) |
| | C16:1n7 | 18.06 (±1.28) | 19.69 (±3.51) | 15.97 (±1.26) | 14.84 (±1.49) |
| | C16:1n6 | 36.59 (±0.48) | 36.81 (±1.51) | 38.16 (±0.97) | 38.41 (±0.82) |

| | | | | |
|---|---|---|---|---|
| C16:1n5 | 2.24 (±0.24) | 2.23 (±0.45) | 2.14 (±0.48) | 2.56 (±0.45) |
| C16 unknown1 | 0.3 (±0.06) | 0.32 (±0.18) | 0.34 (±0.08) | 0.47 (±0.12) |
| C16:2 | 0.31 (±0.08) | 0.57 (±0.5) | 0.52 (±0.04) | 0.64 (±0.18) |
| C16:3 | 0.44 (±0.07) | 0.32 (±0.16) | 0.54 (±0.07) | 0.55 (±0.13) |
| C18 | 0.28 (±0.06) | 0.5 (±0.28) | 0.49 (±0.08) | 0.51 (±0.09) |
| C18:1n9 | 0.18 (±0.14) | 0.22 (±0.32) | 0.11 (±0.16) | 0.05 (±0.11) |
| C18:1n7 | 0.08 (±0.06) | 0.1 (±0.13) | 0.03 (±0.07) | 0.03 (±0.06) |

## B) Supplementary Material for Chapter 4

**Supp. Table B-1**. Number of genes in *Methylomicrobium album* BG8 that were differentially regulated between the test condition and base condition of each comparison. Results shown graphically in Figure 1. All genes counted were significant at log-fold change > |1| and adjusted p-value < 0.01. Darker boxes represent higher proportion of genes differentially regulated in that condition.

| | | Increase | | | | |
|---|---|---|---|---|---|---|
| | **Test Condition** | *Methane-Ammonium* | *Methanol-Nitrate* | *Methanol-Ammonium* | *Methanol-Ammonium* | *Methanol-Ammonium* |
| | **Base Condition** | *Methane-Nitrate* | *Methane-Nitrate* | *Methane-Nitrate* | *Methane-Ammonium* | *Methanol-Nitrate* |
| **A** | RNA processing and modification | 0 | 0 | 0 | 0 | 0 |
| **B** | Chromatin Structure and dynamics | 0 | 1 | 0 | 0 | 0 |
| **C** | Energy production and conversion | 3 | 15 | 0 | 0 | 14 |
| **D** | Cell cycle control and mitosis | 0 | 1 | 0 | 0 | 0 |
| **E** | Amino Acid metabolism and transport | 4 | 33 | 0 | 0 | 4 |
| **F** | Nucleotide metabolism and transport | 0 | 7 | 0 | 0 | 0 |
| **G** | Carbohydrate metabolism and transport | 1 | 11 | 0 | 0 | 3 |
| **H** | Coenzyme metabolis | 0 | 7 | 0 | 0 | 1 |
| **I** | Lipid metabolism | 0 | 14 | 0 | 0 | 2 |
| **J** | Translation | 0 | 75 | 0 | 0 | 1 |
| **K** | Transcription | 0 | 9 | 0 | 0 | 5 |
| **L** | Replication and repair | 0 | 14 | 0 | 0 | 11 |
| **M** | Cell wall/membrane/envelop biogenesis | 0 | 7 | 0 | 0 | 8 |
| **N** | Cell motility | 1 | 1 | 0 | 0 | 16 |
| **O** | Post-translational modification, protein | 0 | 5 | 0 | 0 | 13 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | turnover, chaperone functions | | | | | |
| **P** | Inorganic ion transport and metabolism | 12 | 17 | 0 | 0 | 3 |
| **Q** | Secondary Structure | 0 | 7 | 0 | 0 | 6 |
| **T** | Signal Transduction | 4 | 9 | 0 | 0 | 17 |
| **U** | Intracellular trafficing and secretion | 0 | 8 | 0 | 0 | 10 |
| **Y** | Nuclear structure | 0 | 0 | 0 | 0 | 0 |
| **Z** | Cytoskeleton | 0 | 0 | 0 | 0 | 0 |
| **R** | General Functional Prediction only | 0 | 0 | 0 | 0 | 0 |
| **S** | Function Unknown | 8 | 26 | 0 | 0 | 31 |
| **n/a** | Unclassified | 1 | 23 | 0 | 0 | 47 |

**Decrease**

| | **Test Condition** | *Methane-Ammonium* | *Methanol-Nitrate* | *Methanol-Ammonium* | *Methanol-Ammonium* | *Methanol-Ammonium* |
|---|---|---|---|---|---|---|
| | **Base Condition** | *Methane-Nitrate* | *Methane-Nitrate* | *Methane-Nitrate* | *Methane-Ammonium* | *Methanol-Nitrate* |
| **A** | RNA processing and modification | 0 | 0 | 0 | 0 | 1 |
| **B** | Chromatin Structure and dynamics | 0 | 0 | 0 | 0 | 0 |
| **C** | Energy production and conversion | 1 | 7 | 1 | 3 | 19 |
| **D** | Cell cycle control and mitosis | 0 | 0 | 0 | 0 | 1 |
| **E** | Amino Acid metabolism and transport | 0 | 2 | 0 | 1 | 23 |
| **F** | Nucleotide metabolism and transport | 0 | 0 | 0 | 0 | 12 |
| **G** | Carbohydrate metabolism and transport | 0 | 6 | 0 | 0 | 12 |
| **H** | Coenzyme metabolism | 0 | 1 | 0 | 0 | 5 |
| **I** | Lipid metabolism | 0 | 5 | 2 | 0 | 12 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **J** | Translation | 0 | 1 | 0 | 0 | 67 |
| **K** | Transcription | 0 | 5 | 0 | 0 | 10 |
| **L** | Replication and repair | 0 | 4 | 0 | 0 | 12 |
| **M** | Cell wall/membrane/envelop biogenesis | 0 | 8 | 0 | 0 | 8 |
| **N** | Cell motility | 0 | 1 | 0 | 0 | 1 |
| **O** | Post-translational modification, protein turnover, chaperone functions | 0 | 7 | 0 | 0 | 8 |
| **P** | Inorganic ion transport and metabolism | 0 | 6 | 1 | 7 | 15 |
| **Q** | Secondary Structure | 0 | 6 | 1 | 1 | 3 |
| **T** | Signal Transduction | 0 | 7 | 0 | 1 | 7 |
| **U** | Intracellular trafficing and secretion | 0 | 0 | 0 | 0 | 3 |
| **Y** | Nuclear structure | 0 | 0 | 0 | 0 | 0 |
| **Z** | Cytoskeleton | 0 | 0 | 0 | 0 | 0 |
| **R** | General Functional Prediction only | 0 | 0 | 0 | 0 | 0 |
| **S** | Function Unknown | 1 | 34 | 3 | 4 | 30 |
| **n/a** | Unclassified | 3 | 42 | 6 | 2 | 26 |

**Supp. Table B-2**. Most highly differentially expressed genes in each test comparison, shown in log-fold change. All genes with adjusted p-value < 0.01. Hypothetical genes shown were analysed via BLAST to determine likely orthologs and function. BLAST-suggested functional assignment denoted by italicized font.

| Methane-Ammonium Vs. Methane-Nitrate | | |
|---|---|---|
| METAL_RS06040 | sulfite reductase subunit alpha CDS | 6.59 |
| METAL_RS13065 | nitrate ABC transporter substrate-binding protein CDS | 5.81 |
| METAL_RS15340 | NarK family nitrate/nitrite MFS transporter CDS | 5.75 |
| METAL_RS13060 | ABC transporter permease CDS | 5.72 |
| METAL_RS13080 | formate/nitrite transporter family protein CDS | 5.53 |
| METAL_RS15330 | nitrite reductase large subunit CDS | 5.49 |
| METAL_RS18900 | *alpha-E domain-containing protein CDS* | 5.37 |
| METAL_RS18895 | TonB-dependent hemoglobin/transferrin/lactoferrin family receptor CDS | 5.36 |
| METAL_RS15325 | nitrite reductase (NAD(P)H) small subunit CDS | 5.20 |
| METAL_RS20160 | hypothetical protein CDS | 5.18 |
| METAL_RS13055 | ABC transporter ATP-binding protein CDS | 5.06 |
| METAL_RS15335 | bifunctional protein-serine/threonine kinase/phosphatase CDS | 4.92 |
| METAL_RS11260 | multidrug ABC transporter permease/ATP-binding protein CDS | 4.84 |
| METAL_RS11055 | ammonia channel protein CDS | 4.82 |
| METAL_RS11065 | porin CDS | 4.53 |
| METAL_RS11060 | P-II family nitrogen regulator CDS | 4.42 |
| METAL_RS13050 | hypothetical protein CDS | 4.35 |
| METAL_RS02865 | aliphatic sulfonate ABC transporter substrate-binding protein CDS | 4.32 |
| METAL_RS02875 | ABC transporter ATP-binding protein CDS | 4.10 |
| METAL_RS15320 | PhzF family phenazine biosynthesis protein CDS | 3.63 |
| METAL_RS06045 | chemotaxis protein CheW CDS | 3.56 |
| METAL_RS19360 | IMP dehydrogenase CDS | 3.54 |
| METAL_RS02880 | DUF1989 domain-containing protein CDS | 3.37 |
| METAL_RS08060 | hypothetical protein CDS | -4.70 |

| Methanol-Nitrate Vs. Methane-Nitrate | | |
|---|---|---|
| METAL_RS11250 | hypothetical protein CDS | 6.34 |
| METAL_RS11245 | hypothetical protein CDS | 6.32 |
| METAL_RS11240 | penicillin acylase family protein CDS | 5.95 |
| METAL_RS11220 | hypothetical protein CDS | 5.69 |
| METAL_RS18895 | TonB-dependent hemoglobin/transferrin/lactoferrin family receptor CDS | 5.69 |
| METAL_RS11255 | hypothetical protein CDS | 5.60 |

| | | |
|---|---|---|
| METAL_RS18900 | *peptidase, M36 (Fungalysin) family CDS* | 5.56 |
| METAL_RS11225 | hypothetical protein CDS | 5.55 |
| METAL_RS11205 | MbtH family protein CDS | 5.41 |
| METAL_RS11200 | thioesterase CDS | 5.20 |
| METAL_RS11230 | plastocyanin CDS | 5.15 |
| METAL_RS20160 | hypothetical protein CDS | 5.12 |
| METAL_RS11195 | *syringomycin biosynthesis enzyme, SyrP CDS* | 4.92 |
| METAL_RS11215 | formyl transferase CDS | 4.90 |
| METAL_RS09920 | hemerythrin CDS | 4.82 |
| METAL_RS11235 | lysine/ornithine N-monooxygenase CDS | 4.80 |
| METAL_RS11210 | histone deacetylase CDS | 4.64 |
| METAL_RS11190 | non-ribosomal peptide synthetase CDS | 4.22 |
| METAL_RS01740 | energy transducer TonB CDS | 3.92 |
| METAL_RS18145 | *outer membrane lipoprotein CDS* | -4.24 |
| METAL_RS18140 | hypothetical protein CDS | -4.28 |
| METAL_RS06090 | EthD family reductase CDS | -4.65 |
| METAL_RS06085 | *peroxidase CDS* | -4.66 |
| METAL_RS06080 | *poly(3-hydroxybutyrate) depolymerase CDS* | -4.67 |

Methanol-Ammonium
Vs. Methane-Nitrate

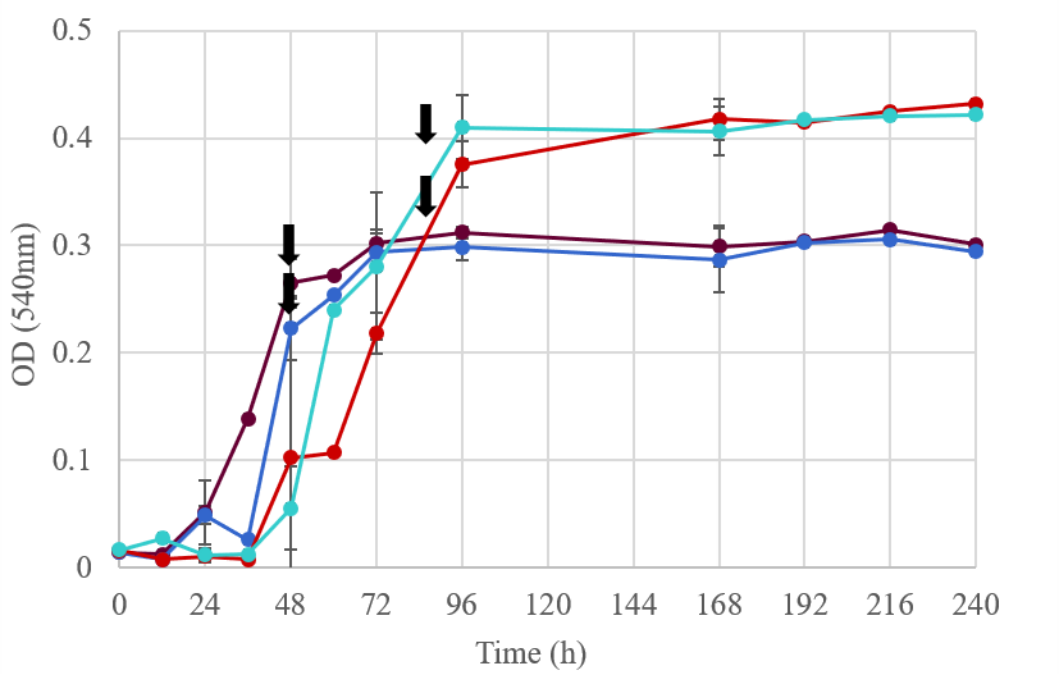
| | | |
|---|---|---|
| METAL_RS13825 | squalene/phytoene synthase family protein CDS | 2.58 |
| METAL_RS13830 | squalene--hopene cyclase CDS | -2.35 |
| METAL_RS06080 | *poly(3-hydroxybutyrate) depolymerase CDS* | -3.19 |
| METAL_RS06000 | hypothetical protein CDS | -3.33 |
| METAL_RS11450 | *NrtR-regulated NrtX CDS* | -3.43 |
| METAL_RS06090 | EthD family reductase CDS | -3.47 |
| METAL_RS06085 | *peroxidase CDS* | -3.47 |
| METAL_RS05990 | formate dehydrogenase CDS | -4.15 |
| METAL_RS05995 | cyanase CDS | -4.26 |
| METAL_RS14450 | hypothetical protein CDS | -4.55 |
| METAL_RS06540 | methyltransferase domain-containing protein CDS | -4.85 |
| METAL_RS08060 | hypothetical protein CDS | -4.92 |
| METAL_RS17295 | hypothetical protein CDS | -5.31 |
| METAL_RS17290 | hypothetical protein CDS | -5.41 |

Methanol-ammonium
Vs. Methane-ammonium

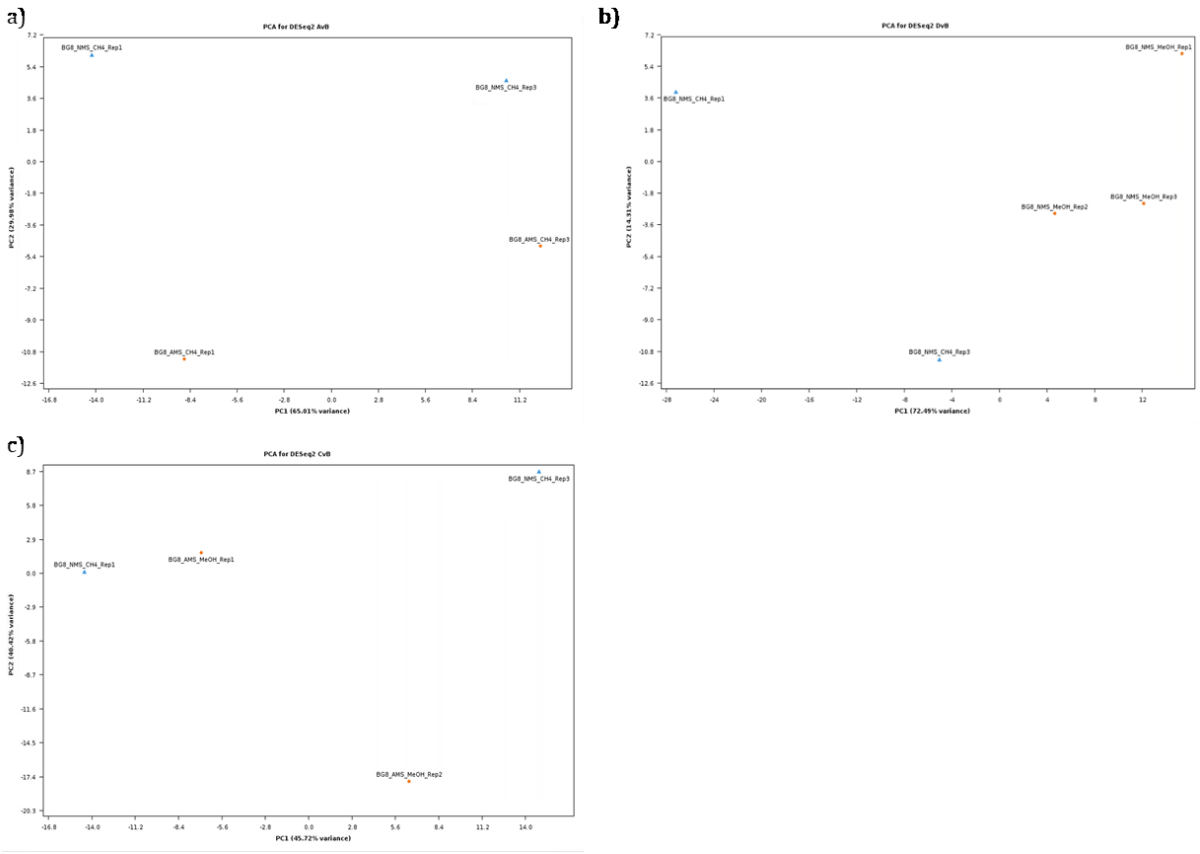
| | | |
|---|---|---|
| METAL_RS06080 | *poly(3-hydroxybutyrate) depolymerase CDS* | -3.22 |
| METAL_RS06085 | *peroxidase CDS* | -3.56 |
| METAL_RS06000 | hypothetical protein CDS | -3.81 |
| METAL_RS06090 | EthD family reductase CDS | -3.84 |
| METAL_RS02875 | ABC transporter ATP-binding protein CDS | -4.47 |

| | | |
|---|---|---|
| METAL_RS11060 | P-II family nitrogen regulator CDS | -4.72 |
| METAL_RS15335 | bifunctional protein-serine/threonine kinase/phosphatase CDS | -4.76 |
| METAL_RS02865 | aliphatic sulfonate ABC transporter substrate-binding protein CDS | -4.80 |
| METAL_RS11055 | ammonia channel protein CDS | -4.99 |
| METAL_RS06540 | methyltransferase domain-containing protein CDS | -5.03 |
| METAL_RS13050 | *lipoprotein CDS* | -5.21 |
| METAL_RS11065 | porin CDS | -5.39 |
| METAL_RS05995 | cyanase CDS | -5.51 |
| METAL_RS15340 | NarK family nitrate/nitrite MFS transporter CDS | -5.61 |
| METAL_RS15325 | nitrite reductase (NAD(P)H) small subunit CDS | -5.68 |
| METAL_RS13055 | ABC transporter ATP-binding protein CDS | -5.74 |
| METAL_RS05990 | formate dehydrogenase CDS | -5.89 |
| METAL_RS15330 | nitrite reductase large subunit CDS | -6.01 |
| METAL_RS13080 | formate/nitrite transporter family protein CDS | -6.05 |

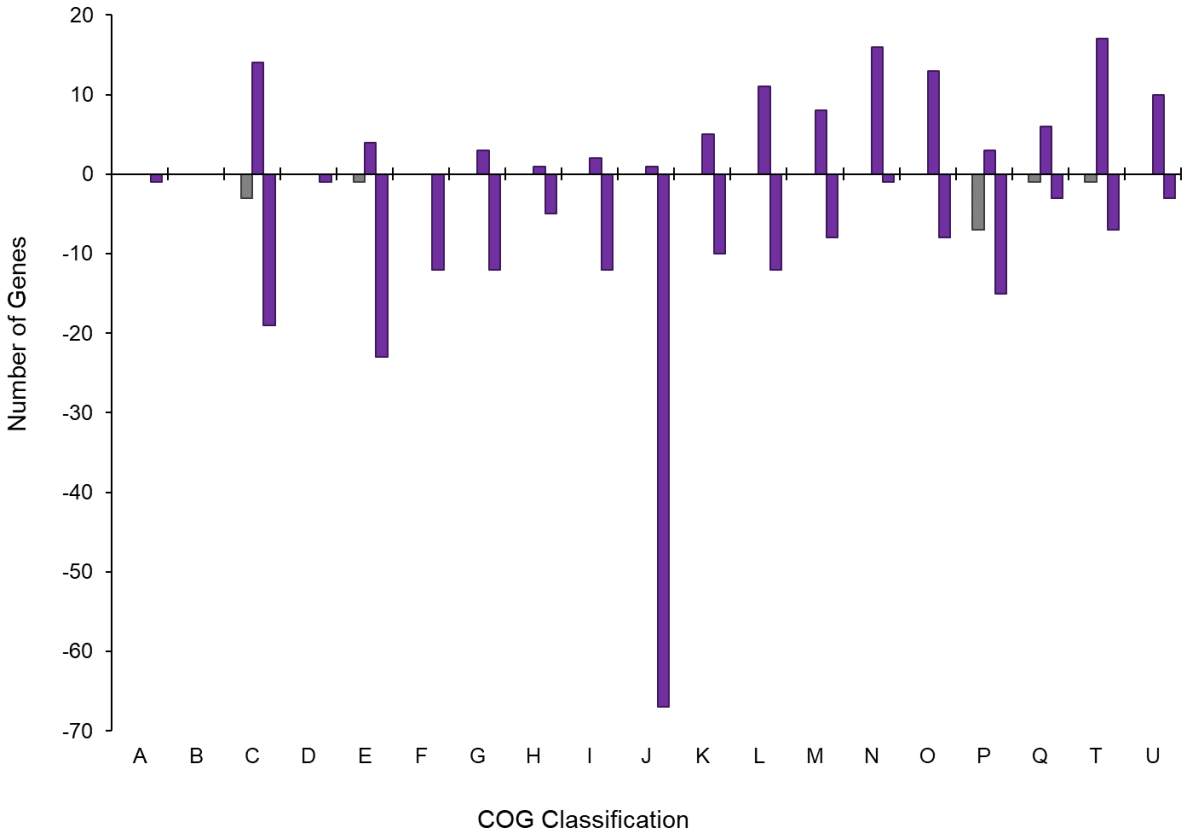
| | Methanol-Ammonium Vs. Methanol-Nitrate | |
|---|---|---|
| METAL_RS03395 | DnaK suppressor protein CDS | 4.25 |
| METAL_RS14090 | competence protein ComEA CDS | 4.19 |
| METAL_RS03390 | hypothetical protein CDS | 3.98 |
| METAL_RS18145 | *outer membrane lipoprotein CDS* | 3.76 |
| METAL_RS18140 | hypothetical protein CDS | 3.62 |
| METAL_RS18150 | hypothetical protein CDS | 3.46 |
| METAL_RS16240 | STAS domain-containing protein CDS | 3.38 |
| METAL_RS17375 | hybrid sensor histidine kinase/response regulator CDS | 3.32 |
| METAL_RS10755 | DUF1264 domain-containing protein CDS | 3.15 |
| METAL_RS03705 | hypothetical protein CDS | 3.14 |
| METAL_RS16245 | PAS domain S-box protein CDS | 3.08 |
| METAL_RS03700 | hypothetical protein CDS | 2.94 |
| METAL_RS06865 | hypothetical protein CDS | 2.87 |
| METAL_RS05280 | 30S ribosomal protein S18 CDS | -2.78 |
| METAL_RS11450 | *sugar kinase CDS* | -2.79 |
| METAL_RS06045 | chemotaxis protein CheW CDS | -2.86 |
| METAL_RS16470 | hypothetical protein CDS | -2.88 |
| METAL_RS05990 | formate dehydrogenase CDS | -2.88 |
| METAL_RS01020 | YfdX protein CDS | -2.92 |
| METAL_RS04640 | 50S ribosomal protein L19 CDS | -2.94 |
| METAL_RS13075 | bifunctional protein-serine/threonine kinase/phosphatase CDS | -2.95 |
| METAL_RS07895 | rpmF CDS | -3.06 |
| METAL_RS06000 | hypothetical protein CDS | -3.36 |
| METAL_RS06540 | methyltransferase domain-containing protein CDS | -5.58 |



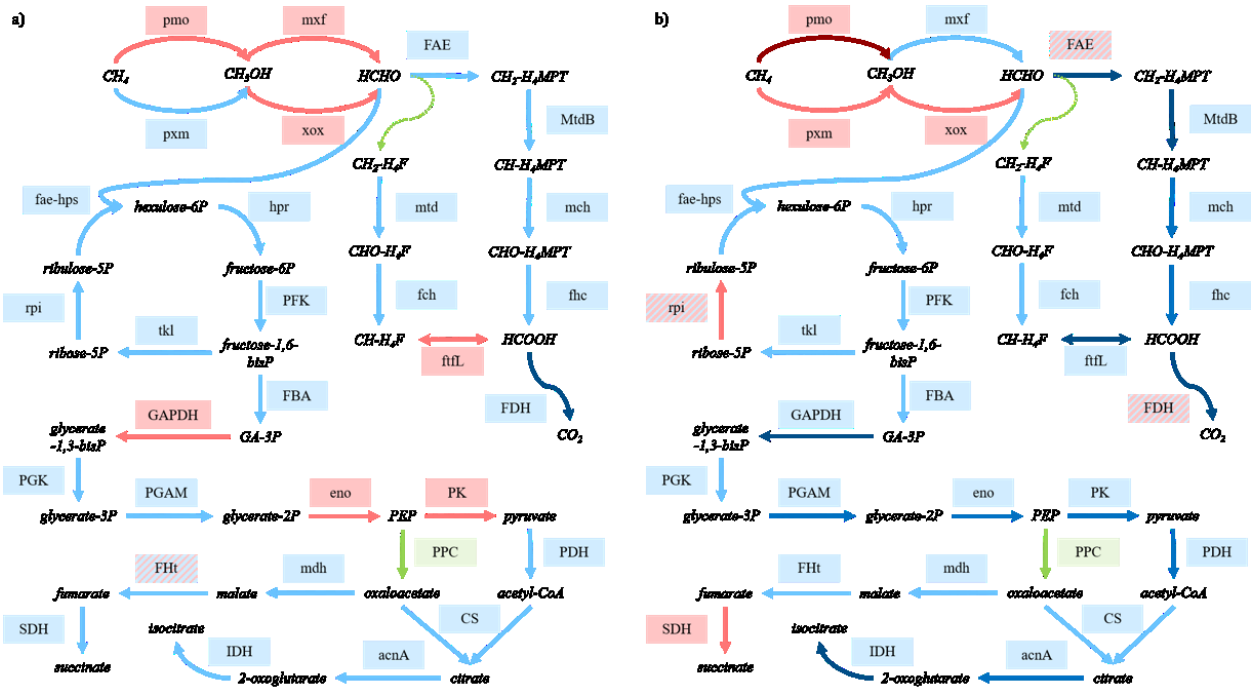

**Supp. Figure B-1**. Growth curve of *M. album* BG8 under extraction conditions, in 100 mL media in 250-mL Wheaton bottles. Methane-ammonium is purple, methane-nitrate is blue, methanol-ammonium is red, methanol-nitrate is teal. Arrows indicate time at which RNA was extracted from subsequent cultures.



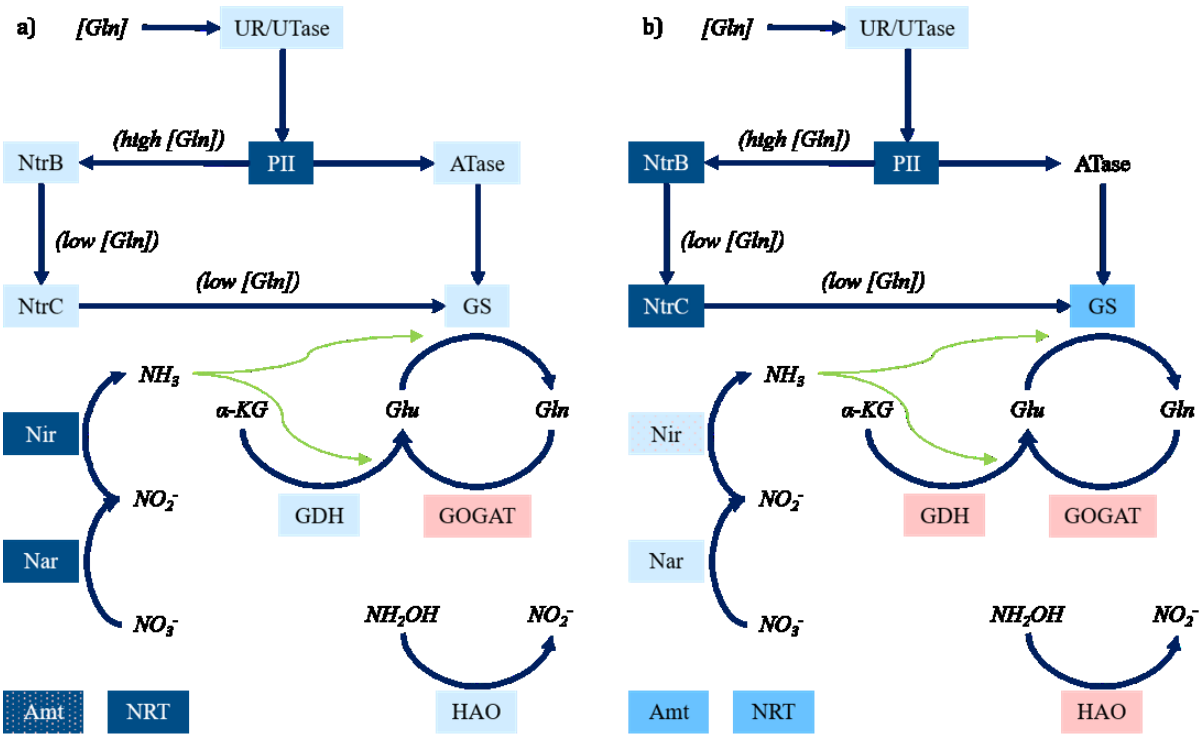

**Supp. Figure B-2**. Principal component analyses (PCA) of test conditions, grouped against base condition of methane-nitrate, determined using DESeq2 analysis on Geneious software. Relative distance from other replicates in PCA was used to determine presence or absence of outliers in data. Replicate 2 from methane-ammonium condition and Replicate 2 from methane-nitrate conditions were excluded from further analysis. Methane-ammonium vs. methane-nitrate is shown in (a), methanol-nitrate vs. methane-nitrate in (b), and methanol-ammonium vs. methane-nitrate in (c).




**Supp. Figure B-3**. Classification of significant differential gene expression (DGE) in *M. album* BG8, based on COG classification according to EggNOG database. Grey represents methanol-ammonium vs. methane-ammonium, and purple represents methanol-ammonium vs. methanol-nitrate. A = RNA processing and modification; B = Chromatin structure and dynamics; C = Energy production and conversion; D = Cell cycle control and mitosis; E = Amino acid metabolism and transport; F = Nucleotide metabolism and transport; G = Carbohydrate metabolism and transport; H = Coenzyme metabolism; I = Lipid metabolism; J = Translation; K = Transcription; L = Replication and repair; M = Cell wall/membrane/envelop biogenesis; N = Cell motility; O = Post-translational modification, protein turnover, chaperone functions; P = Inorganic ion transport and metabolism; Q = Secondary structure; T = Signal transduction; U = Intracellular trafficking and secretion.




**Supp. Figure B-4**. Differential regulation of methane oxidation and carbon assimilation *via* the RuMP pathway in *M. album* BG8. Methanol-ammonium vs. methane-ammonium (a), and methanol-ammonium vs. methanol-nitrate (b). Genes shown in red are comparatively upregulated in the experimental conditions, blue denotes downregulation. Dark arrows represent significant differential regulation (log-fold change > 1, adj. p-value < 0.01), medium-coloured represent differential regulation under adj-p-value < 0.05. Where striped, multiple genes showed both up- and down-regulation in test condition and the overall expression difference was used to determine the colour of the arrow.




**Supp. Figure B-5**. Differential regulation of nitrogen uptake and assimilation in *M. album* BG8. Methanol-ammonium vs. methane-ammonium (a), and methanol-ammonium vs. methanol-nitrate (b). Genes shown in red are comparatively upregulated in the experimental conditions, blue denotes downregulation. Dark arrows represent significant differential regulation (log-fold change > 1, adj. p-value < 0.01), medium-coloured represent differential regulation under adj-p-value < 0.05. Where striped, multiple genes showed both up- and down-regulation in test condition and the overall expression difference was used to determine the colour of the arrow.

## C) Supplementary Material for Chapter 5

**Supp. Table C-1**. Number of genes in *Methylocystis* sp. Rockwell that were differentially regulated between the test condition and reference base condition for each comparison. Results shown graphically in Figure 1. All genes counted were significant at log-fold change > |1| and adjusted p-value < 0.01. Darker boxes represent higher proportion of genes differentially regulated in that condition.

| | | Increase | | | | |
|---|---|---|---|---|---|---|
| | **Test Condition** | *Ammonium-Methane* | *Ammonium-Methanol* | *Nitrate-Methanol* | *Ammonium-Methanol* | *Ammonium-Methanol* |
| | **Base Condition** | *Nitrate-Methane* | *Nitrate-Methane* | *Nitrate-Methane* | *Ammonium-Methane* | *Nitrate-Methanol* |
| **A** | RNA processing and modification | 0 | 0 | 0 | 0 | 0 |
| **B** | Chromatin Structure and dynamics | 0 | 0 | 0 | 0 | 0 |
| **C** | Energy production and conversion | 17 | 24 | 3 | 19 | 10 |
| **D** | Cell cycle control and mitosis | 0 | 1 | 0 | 0 | 0 |
| **E** | Amino Acid metabolism and transport | 11 | 23 | 1 | 10 | 10 |
| **F** | Nucleotide metabolism and transport | 0 | 2 | 0 | 3 | 0 |
| **G** | Carbohydrate metabolism and transport | 1 | 6 | 0 | 3 | 3 |
| **H** | Coenzyme metabolis | 1 | 2 | 0 | 2 | 2 |
| **I** | Lipid metabolism | 2 | 7 | 1 | 6 | 0 |
| **J** | Translation | 0 | 2 | 0 | 3 | 0 |
| **K** | Transcription | 4 | 13 | 2 | 9 | 4 |
| **L** | Replication and repair | 32 | 110 | 6 | 61 | 19 |
| **M** | Cell wall/membrane/envelop biogenesis | 7 | 38 | 1 | 24 | 27 |
| **N** | Cell motility | 0 | 26 | 5 | 14 | 0 |
| **O** | Post-translational modification, protein | 3 | 12 | 1 | 9 | 3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | turnover, chaperone functions | | | | | |
| **P** | Inorganic ion transport and metabolism | 13 | 21 | 2 | 12 | 11 |
| **Q** | Secondary Structure | 2 | 7 | 1 | 8 | 2 |
| **T** | Signal Transduction | 2 | 18 | 2 | 9 | 8 |
| **U** | Intracellular trafficing and secretion | 10 | 22 | 1 | 3 | 0 |
| **Y** | Nuclear structure | 0 | 0 | 0 | 0 | 0 |
| **Z** | Cytoskeleton | 0 | 0 | 0 | 0 | 0 |
| **R** | General Functional Prediction only | 0 | 0 | 0 | 0 | 0 |
| **S** | Function Unknown | 44 | 92 | 17 | 47 | 33 |
| **n/a** | Unclassified | 77 | 223 | 9 | 135 | 67 |

**Decrease**

| | **Test Condition** | *Ammonium-Methane* | *Ammonium-Methanol* | *Nitrate-Methanol* | *Ammonium-Methanol* | *Ammonium-Methanol* |
|---|---|---|---|---|---|---|
| | **Base Condition** | *Nitrate-Methane* | *Nitrate-Methane* | *Nitrate-Methane* | *Ammonium-Methane* | *Nitrate-Methanol* |
| **A** | RNA processing and modification | 0 | 0 | 0 | 0 | 0 |
| **B** | Chromatin Structure and dynamics | 0 | 0 | 0 | 1 | 1 |
| **C** | Energy production and conversion | 3 | 30 | 2 | 23 | 16 |
| **D** | Cell cycle control and mitosis | 1 | 6 | 0 | 5 | 2 |
| **E** | Amino Acid metabolism and transport | 5 | 36 | 0 | 32 | 3 |
| **F** | Nucleotide metabolism and transport | 0 | 3 | 0 | 4 | 1 |
| **G** | Carbohydrate metabolism and transport | 0 | 8 | 0 | 6 | 3 |
| **H** | Coenzyme metabolism | 2 | 19 | 1 | 20 | 3 |
| **I** | Lipid metabolism | 1 | 12 | 0 | 11 | 15 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **J** | Translation | 0 | 12 | 0 | 16 | 1 |
| **K** | Transcription | 3 | 16 | 1 | 15 | 6 |
| **L** | Replication and repair | 2 | 21 | 0 | 29 | 1 |
| **M** | Cell wall/membrane/envelop biogenesis | 0 | 15 | 0 | 15 | 14 |
| **N** | Cell motility | 1 | 1 | 0 | 0 | 0 |
| **O** | Post-translational modification, protein turnover, chaperone functions | 2 | 48 | 0 | 33 | 10 |
| **P** | Inorganic ion transport and metabolism | 3 | 27 | 0 | 32 | 16 |
| **Q** | Secondary Structure | 1 | 4 | 0 | 6 | 7 |
| **T** | Signal Transduction | 1 | 12 | 0 | 7 | 0 |
| **U** | Intracellular trafficing and secretion | 1 | 9 | 0 | 8 | 5 |
| **Y** | Nuclear structure | 0 | 0 | 0 | 0 | 0 |
| **Z** | Cytoskeleton | 0 | 0 | 0 | 0 | 0 |
| **R** | General Functional Prediction only | 0 | 0 | 0 | 0 | 0 |
| **S** | Function Unknown | 19 | 103 | 4 | 84 | 39 |
| **n/a** | Unclassified | 20 | 114 | 1 | 67 | 50 |

**Supp. Table C-2**. Most highly differentially expressed genes in each test comparison, shown in log-fold change. All genes with adjusted p-value < 0.01. Hypothetical genes shown were analysed via BLAST to determine likely orthologs and function, BLAST-suggested functional assignment denoted by italicized text.

| Ammonium-Methane Vs. Nitrate-Methane | | |
|---|---|---|
| MET49242_RS00375 | NAD(P)/FAD-dependent oxidoreductase CDS | 6.70 |
| MET49242_RS00380 | nitrite reductase (NAD(P)H) small subunit CDS | 6.36 |
| MET49242_RS00390 | NarK/NasA family nitrate transporter CDS | 5.95 |
| MET49242_RS24345 | *AmP-dependent synthetase and ligase CDS* | 5.15 |
| MET49242_RS07865 | nitrogen regulatory protein P-II 1 CDS | 5.02 |
| MET49242_RS04530 | nitrogen regulatory protein P-II 1 CDS | 5.00 |
| MET49242_RS08475 | hypothetical protein CDS | 4.97 |
| MET49242_RS01005 | hypothetical protein CDS | 4.93 |
| MET49242_RS07870 | ammonium transporter CDS | 4.79 |
| MET49242_RS06005 | hypothetical protein CDS | 4.78 |
| MET49242_RS20365 | nitrogenase iron protein CDS | 4.65 |
| MET49242_RS20320 | nitrogen fixation protein NifQ CDS | 4.62 |
| MET49242_RS19120 | circularly permuted type 2 ATP-grasp protein CDS | 4.58 |
| MET49242_RS20435 | iron-sulfur cluster assembly accessory protein CDS | 4.56 |
| MET49242_RS20310 | Fe-S cluster assembly protein NifU CDS | 4.49 |
| MET49242_RS10595 | hypothetical protein CDS | 4.49 |
| MET49242_RS08470 | *bifunctional DNA primase/polymerase, N-terminal CDS* | 4.47 |
| MET49242_RS20315 | iron-sulfur cluster assembly accessory protein CDS | 4.47 |
| MET49242_RS04525 | ammonium transporter CDS | 4.46 |
| MET49242_RS20430 | tetratricopeptide repeat protein CDS | 4.45 |
| MET49242_RS23460 | hypothetical protein CDS | 4.40 |
| MET49242_RS20300 | homocitrate synthase CDS | 4.38 |
| MET49242_RS16280 | hypothetical protein CDS | -5.33 |
| MET49242_RS05210 | hypothetical protein CDS | -6.59 |

| Nitrate-Methanol Vs. Nitrate-Methane | | |
|---|---|---|
| MET49242_RS18895 | *M15 family peptidase CDS* | 3.70 |
| MET49242_RS18865 | hypothetical protein CDS | 3.70 |
| MET49242_RS01005 | hypothetical protein CDS | 3.55 |
| MET49242_RS23330 | *terminase CDS* | 3.50 |
| MET49242_RS21355 | flagellar biosynthesis regulator FlhF CDS | 3.41 |

| | | |
|---|---|---|
| MET49242_RS18885 | hypothetical protein CDS | 3.40 |
| MET49242_RS06005 | hypothetical protein CDS | 3.35 |
| MET49242_RS04660 | chemotaxis protein CheA CDS | 3.34 |
| MET49242_RS03010 | DNA-binding protein CDS | 3.33 |
| MET49242_RS21350 | flagellar biosynthesis repressor FlbT CDS | 3.29 |
| MET49242_RS24490 | transposase CDS | 3.23 |
| MET49242_RS14440 | hypothetical protein CDS | 3.23 |
| MET49242_RS20285 | electron transfer flavoprotein subunit beta/FixAfamily protein CDS | 3.22 |
| MET49242_RS12690 | IS66 family insertion sequence hypothetical protein CDS | 3.22 |
| MET49242_RS04665 | protein-glutamate O-methyltransferase CheR CDS | 3.22 |
| MET49242_RS12235 | IS110 family transposase CDS | 3.21 |
| MET49242_RS15040 | antirestriction protein ArdA CDS | 3.21 |
| MET49242_RS10245 | *phage conserved hypothetical protein, phiE125 gp8 family CDS* | 3.20 |
| MET49242_RS14460 | *transcriptional regulator, lysR family CDS* | 3.19 |
| MET49242_RS18870 | DUF2730 domain-containing protein CDS | 3.17 |
| MET49242_RS20735 | hypothetical protein CDS | 3.17 |
| MET49242_RS17560 | DUF2852 domain-containing protein CDS | -3.64 |
| MET49242_RS05285 | quinolinate synthase NadA CDS | -3.67 |
| MET49242_RS11240 | TetR family transcriptional regulator CDS | -4.02 |

| Ammonium-Methanol Vs. Nitrate-Methane | | |
|---|---|---|
| MET49242_RS00390 | NarK/NasA family nitrate transporter CDS | 5.18 |
| MET49242_RS20435 | iron-sulfur cluster assembly accessory protein CDS | 5.10 |
| MET49242_RS00380 | nitrite reductase (NAD(P)H) small subunit CDS | 5.05 |
| MET49242_RS18290 | hypothetical protein CDS | 4.93 |
| MET49242_RS18460 | glyoxalase/bleomycin resistance/dioxygenase family protein CDS | -4.64 |
| MET49242_RS07545 | Holliday junction branch migration DNA helicase RuvB CDS | -4.69 |
| MET49242_RS15970 | hypothetical protein CDS | -4.72 |
| MET49242_RS08560 | hypothetical protein CDS | -4.76 |
| MET49242_RS11240 | TetR family transcriptional regulator CDS | -4.82 |
| MET49242_RS06695 | molecular chaperone CDS | -4.90 |
| MET49242_RS17465 | DUF1150 domain-containing protein CDS | -5.04 |
| MET49242_RS17460 | (2Fe-2S)-binding protein CDS | -5.05 |
| MET49242_RS13195 | META domain-containing protein CDS | -5.20 |
| MET49242_RS17560 | DUF2852 domain-containing protein CDS | -5.21 |

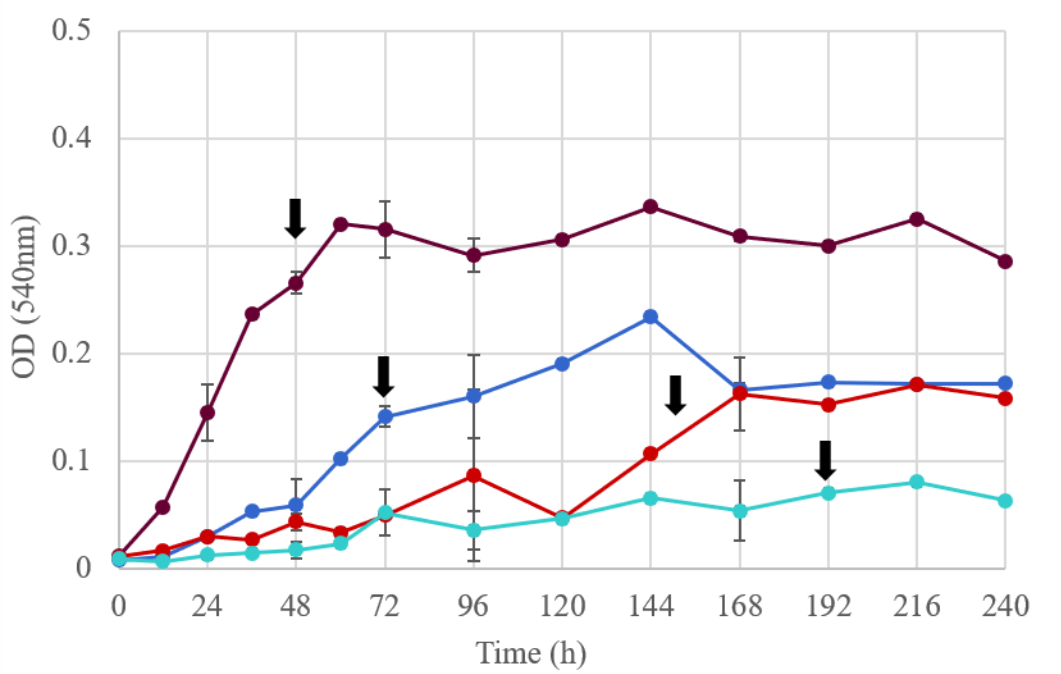
| | | |
|---|---|---|
| MET49242_RS02470 | histidine kinase CDS | -5.23 |
| MET49242_RS02740 | membrane protein CDS | -5.26 |
| MET49242_RS05285 | quinolinate synthase NadA CDS | -5.50 |
| MET49242_RS16395 | iron oxidase CDS | -5.96 |
| MET49242_RS05210 | hypothetical protein CDS | -5.99 |
| MET49242_RS22920 | MarR family transcriptional regulator CDS | -6.31 |
| MET49242_RS06865 | hypothetical protein CDS | -6.35 |
| MET49242_RS21260 | hypothetical protein CDS | -6.36 |
| MET49242_RS22915 | thioredoxin CDS | -7.43 |
| MET49242_RS09605 | hypothetical protein CDS | -7.90 |

| Ammonium-Methanol Vs. Ammonium-Methane | | |
|---|---|---|
| MET49242_RS19810 | hemerythrin CDS | 5.52 |
| MET49242_RS12215 | IS110 family transposase CDS | 5.42 |
| MET49242_RS02170 | hypothetical protein CDS | 4.90 |
| MET49242_RS12735 | *polyketide cyclase CDS* | 4.03 |
| MET49242_RS12745 | *NADH:ubiquinone oxidoreductase CDS* | 3.99 |
| MET49242_RS04525 | ammonium transporter CDS | -4.00 |
| MET49242_RS17335 | radical SAM protein CDS | -4.03 |
| MET49242_RS06865 | *LPXTG cell wall anchor domain-containing protein CDS* | -4.03 |
| MET49242_RS05285 | quinolinate synthase NadA CDS | -4.04 |
| MET49242_RS16395 | iron oxidase CDS | -4.17 |
| MET49242_RS17265 | hypothetical protein CDS | -4.27 |
| MET49242_RS02470 | histidine kinase CDS | -4.28 |
| MET49242_RS09605 | hypothetical protein CDS | -4.36 |
| MET49242_RS18460 | glyoxalase/bleomycin resistance/dioxygenase family protein CDS | -4.37 |
| MET49242_RS15965 | DNA polymerase Y family protein CDS | -4.43 |
| MET49242_RS23850 | ArsR family transcriptional regulator CDS | -4.68 |
| MET49242_RS18465 | transcriptional regulator CDS | -4.68 |
| MET49242_RS04530 | nitrogen regulatory protein P-II 1 CDS | -4.69 |
| MET49242_RS08560 | hypothetical protein CDS | -4.83 |
| MET49242_RS02940 | glyoxalase/bleomycin resistance/dioxygenase family protein CDS | -4.85 |
| MET49242_RS15970 | hypothetical protein CDS | -5.12 |
| MET49242_RS03285 | MarR family transcriptional regulator CDS | -5.20 |
| MET49242_RS22915 | thioredoxin CDS | -5.90 |
| MET49242_RS22920 | MarR family transcriptional regulator CDS | -6.06 |

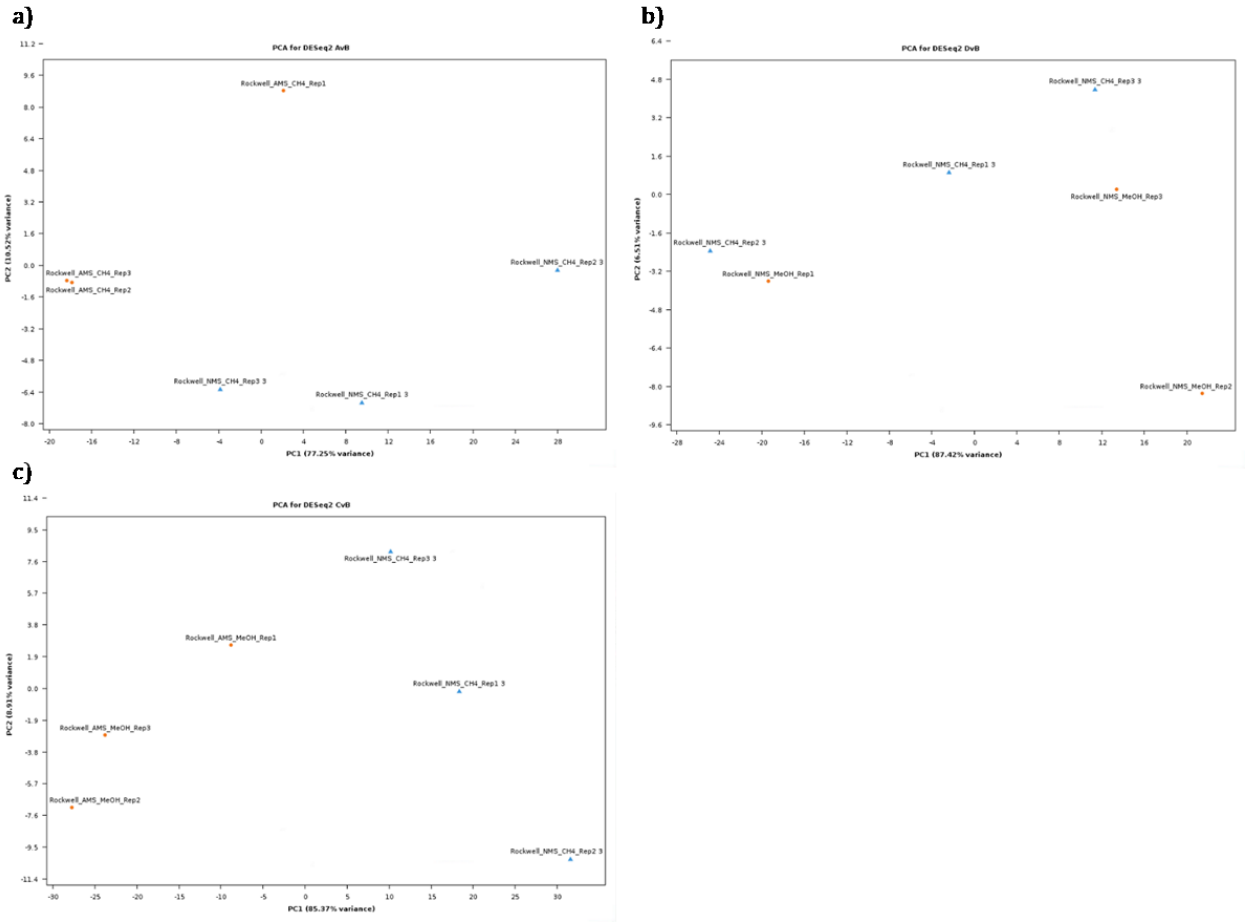
| | | Ammonium-Methanol Vs. Nitrate-Methanol |
|---|---|---|
| MET49242_RS00375 | NAD(P)/FAD-dependent oxidoreductase CDS | 5.81 |
| MET49242_RS00380 | nitrite reductase (NAD(P)H) small subunit CDS | 5.75 |
| MET49242_RS18290 | hypothetical protein CDS | 5.70 |
| MET49242_RS00390 | NarK/NasA family nitrate transporter CDS | 5.57 |
| MET49242_RS06355 | methylamine utilization protein MauG CDS | 5.34 |
| MET49242_RS02170 | hypothetical protein CDS | 5.18 |
| MET49242_RS05625 | TonB-dependent siderophore receptor CDS | 5.05 |
| MET49242_RS00385 | nitrate reductase CDS | 4.29 |
| MET49242_RS20450 | nif-specific transcriptional activator NifA CDS | 4.21 |
| MET49242_RS07865 | nitrogen regulatory protein P-II 1 CDS | 4.17 |
| MET49242_RS20645 | hypothetical protein CDS | 4.02 |
| MET49242_RS20435 | iron-sulfur cluster assembly accessory protein CDS | 3.86 |
| MET49242_RS20650 | alpha-amylase CDS | 3.78 |
| MET49242_RS11605 | porin CDS | 3.74 |
| MET49242_RS09945 | hypothetical protein CDS | -4.19 |
| MET49242_RS23635 | hypothetical protein CDS | -4.53 |
| MET49242_RS22045 | hypothetical protein CDS | -4.57 |
| MET49242_RS08430 | hypothetical protein CDS | -4.93 |
| MET49242_RS19820 | *dna ligase LigA CDS* | -5.37 |
| MET49242_RS05210 | hypothetical protein CDS | -6.10 |
| MET49242_RS22920 | MarR family transcriptional regulator CDS | -6.30 |
| MET49242_RS22915 | thioredoxin CDS | -6.64 |
| MET49242_RS21260 | hypothetical protein CDS | -6.87 |
| MET49242_RS09605 | hypothetical protein CDS | -8.87 |

**Supp. Table C-3**. Differential regulation of polyhydroxybutyrate (PHB) biosynthesis cycle in *Methylocystis* sp. Rockwell. Additional comparisons were done to provide context to methanol-ammonium condition: methanol-ammonium vs. methane-ammonium, and methanol-ammonium vs. methanol-nitrate. Genes shown in red are comparatively upregulated in the experimental conditions, blue denotes downregulation. Bold values represent significant differential regulation (log-fold change > 1, adj. p-value < 0.01), italicized are significant under adj. p-value < 0.05.

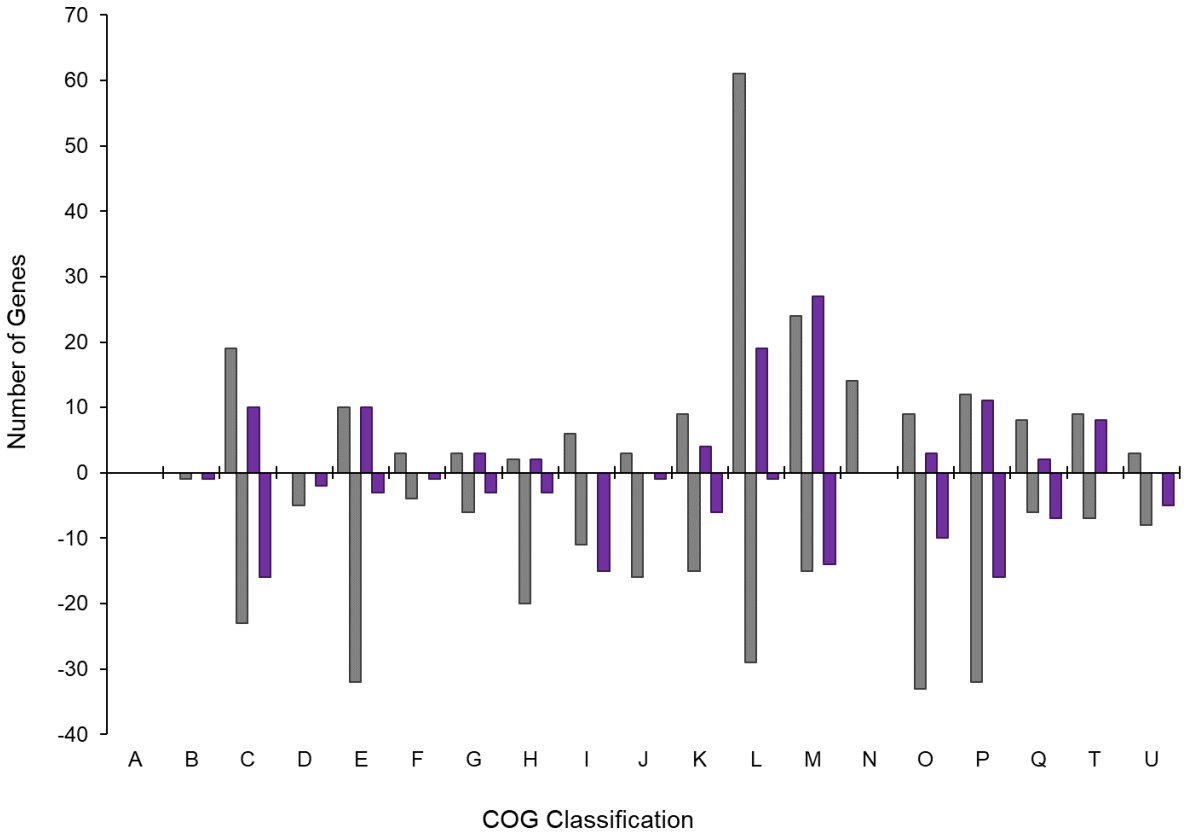
| | | Base Condition | Ammonium-Methanol | Ammonium-Methanol |
|---|---|---|---|---|
| | | Test Condition | Ammonium-Methane | Nitrate-Methanol |
| MET49242_RS21865 | phbA | acetyl-CoA C-acetyltransferase | **-2.22** | **-1.77** |
| MET49242_RS20855 | phbB | 3-hydroxyacyl-CoA dehydrogenase | *-0.96* | *-1.21* |
| MET49242_RS21780 | phbC | poly-beta-hydroxybutyrate polymerase | *-0.94* | **-2.11** |
| MET49242_RS13165 | phaZ | poly-hydroxyalkanoate depolymerase | **-2.71** | -1.23 |
| MET49242_RS16490 | bdhA | 3-hydroxybutyryl-CoA dehydrogenase | **-1.98** | -0.89 |
| MET49242_RS16295 | acsA2 | CoA-transferase | 0.66 | -0.15 |
| MET49242_RS21870 | phaR | poly-hydroxyalkanoate synthesis repressor PhaR | *0.84* | 0.39 |



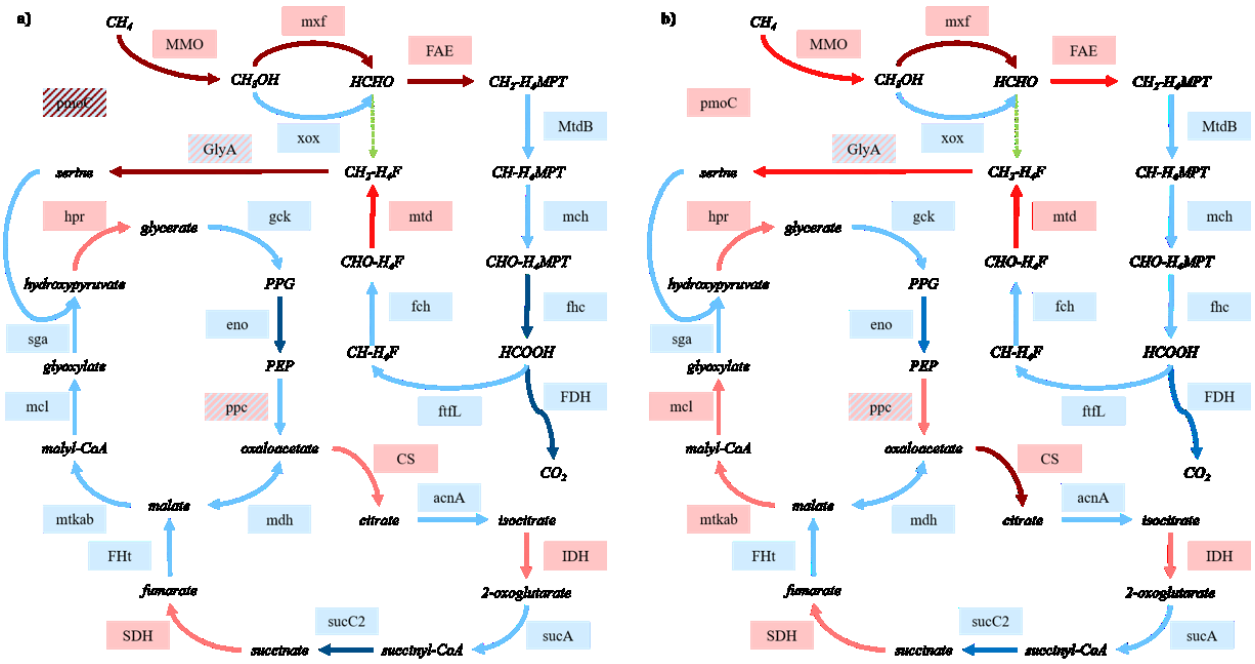

**Supp. Figure C-1**. Growth curve of *Methylocystis* sp. Rockwell under extraction conditions, in 100mL media in 250 mL Wheaton bottles. Methane-ammonium is purple, methane-nitrate is blue, methanol-ammonium is red, methanol-nitrate is teal. Arrows indicate time at which RNA was extracted from subsequent cultures.



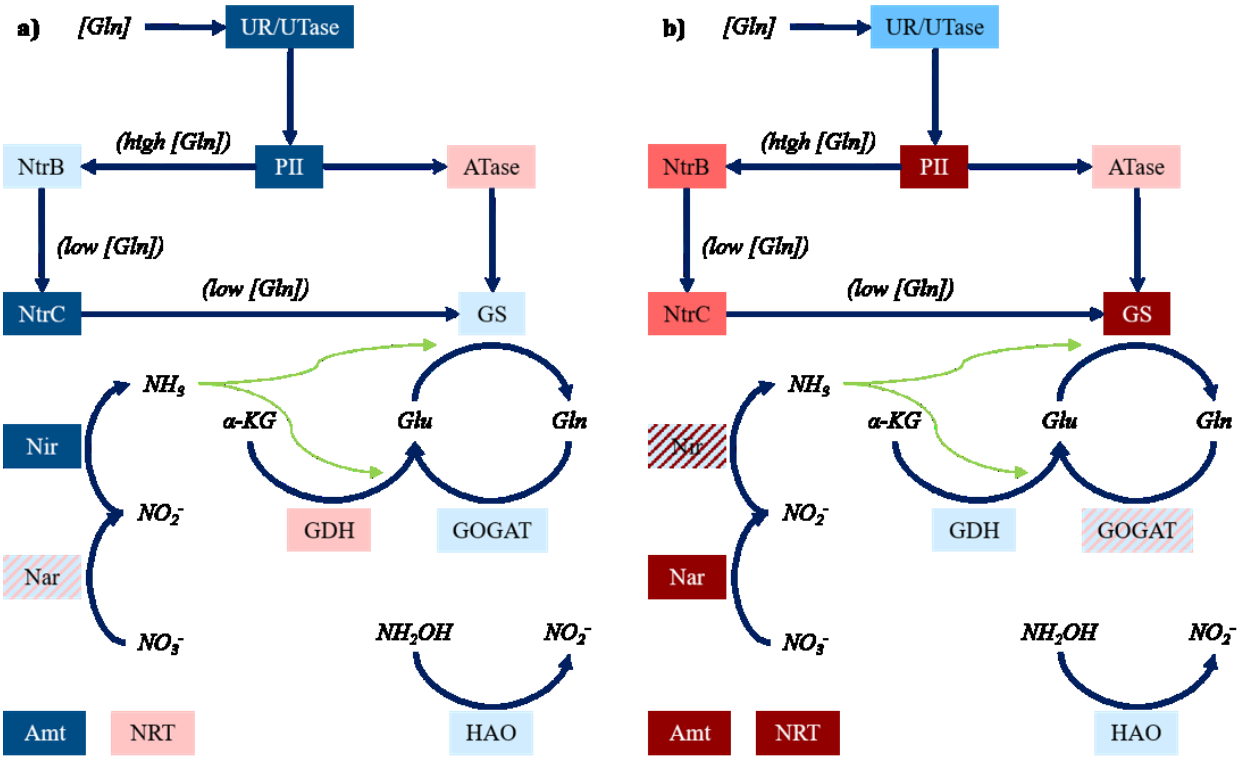

**Supp. Figure C-2**. Principal component analyses (PCA) of test conditions, grouped against base condition of methane-nitrate, determined using DESeq2 analysis on Geneious software. Relative distance from other replicates in PCA was used to determine presence or absence of outliers in data. From these data, Replicate 3 from methane-ammonium condition and Replicate 3 from methanol-nitrate conditions were excluded from further analysis.




**Supp. Figure C-3**. Classification of significant differential gene expression (DGE) in *Methylocystis* sp. strain Rockwell, based on COG classification according to EggNOG database. Grey represents methanol-ammonium vs. methane-ammonium, and purple represents methanol-ammonium vs. methanol-nitrate. A = RNA processing and modification; B = Chromatin Structure and dynamics; C = Energy production and conversion; D = Cell cycle control and mitosis; E = Amino Acid metabolism and transport; F = Nucleotide metabolism and transport; G = Carbohydrate metabolism and transport; H = Coenzyme metabolism; I = Lipid metabolism; J = Translation; K = Transcription; L = Replication and repair; M = Cell wall/membrane/envelop biogenesis; N = Cell motility; O = Post-translational modification, protein turnover, chaperone functions; P = Inorganic ion transport and metabolism; Q = Secondary structure; T = Signal transduction; U = Intracellular trafficking and secretion.




**Supp. Figure C-4**. Differential regulation of methane oxidation and carbon assimilation *via* the serine cycle in *Methylocystis* sp. Rockwell. Methanol-ammonium vs. methane-ammonium (a), and methanol-ammonium vs. methanol-nitrate (b). Genes shown in red are comparatively upregulated in the experimental conditions, blue denotes downregulation. Dark arrows represent significant differential regulation (log-fold change > 1, adj. p-value < 0.01), medium-coloured represent differential regulation under adj-p-value < 0.05. Where striped, multiple genes showed both up- and down-regulation in test condition and the overall expression difference was used to determine arrow colour.




**Supp. Figure C-5**. Differential regulation of nitrogen uptake and assimilation in *Methylocystis* sp. strain Rockwell. Methanol-ammonium vs. methane-ammonium (a), and methanol-ammonium vs. methanol-nitrate (b). Genes shown in red are comparatively upregulated in the experimental conditions, blue denotes downregulation. Dark arrows represent significant differential regulation (log-fold change > 1, adj. p-value < 0.01), medium-coloured represent differential regulation under adj-p-value < 0.05. Where striped, multiple genes showed both up- and down-regulation in test condition and the overall expression difference was used to determine arrow colour.