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Bio-conversion of Methane to Liquid Transportation Fuel Using Methanotrophic Bacteria

Ph.D. Thesis

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2018

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy, November 2018

Acknowledgments

Firstly, I would like to thank my supervisors Dr Ying Zhang and Professor Nigel Minton for their time, support and enthusiasm that made my Ph.D. an especially enriching experience. I am particularly grateful for the excellent guidance and mentoring I received that gave me confidence to improve and progress throughout my project.

I would like to express my sincere gratitude to Bashir Rumah and Emily Harding who not only assisted greatly with isolation and characterisation of methanotrophic bacteria but made the process such an enjoyable experience. Further to this I would like to thank Dr Naglis Malys, Erik Hanko, Denise Mclean, Dr Andrew Crombie and Dr Gareth Little for valuable input within the project. I would especially like to thank Bashir and Erik, in addition to my friends and colleagues from C51/B67, for the good times and making my time at the SBRC so enjoyable. Aside from my friends and colleagues I would like to express my gratitude to the BBSRC for the funding that enabled the research.

I would like to express my upmost appreciation for the people who have unrelentingly supported and encouraged me through my PhD and beyond. I give thanks to my partner Jennifer Cooke for unwavering encouragement through difficult times. Last but by no means least, I would like to thank my parents John and Judith Stead for being a constant source of inspiration for me and supporting me every step of the way.

Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

Chistopher Edward Stead.

Abstract

Methane is the second largest contributor to climate radiative warming with a global warming potential of ~34 times greater than that of carbon dioxide. Capturable methane from anthropogenic sources includes mainly natural gas and bio-gas and represents a flux of around 162 Mt yr^{-1}. Methane is often flared or vented due to difficulties with storage and transportation. Conversion technologies have poor scalability so are not viable at small or geographically isolated methane sources. It is envisaged that microbial bio-conversion of methane using methane oxidising bacteria (methanotrophs) can be used to biologically upgrade methane to liquid transportation. The present study set out to develop and explore a scalable bioconversion technology for conversion of methane to liquid transportation fuel. To do this an end-to-end approach was taken than included isolation of environmental methanotrophs, characterisation of isolates and metabolic engineering of isobutanol biosynthesis. Using bacterial isolation techniques such as extinction dilution plating and miniaturised extinction dilution methanotrophs were isolated from a variety of methane rich environmental samples such as freshwater sediment, soil and manure. These methanotrophic isolates were characterised and identified within established genera/species of which some are suspected to be novel species. Type I isolates included: Isolate 01; *Methylocystis* sp., isolate 03; *Methylocystis* sp., isolate 3*; *Methylocystis* nov sp. and isolate 6; *Methylocystis* SB2. Novel characteristics of the *Methylocystis* genus such as fimbriae were viewed using TEM in isolate 3*. Type I isolates included, isolate 14 *Methylococcus capsulatus* (isolated from the same sampling site as the type strain *Methylococcus capsulatus* (Bath)) and isolate 10 *Methylocaldum* nov sp. of which isolate 10 exhibited novel characteristics for the genus. Isolate 14 and 6 had their genome sequenced using a PacBio method and genomes were compared to closely related established strains genomes including *Methylocystis rosea* and *Methylococcus capsulatus* (Bath). Isolate 14 and isolate 6 were chosen for engineering and A molecular toolbox was developed including expression vectors, promoters and selection markers to facilitate the metabolic engineering of isolates and established strains. In addition to this an attempt was made to knockout carbon storage production within the isolates via allelic exchange. Metabolic engineering of an isobutanol biosynthetic pathway was employed by diverting flux from native valine biosynthesis to isobutanol. To do this a selection of heterologous keto-acid decarboxylases and alcohol dehydrogenases were overexpressed which, along with 2-ketoisovalerate feeding, yielded an isobutanol titre of 0.53 mM in *M. parvus*, 0.117 mM in *M. capsulatus* (Bath) and 0.27 mM in isolate 6 (*Methylocystis* SB2).

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Abbreviations

2,3BD	2,3-Butandiol
AD	Anaerobic Digestion
ADH	Alcohol Dehydrogenase

Amp	Ampacillin
AMS	Ammonium Mineral Salts
aTc	Anhydrotetracycline
CBB	Calvin Benson Bassham
CDW	Cell Dry Weight
Cm	Chloramphenicol
DNA-SIP	Deoxyribonucleic Acid Stable Isotope Probing
dNMS	Dilute Nitrate Mineral Salts
Ery	Erythromycin
EYFP	Enhanced Yellow Fluorescent Protein
FC	Flow Cytometery
FT	Fischer Tropsch
GC-MS	Gas Chromatography Mass Spectrometery
GTL	Gas To Liquid
ICM	Intracytoplasmic Membrane
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KDC	Keto-acid Decarboxylase
Km	Kanamycin
KmR	Kanamycin Resistance
LB	Luria Bertani
LDH	Lactate Dehydrogenase
MDH	Methanol Dehydrogenase
NMS	Nitrate Mineral Salts
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PHA	Polyhydroxyalkonate
PHB	Poly-3-hydroxybutyrate
pMMO	Particulate Methane Monooxygenase
RMS	Restriction Modification Systems
RuMP	Ribulose Monophosphate
SCP	Single Cell Protein
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sMMO	Soluble Methane Monooxygenase
TEM	Transmission Electron Microscopy
TSA	Tryptic Soy Agar

1 *Introduction*

1.1 Context

After carbon dioxide methane is the most abundant greenhouse gas with a global warming potential of ~34 times greater than carbon dioxide over a 100 year period [1]. Capturable waste methane is often released into the environment via: Petroleum extraction (92 Mt yr^{-1}), landfills (38 Mt yr^{-1}), wastewater treatment (21 Mt yr^{-1}) and manure management (11 Mt yr^{-1}) [2]. Methane from petroleum extraction exists as natural gas which contains a high methane content, over 90%, and the remaining mostly carbon dioxide. Other sources of methane are biologically derived from anaerobic digestion of organic matter known as biogas. This gas composition varies greatly though biogas derived from agricultural waste, for example, is composed of 50-80% methane and 30-50% carbon dioxide [3]. Both methane sources represent an underutilised feedstocks currently causing significant damage to the environment.

1.1.1 *Natural gas*

Natural gas forms in sub surface petroleum reservoirs and is released during petroleum extraction by means of drilling and hydraulic fracturing, or "fracking", of shale formations. Natural gas is commonly used as cheap gas for heating and cooking. Despite its value natural gas is often vented or flared into the atmosphere with global figures describing almost consistent rates of gas flaring from 2008-2016 of around 130 billion cubic metres (bcm) [4]. The factors causing flaring/venting include [5]:

- Natural gas is extracted in isolated regions (e.g. offshore sites) and is difficult to transport to the market
- Impurities existing in the natural gas are expensive to remove

- Storing large amounts of flammable gas is a substantial safety risk

To prevent flaring/venting though also avoiding the risks associated with methane, natural gas can be upgraded to liquid hydrocarbons via the Fischer-Tropsch (FT) method. This method is a multi-step process converting methane to syngas of which the syngas is then converted too long chain hydrocarbons using high pressures, temperatures and chemical catalysts. Due to these requirements of FT the process is capital intensive and so is only economically viable with a plant upwards of $20 billion [6]. Scalability and geographical isolation and finite lifespan of the reservoir greatly reduce the amount of natural gas reserves that can be exploited using this technique. Further methods to utilise the natural gas include liquefication which involves cooling the gas to around -161°C [7] to liquid form for transportation though this is energy and cost intensive especially when considering the economic value of the gas.

Conversion of natural gas too liquid fuel as a core concept is economically viable due to the comparable low price of natural gas and high price of gasoline, as seen a case study of natural gas in the US market as described in Figure 1. The presence of a one step, scalable, non-capital intensive GTL technology would improve the viability of capture and utilisation of natural gas. The resulting liquid fuel can be introduced into the existing petroleum infrastructure.

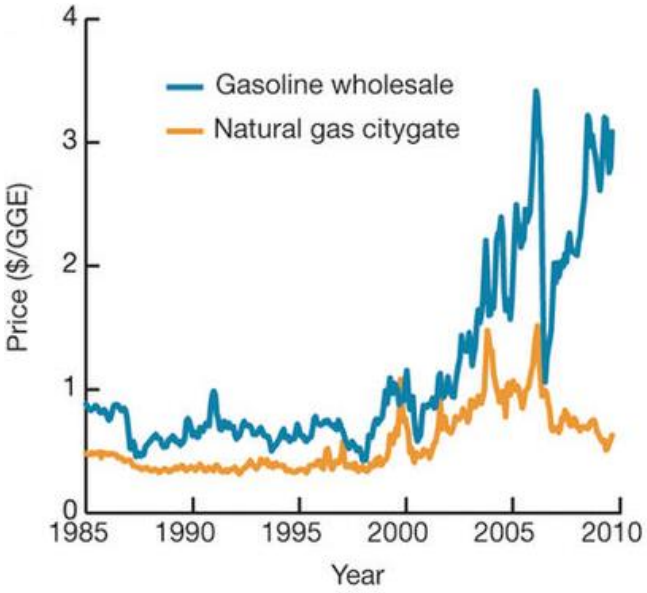


Figure 1 - US interstate/intrastate pipeline natural gas (citygate) wholesale price compared to gasoline price when based of Gallons of Gasoline Equivalent on energy basis (GGE) [6].

1.1.2 *Bio-gas*

Bio-gas from landfill and waste management has low incentive for capture due to low economic value. Low economic value is due to the high amounts of carbon dioxide present in the gas reducing its energy density. Bio-gas is a useful renewable feedstock and can be used to generate combined heat and power, though the heat/energy produced has to be used on site so in many cases is not appropriate [8]. Biogas can be upgraded to bio-methane to remove carbon dioxide, water and hydrogen sulphide via pressure swing adsorption. Upgrading the gas from around 40-70% to 97% methane allows for use on the gas grid though again this process suffers from high capital expenditure and so is only feasible at large scale which does not reflect the scale of many biogas sources. The process of upgrading to bio-methane often raises the price of bio-methane to exceed that of natural gas making the purification rarely economically feasible [9]. Other methods such as previously described GTL-FT technology also cannot economically convert these low quality/quantities.

Improvement on current GTL technology would allow for these significant but fragmented sources of low quality methane to be upgraded to liquid fuel.

1.1.3 *Scalable Methane Mitigation Solution*

An existing example of a scalable liquid fuel production technology can be seen in bioethanol. Bioethanol is a drop-in biofuel and so can be blended up to 5% in petrol vehicles. It is produced by bio-conversion of feedstocks such as corn, wheat, rice (first generation biofuel) or woods straws and grass (second generation biofuel) at ambient pressures and temperatures [10]. As seen in Figure 2, ethanol production from corn grain using yeast as a biocatalyst allows for much smaller, scalable, lower production of a liquid fuel compared to GTL-FT. The reason for this difference is that enzyme catalysis of the feedstock forgoes the need for high temperature high pressures or metal catalysts associated with chemical catalysis that increase capital expenditure.

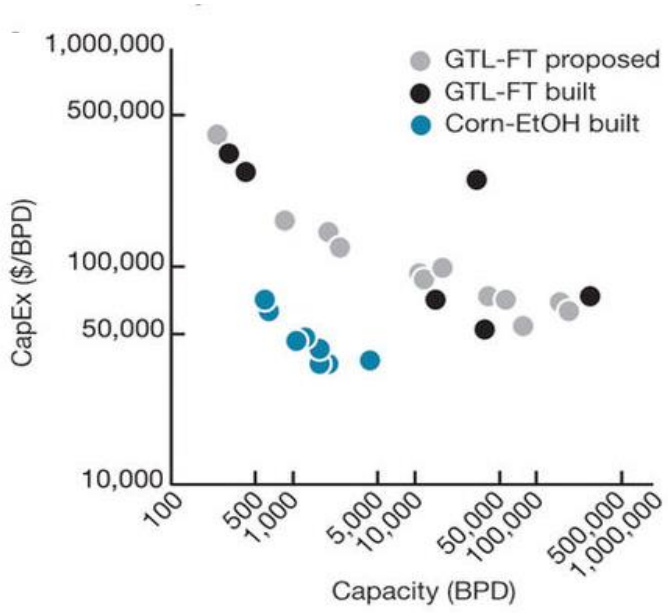


Figure 2 – Comparison of capital expenditure versus capacity output of, on an energy equivalent basis of gas to liquid fischer-tropsch proposed plants, gas to liquid fischer-tropsch currently built plants and corn to ethanol bioconversion plant [6]. CapEx, Capital Expenses . BPD, Barrel Per Day.

Using this concept, it is foreseeable that bioconversion of methane may provide a scalable method to convert methane gas to liquid fuel. Improved scalability would allow small scale conversion systems to be implemented on small, low grade or isolated methane reserves for example off shore oil rigs or sewage treatment plants. A group of microorganisms that can utilise methane are methane oxidising bacteria, or methanotrophs [11], these organisms use methane as a sole source of carbon and energy but are not known to endogenously produce a drop-in liquid fuel. This however could be overcome using well established metabolic engineering of heterologous metabolic pathways for drop-in fuel. This concept suggests a method where in which methane can be scaled appropriately to convert methane to drop-in liquid fuel.

1.2 Methanotrophic bacteria

Methanotrophy is a metabolism allowing a subsection of microorganisms to grow on methane as their sole source of carbon and energy. This ability is seen in both gram negative bacteria and also archaea. Methanotrophic archaea oxidise methane anaerobically in a consortia coupled with bacterial sulphate or Fe/Mn-oxide reduction, or alternatively in pure culture with nitrite/nitrate reduction [12]. Anaerobic methanotrophy will not be discussed further due to its less explored application for biotechnology.

1.2.1 *Metabolism and Taxonomy*

Methylotrophy is the ability of an organism to grow using one carbon compounds more reduced than formic acid as a source of carbon and energy e.g. methane, methanol methylated amines, halomethanes, and methylated compounds. Obligate methanotrophy is a subset of this group as it is defined as an organisms ability to

utilize methane as a sole source of carbon and energy [11]. In addition to obligate methanotrophy, facultative methanotrophy has also been observed which has proved to be controversial topic due to mis-identification of syntrophic consortia of heterotrophs and methanotrophs as facultative methanotrophs [13]. Facultative methanotrophy includes the ability to use methane but also acetate, pyruvate, succinate, malate or ethanol as an alternate carbon source. Often true facultative methanotrophic growth on alternate substrates is very slow (*M.silvestris* growth rate on acetate is $0.033h^{-1}$) and so methane is considered the primary metabolism in methanotrophs [13].

Methane is converted to carbon dioxide through a four step reaction with methanol, formaldehyde and formate as intermediates. This first step of this pathway is catalysed by one of two isozymes of methane monooxygenases (MMO) which include a particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO). The pMMO is located in a cytoplasmic membrane and is composed of three polypeptides: *pmoA* (β subunit, 24kDA), *pmoB* (α subunit, 47 kDA) and *pmoC* (γ subunit, 22kDa) [14]. *pmoA* and *pmoC* are transmembrane helices whereas *pmoB* has both a transmembrane and periplasmic domain of which the latter contains a Cu active site. In-contrast sMMO is an enzyme system located in the cytoplasm and consists of a hydroxylase, a regulatory protein and a reductase. The hydroxylase (MMOH) has a diiron centre and is composed of 3 polypeptide sub units MmoX (α subunit, 60.6 kDa) MmoY (β subunit, 45 kDa) and MmoZ (γ subunit, 19.8 kDa). It is coupled with a reductase, MmoC (or referred to as MMOR), which has a 2Fe-2S cluster. MmoC transfers electrons from NADH to the hydroxylase B (also known as MmoB) which has a regulatory function where it affects the access of the hydroxylase active site [15]. Most methanotrophs contain pMMO with the exception of

Methyloferula stellata [16] and *Methylocella silvestris* [17] which contain only sMMO. Other species from genera such as *Methylococcus*, *Methylosinus*, *Methylocystis*, *Methylomonas*, *Methylomicrobium* and *Methylovulum* contain both sMMO and pMMO. The membrane that pMMO exists in is termed the intracytoplasmic membrane (ICM). The physiological arrangement of the ICM differs in Type I methanotrophs (of the class *Gammaproteobacteria*) and type II (of the class *Alphaproteobacteria*). As seen in Figure 3B+C Type I formations exist as bundled vesicles anti-parallel to the cell wall whereas type II ICMs run in parallel along the periphery of the cell.

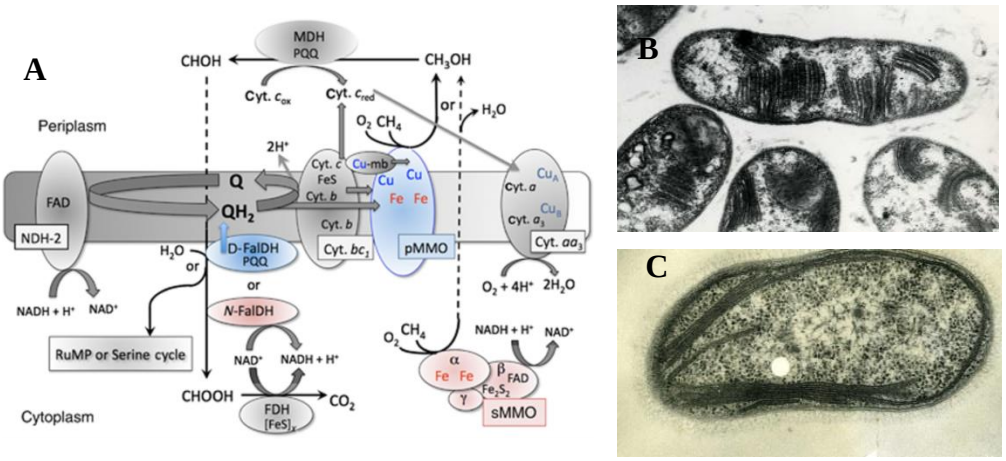


Figure 3 – (A) Methane oxidation in high and low Cu conditions. Proteins expressed under high Cu in blue, and low Cu red. Cyt, cytochrome; D-FalDH, dye-linked/quinone-linked formaldehyde dehydrogenase; FDH, formate dehydrogenase; N-FalDH, NAD(P)-linked formaldehyde dehydrogenase; NDH-2, type 2 NADH dehydrogenase; pMMO, membrane-associated or particulate methane monooxygenase; Q, ubiquinone; FAD, flavin adenine dinucleotide; MDH, methanol dehydrogenase; PQQ, pyroloquinoline quinone; sMMO, cytoplasmic or soluble methane monooxygenase; RuMP, ribulose monophosphate [18]. (B) Typical type I ICM [19] (C)Typical type II ICM [19].

Carbon is assimilated to biomass at formaldehyde and occurs via distinct pathways, in Type I organisms by the ribulose monophosphate (RuMP) cycle and Type II by the serine cycle as seen in Figure 4. A sub-division of the Type I group is the Type X

group which contains *Methylococcus capsulatus* which primarily uses the RuMP cycle but also possesses enzymes for the serine and Calvin-Benson-Bassham (CBB) for carbon dioxide fixation. Type III methanotrophs, *Verrucomicrobia*, are extreme acidophiles and thermophiles that use the CBB cycle as their sole carbon fixation pathway [20].

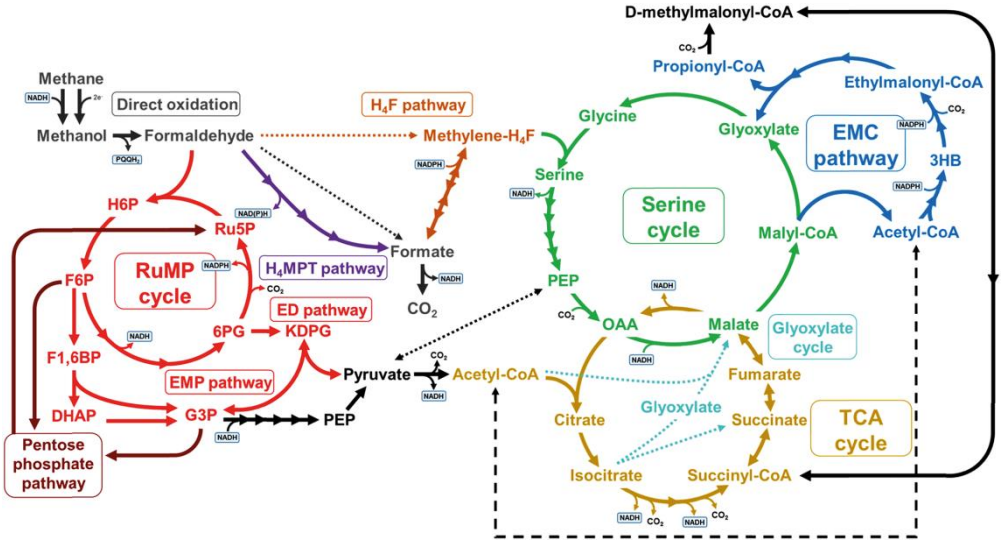


Figure 4 – A simplified metabolic network of type describing the RuMP pathway (type I methanotrophs) and serine cycle and EMC cycle (type II methanotrophs) [21].

Further differences between these classifications include carbon storage. Type II organisms accumulate poly-3-hydroxybutyrate (PHB) as a carbon storage compound [22] whereas type I organisms produce exopolysaccharides or glycogen [23] under nitrate/nutrient limiting conditions.

1.2.2 *Ecology*

Methane is produced in the environment by anaerobic digestion (AD) (which consists of hydrolysis, acidogenesis, acetogenesis and methanogenesis) of organic compounds is catalysed by a consortia of anaerobic bacteria and archaea. The significant sources of methane of this manner include: wetlands, rice paddies, ruminant animals, landfill,

oceans, and hydrates (e.g. lakes). These respectively contribute 145, 60, 93, 50, 10 and 5 Tg yr^{-1} [24]. Methanotrophs exist at the aerobic region of the aerobic anaerobic interface where the AD derived methane can be utilised by the methanotrophs. It is estimated that around 60-90% of methane produced in anaerobic zones of wetlands can be re-oxidised in the aerobic rhizosphere soil-water interface [25].

1.3 Isolation of Methanotrophic Bacteria

1.3.1 *Isolating pure cultures of methanotrophic bacteria*

Isolating pure cultures of methanotrophic species is essential for strain characterisation and understanding the methane cycle in its broader context. Aside from this, pure cultures are essential in an industrial sense as strain development is only effective using a single species.

The first ever isolated methanotroph was that of *Bacillus methanica* made by N.L. Sohngen in 1906 [26]. After this little progress was made in the field until the isolation and characterisation of *Methylococcus capsulatus* in 1966 [27]. Shortly after this in 1970 an expansion of the study of methanotrophs occurred when R. Whittenbury [28] isolated more than 100 pure cultures of methanotrophs. Whittenbury's method was based on enrichment of environmental samples suspected to contain methanotrophs, using Nitrate Mineral Salts (NMS) medium and methane, followed by dilution and plating onto respective solid growth media and colony picking and re-streaking to purity.

While using this method Whittenbury encountered two scavenging activities of non-methanotrophic colonies and contamination of methane consuming colonies. This syntrophic relationship of heterotrophs scavenging carbon and stimulation of methanotroph growth by heterotrophs has been characterised in much more detail

since its discovery. Carbon scavenging is an effective strategy for heterotrophs as it provides them with a source of carbon compounds when suitable carbon sources are not available. Scavenging compounds derived from methane oxidation has been seen in rice paddy rhizospheres using DNA-stable isotope probing (DNA-SIP). Using ^{13}C-labelled methane with subsequent analysis of 16S rDNA sequences, it was revealed that a variety of non methanotrophic bacteria, some of which included *Sphingomonadales, Rhozobiales* and *Rhodospirillales* (that cannot consume methane directly), were found to have metabolised methane derived carbon strongly suggesting cross-feeding of metabolites secreted from methanotrophic bacteria. Evidence of cross feeding of *Hyphomicrobium* methylotrophs and other heterotrophs including *Bacteroidetes* [29, 30] have been known to contaminate batch culture fermentations, the contamination has been suggested to remove excess methanol from the culture which is believed to have an inhibitory effect to methanotrophs due to the increased concentration of toxic formaldehyde [31, 32]. Artificial communities of a *Methylomonas spp.* with increasing heterotroph richness show an increase in methane oxidation [33]. A clearly elucidated mechanism to explain this was demonstrated when *Methylovulum miyakonense* was co co-cultured with common contaminating species during isolations, the contaminants that improved growth had there spent media (grown in pure culture) analysed and it was shown that cobalamin excreted by a *Rhizobia* species significantly improved growth of *M.miyakonese* [34].

Whittenbury's method for successfully overcoming this syntrophic bond and isolating pure methanotrophic cultures from mixed cultures laid the foundation for subsequent isolations and although many methods have been developed the fundamental principle remains. An overview of key points from methods and outcomes are described below:

Enrichment dilution isolation, Over 100 methanotrophs [28] – Samples were taken from ponds, rivers, streams and ditches. Environmental samples were used to NMS and ammonium mineral salts (AMS) media and enriched at 3:7 (methane:air) and growth was seen in 3 to 4 days. Cultures were then serially diluted onto respective solid agar and further enriched with methane. Non-methanotrophic colonies were seen at 3 days and methanotrophs typically seen at 5 to 7 days which were re-streaked onto fresh medium and assessed for purity by growing with and without the presence of methane and assessing growth.

Enrichment dilution isolation, *Methylovulum miyakonense* [35] – Soil samples were mixed in NMS media and enriched in a 1:4 (methane:air) headspace, incubated at 28°C until turbid, sub cultured into fresh NMS media and the same step repeated. Cultures then serially diluted and spread onto NMS agar plates in methane atmosphere. Single colonies were picked and inoculated into NMS medium with 0.01% Bacto tryptone. This final step was repeated and purity was assessed on LB agar and cell uniformity using light microscopy.

Opposing methane oxygen gradient isolation, *Methylomonas* sp. and *Methylobacter* sp. [36] *Methyloglobulus morosus [37]* – The enrichment step was performed in glass tubes (8 mm diameter and 12 cm length) and sealed with polytetrafluoroethylene filters and held by silicone septa. A semi-solid dilute Nitrate Mineral Salts (dNMS) medium with 0.2% agarose mixture was inoculated (inoculum was sediment dissolved in dNMS and diluted up to 1×10^{-7}), one end of the tube was exposed to 2% CO_2, 24% CH_4 and balance N_2 whilst the other end air. This was designed to recreate the natural anaerobic/aerobic interface where methanotrophs are usually present. Growth was seen as bands forming in the gel and were excised,

serially diluted, grown in media, and the most dilute culture to grow was streaked on agar. Smaller colonies were extracted using binocular microscope and restreaked. Purity was assessed on rich media. Strains were assessed using *pmoA, mmoX* and 16S rRNA analysis.

Miniaturized extinction isolation, [38] *Methyloparacoccus murrellii* gen. Nov. sp. nov. [39] and *Methylomonas lenta* sp. nov. [40] – Homogenised sample in 27ml of NMS medium with 0.8 µM Cu^{2+}, and 2 mM phosphate buffer. The samples were diluted 10^{-2} to 10^{-11} and enriched with methane within vials for 5 weeks. Methanotrophic positive cultures were identified by testing methane/oxygen/carbon dioxide concentrations using GC. Followed by extinction dilution culturing (10^{-2} to 10^{-9} dilution) in a 96 well plate containing dNMS, enriched with methane. Growth was observed by change in OD at 600nm. The highest dilution was plated on dNMS and sub-cultured to purity. Purity assessed with phase contrast microscopy, and absence of growth on rich media agar. This technique was run in parallel with traditional plating dilution to extinction methods and yielded 22 methanotrophs compared to more tradition plating methods which did not yield any pure colonies.

Enrichment isolation using variation in culture conditions, *Methylocystis* sp., *Methylomonas* spp,, *Methylobacter* spp., *Methylosinus* spp.. [41]**–** Lake sediment samples were enriched using NMS medium and nitrate-free mineral salts containing no added copper, headspace (methane:air) of 50:50, 80:20, 5:95 and incubated at room temperature at 200 rpm. Serial dilutions were plated onto NMS agar with 10 µM of $CuSO_4$ and incubated at 50:50 (methane:air). This yielded the isolation of five methanotrophs *Methylocystis* and *Methylosinus* were produced from 5 and 50% methane, only *Methylosinus* was obtained from 80% methane. Similar enrichment

isolations were performed though enrichments used lake sediment and filter sterilised lake water at 1:1 (v/v) then inoculated into NMS with 10, 20 and 40 µM of $CuSO_4$. Also a final enrichment using unfiltered pore water inoculated into NMS supplemented with 10 µM $CuSO_4$. 50:50 (methane:air) was used for all and incubated at 25°C at 200 rpm. Serial dilution was done on the samples and inoculated onto NMS agar plates supplemented with 10 µM $CuSO_4$ under similar conditions. A further six methanotrophs were isolated including *Methylobacter*, *Methylomonas* and *Methylosinus*. Isolates were characterised for *pmoA* and *mmoX* also 16S rRNA genes were sequenced. A range of isolates were found though no correlation to methane ratio and the presence of copper could be concluded.

1.3.2 *Characterisation of novel species*

Characterisation of bacteria is essential for taxonomic identification within existing or novel taxons. Taxonomy is a rank based classification system for organisms consisting of ranks Domain, Kingdom, Phylum, Class, Order, Family, Genus and species. Taxonomic ranks used to name an organism in its most precise terms are genus and species. This is known as binary nomenclature. To ascertain which genus or species an isolated bacterium belongs to a variety of phenotypic and genotypic characterisations such as 16S rRNA comparison, GC content, cell morphology and fatty acid profile are assessed [42]. Characterisation of methanotrophs requires these traits in addition to other methanotroph specific traits. Additional characteristics refer to traits connected to methane metabolism for example the specific type of MMO or ICM. Methanotrophs should be characterised using the approach described by [43] as a reference. A high quality description of novel methanotrophs can be seen in Table 1.

Table 1 - Characteristics for a high quality description for identification of a novel methanotroph [43].

Characteristics	Examples in Methanotrophs
Morphology and ultrastructure	Cocci, Rod etc.
Cell shape, cell division, capsules	Rosettes and budding cell division (*Methylosinus* spp., some *Methylocella* spp.); sarcinal packets (*Methylosarcina spp*.)
Mode of motility	polar flagella otherwise
Resting cells—type and relation to survival of desiccating conditions and heating	Cysts (most type I methanotrophs; *Methylocystsis* spp.); exospores (*Methylosinus* spp.)
Cell wall stability based on lysis in 2% sodium dodecyl sulfate solution	Type II methanotrophs are more stable and resist lysis in 2% SDS
Intracytoplasmic membrane (ICM) presence and morphology	Absent in *Methylocella* spp. Seen aligned parallel to the membrane and in antiparallel stacks
Spinae (extensions of the S-layer)	Formed by some *Methylocystis* spp. (can be up to 150 nm)
Intracellular granules—polyhydroxyalkanoate (PHA) and polyphosphate bodies	Large PHA granules may occur depending on the strain (possibly can be confused with cysts)
Pigmentation	*Methylocystis rosea* shows pink pigmentaation
pH range; NaCl tolerance; temperature range for growth	*Methylocella* and *Methylocapsa* are acidophilic; various type I methanotrophs show preferences for different pH/ salinity/ temperature
Naphthalene oxidation assay for sMMO in the presence and absence of copper	Methylocella spp. possess only sMMO and Methylococcus capsulatus contains both pMMO and sMMO
Formaldehyde fixation pathway	Ribulose monophosphate pathway (type I methanotrophs); Serine pathway (type II methanotrophs); Calvin Benson Bassham cycle (type III)
Catalase, cytochrome *c* oxidase	
Completeness of the TCA cycle; presence of glyoxylate cycle, glycolysis, and pentose phosphate pathway key enzymes	2-Oxoglutarate dehydrogenase may be absent in some type II methanotrophs
Other enzymatic activity—phosphatases, nitrate reduction, urease	
Carbon/nitrogen source tests	Many strains can use a range of amino acids as nitrogen sources
Nitrogen fixation – resting cell acetylene reduction assay; *nifH* gene sequencing	May vary between strains of the same species

Although there is no specific cut-off for defining a novel isolate there has to be genotypic and/or phenotypic evidence in divergence from the existing species [42]. As a result a novel species can usually be described when one or multiple characteristics seen in Table 1 differ from existing characterised species along with evidence of 16S rRNA sequence divergance. The 16S rRNA plays a structural role in ribosomes and so the corresponding DNA is a conserved sequence found in all prokaryotes making it ideal for resolving phylogenetic relationships between organisms.

1.4 Methanotroph Biotechnology

Due to the unique capability of methanotrophs to utilise methane, methanotrophs have been proposed and implemented in variety biotechnological applications. The applications reviewed below and have been separated into use of naturally occurring methanotrophs with no genetic modifications and also engineered methanotrophs that are genetically recombinant strains with capabilities to produce compounds not found in nature.

1.4.1 *Use of naturally occurring methanotroph strains*

1.4.1.1 Bioremediation

The broad substrate specificity of pMMO (particulate methane monooxygenase) and especially sMMO (soluble methane monooxygenase) allow for a range of toxic compounds, such as halogenated and aromatic compounds, to be broken down into less toxic compounds [44]. *In-situ* remediation of these compounds can be done by stimulating the growth of native methanotrophs within the ecosystem [45]. Examples of *ex-situ* implementation include methanotrophic co-metabolism of a mixed consortia in a two stage bioreactor system for trichloroethylene degradation [46].

1.4.1.2 Bio-filtration

The ability of methanotrophs to consume low grade methane in the form of bio-gas [47] enables the bacteria to utilise waste off-gas from methanogenic sources such as landfills or coal mines. Bio-filtration technology mitigates emissions by diffusing the methane rich gas through soil (containing native or engineered consortia of methanotrophs) enriched with nutrients or controlled temperature to optimise methane to be consumed by the methanotrophs. There are many examples of effective bio-filtration [48], one example by Park *et al* showed by using initial methane concentrations of between 5-53% (v/v) with an optimum temperature of 30°C can

achieve up to 83% methane consumption proving it to be an extremely cost-effective means of methane mitigation [49].

1.4.1.3 Bio-products

A variety of value added products can be produced by non-engineered methanotrophs using methane at a yield competitive with heterotrophic bacteria. A variety of examples can be seen in Table 2:

Table 2 – Value added bio-products produced by non-engineered methanotrophs (adapted from [50]). Poly-3-hydroxybutyrtate (PHB), Single Cell Protein (SCP), Exopolysaccharides (EPS).

Product	Use of bio-product in methanotrophs	Use of bio-product in industry	Heterotrophic yield (mg g biomass−1)	Methanotrophic yield		Main methanotrophic producer
				mg g biomass−1	g/L	
Ectoine	Osmotic balance in halophiles	Medicine, cosmetology, dermatology	120–270	70–230		*Methylomicrobium*
PHB	Carbon storage compound	Bio-plastics	300–850	100–500		*Methylocystis, Methylosinus*
SCP	Biomass	High protein content for animal feed	680–710	690–730		*Methylomonas, Methylococcus*
Lipids	Biomass	Chemical transformation to biofuel	300–510	200–500		*Methylococcus, Methylosinus, Methylocystis*
EPS	Structural and protective use [51]	Foods, pharmaceutical or textiles due to adhesive properties	600–2775	300–1800		*Methylobacter, Methylomonas, Methylomicrobium*
Methanol [47]	Intermediate in methane oxidation	Liquid fuel or platform chemical	N/A		1.1	*Methylosinus, Methylocystis, Methylocaldum, Methylocella*

Due to the economic feasibility of the production, private companies are currently engaged in production and development of these technologies. Key examples include Calysta and Uni bio for production of SCP and also Mango Materials and Newlight for production of biodegradable plastics. It is envisaged that future success of this

technology will lie in separation of multiple fractions of value added products from single fermentation batch [2].

1.4.2 *Use of engineered methanotroph strains*

With the high viability of methane as a feedstock and improved genetic tools for methanotrophs a range of recombinant methanotrophic strains have been engineered to produce exogenous compounds and overproduce endogenous ones. A review by Lee *et al* [21] has been adapted below detailing the products and approach:

1.4.2.1 Carotenoids

Canthaxin and astaxanthin are carotenoids that are used as feed additives for aquiculture [52] and are increasingly being shown to have pharmaceutical applications [53]. The obligate methanotroph *Methylomonas* sp. strain 16a was engineered to heterologously produce canthaxin and astaxanthin [54]. This was achieved by disruption of its native C30 carotenoid biosynthesis pathway and an alternative C40 pathway was introduced. The approach taken was to integrate three carotenogenic promoterless gene clusters into the genome via a Tn5 transposon in order for native promoters to drive the expression of each operon. Kanamycin resistant transposon colonies were screened by observing orange to dark red colouration indicating the presence of C40 carotenoids. Carotenoid quantification showed five chromosomal regions were giving high carotenoid production (*fliCS*, *hsdM*, *ccp-3*, *cysH*, and *nirS*). The native promoters for each of these Open Reading Frames (ORFs) gave between 10 and 20 fold carotenoid synthesis increase over an exogenous chloramphenicol promoter at a neutral genome location. This work was furthered by increasing in the proportion of astaxanthin in overall caretonoid profile by improving oxygen availability from chromosomally integrated recombinant cyanobacterial haemoglobin *thbN1* [55].

1.4.2.2 Lactic acid

Lactic acid is an attractive platform chemical for the production of derivatives such as polylactic acid which can be used to make biodegradable plastics [56]. A haloalkaphilic methanotroph isolated on natural gas with a doubling time of 3 hours [57], *Methylomicrobium buryatense,* was used to overexpress L-lactate dehydrogenase yielding 0.8 g/L of lactate [58]. The approach taken for this was to assess the minimum inhibitory concentration of lactate which significantly inhibited growth at around 1g/L. To regulate heterologous gene expression a tetracycline promoter/operator was taken from pASK75 and was using a green fluorescent protein (GFP) as a reporter and strongly tightly regulated expression was seen under the minimum inhibitory concentration of the effector molecular anhydrotetracycline. Expressing lactate dehydrogenases (LDH) from *Bifidobacterium longum*, *Lactobaccilus helveticus*, *E. coli* and native *M. buryatense* were overexpressed and showed that the heterologous codon-optimised *L. helveticus* LDH produced the greatest amount of lactate. The process was up-scaled to a bioreactor to improve lactate yield and in a 5.0L continuously stirred bioreactor 0.8 g/L was produced over a 96 hour period.

1.4.2.3 Succinic acid

Succinic acid is an industrially relevant chemical used in the production of detergents, surfactants, resins, inks and bioplastics [59]. Engineering of *M. capsulatus* (Bath) allowed a >50% improved succinic acid production when compared to the native strain [60]. This was done by constitutive expression of pyruvate carboxylase using a variant of plasmid pMHA201 [61]. The variant of pMHA201, psB108, is a broad host range vector containing an Origin of replication (OriV), Origin of transfer (OriT) used for conjugative transfer, and a kanamycin marker (Km).

1.4.2.4 Isoprene

Isoprene is a feedstock used in manufacturing of rubber, adhesives and perfumes also has potential for oligomerisation to generate supplement blends for gasoline, diesel or jet fuel [62]. Isoprene was produced in *M. capsulatus* by expressing heterologous isoprene synthase (*IspS*) [63]. The one step reaction catalysed the conversion of endogenous dimethylallyl-DP to isoprene. The gene was expressed using a plasmid based expression system driven by a methanol dehydrogenase (MDH) promoter or IPTG-inducible (*LacIq*). Two isozymes of *IspS* from *Pueria montana* and *Salix* sp. of which *P.montana* outperformed the *Salix* sp. *IspS*. Furthermore, the increase of induction (0.1-10mM IPTG) of *IspS* with *LacIq* proportionally increased the production of isoprene.

To further improve isoprene production a heterologous 1-deoxy-D-xylulose-5-phosphate (DXP) pathway consisting of *IspD*, *IspE*, *IspF*, *IspG* and *IspH* which increases the amount of isopentenyl-DP and dimethylallyl-DP precursors for isoprene. The pathway was optimised by subjecting the encoded pathway to error prone PCR, and polycistronically coupled with a lycopene biosynthesis operon (*ggpps*, *crtB* and *crtI* genes) which produces a quantifiable colorimetric output proportional to the flux of the DXP pathway. The mutant pathways with high flux were selected and subject to the mutagenesis further, this was cycled until the colorimetric output did not increase any further. The new optimised DXP pathway was then coupled back with *IspS* to produce an improved isoprene yield.

1.4.2.5 2,3-butandiol

The diol 2,3-butandiol (2,3BD) is a platform chemical used for the application of manufacturing printing inks, perfumes, plasticizers and pharmaceuticals [64].

Methylomicrobium alcaliphilum 20Z was engineered to produce 86.2 mg/L of 2,3BD from methane.

Initially the plasmid pAWP89 derived from the incP group plasmid containing *oriV*, *OriT* and *trfA* and KmR was used to express a gene cluster *budABC* from *Klebsiella pneumoniae.* Using the host *M. alcaliphilum* 35.66 mg/L 2,3-BDO was accumulated in a shake flask. A variety of promoters, ribosome binding sites and homologs of the genes from different sources were tested for 2,3BD production. The strain harbouring *budA* from *K.pneumoniae* and *budB* from *Bacillus subtilis* driven by the constitutive promoter Tac which produced a titre of 57.7 mg/L 2,3BD. Further to this *in silico* genome scale metabolic network of the strain was used to identify genes for knockout in which a triple knockout mutant was made, *Δldh Δack Δmdh*, by linearized DNA containing homology arms (600-800 bp) KmR and SacB for alleic exchange selection followed by sucrose counter selection. The triple mutant with the optimised expression vector increased the titre to 68.8 mg/L. The highest titre, 86.2 mg/L was then seen in this strain in a fed batch stirred tank.

1.4.2.6 Isobutanol

Isobutanol is a promising drop-in liquid transportation fuel that is compatible with current combustion engines. *M. capsulatus* (Bath) was engineered to produce 0.001 g/L of isobutanol using methane as a carbon source [65].

This was achieved by first expressing the exogenous two-step pathway from endogenously produced 2-ketoisovalerate to isobutyraldehyde and then to isobutanol catalysed by a keto acid decarboxylase (KDC) and an alcohol dehydrogenase (ADH). Various KDCs and ADHs were obtained from *Methylosinus trichosporium* (OB3B), *Methylococcus capsulatus* (Bath), *Lactococcus lactis*, *Saccharomyces cerevisiae* and

Clostridium acetobutylicum were cloned into pCM132 (which contains: Col E1 origin of replication, oriV/IncP origin or replication, OriT/Incp origin of transfer, TraJ conjugative transfer gene, trfA replication initiation protein and a kanamycin resistance marker) under the control of a constitutive promoter (J23115) or an inducible promoter (*Ptrc*, IPTG inducible). The endogenous pool of 2-ketoisovalerate was not high enough to produce isobutanol so pre-curser was added at a variety of concentrations. Of the gene combinations *M. capsulatus* KDC and *S.cereivisiae* ADH6 expressed constitutively performed the best producing 3 mM (0.22 g/L) isobutanol. It was seen that the optimum concentration of 2-ketoisovalerate feeding was 2 g/L due to toxicity [65].

Once the activity of the two step pathway was confirmed a further 3 steps of the native metabolism were overexpressed to increase flux from pyruvate. This was done by constitutive expression of *M. capsulatus ilvK ilvC* and *ilvD* which catalyse pyruvate to Acetolactate, to 2,3-dihydroxy-isovalerate then to 2-ketoisovalerate respectively. In addition to this previously established *M. capsulatus* KDC, and *S. cerevisiae* ADH6 were also overexpressed constitutively. This construct allowed the *M. capsulatus (Bath)* to produce 0.001 g/L isobutanol (without 2-ketoisovalerate feeding) when compared to no isobutanol production in the wildtype strain [65].

1.4.3 *Tools for metabolic engineering in methanotrophs*

As described above replicable plasmids are a key tool in metabolic engineering that allows recombinant expression of genes. As reviewed by (http://methanotroph.org/wiki/genetics/) there are a variety of replicable vectors for methanotrophs available. In general most replicating vectors in methanotrophs are incP type vectors [66] with a kanamycin selection marker. A specific example of such a plasmid can be seen in the plasmid pMHA199 which was used in *M. capsulatus* (Bath) as a broad host range promoter probe vector [61]. The plasmid contained a variety of components which gave it function which include: A Col E1 origin of replication functional in *E. coli*, An *oriV*/*IncP* origin of replication required for replication in *M. capsulatus* , A nick site for conjugal transfer (origin of transfer) *oriT*/*IncP*, A transcriptional activator protein *TraJ* that initiates a cascade of protein expression which activate characteristics for conjugation (e.g. pilus growth, outer-membrane fusion, surface exclusion proteins), A replication initiation protein *trfA* of plasmid RK2 that interacts with *oriV*, and finally a kanamycin resistance marker for antibiotic selection.

Other tools essential for metabolic engineering include promoters for expression of heterologous genes and plasmids for the deletion of competing metabolic genes. As described in the Use of engineered methanotroph strains section a variety of constitutive promoters are used for metabolic engineering, non-native promoters such as Tac are used but also endogenous native promoters such as the MDH promoter are often used. For gene knockout the most common method is allelic exchange with a non-replicative plasmid (suicide plasmid)/DNA fragment with KmR and SacB. Allelic exchange knockout works on the basis of DNA recombination and integrates into the genome via homologous regions flanking the gene. This single crossover event is

selected for by resistance to Km. Following integration counter selection occurs by means of levansucrase (*sacB*) that conveys toxicity in the presence of sucrose causing cells. Cells that have undergone a second recombination event lose the cassette containing *sacB*, KmR and the targeted gene. Cells that have lost the cassette can be selected for by growing in the presence of sucrose.

1.5 Bio-production of Transportation Fuel

1.5.1 *Microbial chassis for transportation fuel production*

Microbial hosts for the production of biofuels are proposed as a sustainable and effective method of obtaining fuels. The nature of the feedstock ultimately underpins weather the process is economical and sustainable. Microbial hosts have an ability to use a variety of renewable, cheap, waste compounds. Such examples include Clostridial bacteria consuming lignocellulosic biomass (agricultural waste) and carbon monoxide (off-gas from steel production), cyanobacteria species utilising carbon dioxide (atmospheric carbon dioxide) and methanotrophic bacteria metabolising methane [67]. The metabolic diversity of bacteria allows a variety of compounds to be used as a feedstock. The improvement of metabolic engineering expands the variety of chemicals, including transportation fuel, that can be synthesised via bacteria. Higher chain alcohols from C3 to C5 have high energy densities and can be blended with existing fuels and include: isopropanol, 1-propanol, 1-butanol, isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol and isopentenol [68]. These alcohols have a number of biological routes for production as seen in Figure 5.

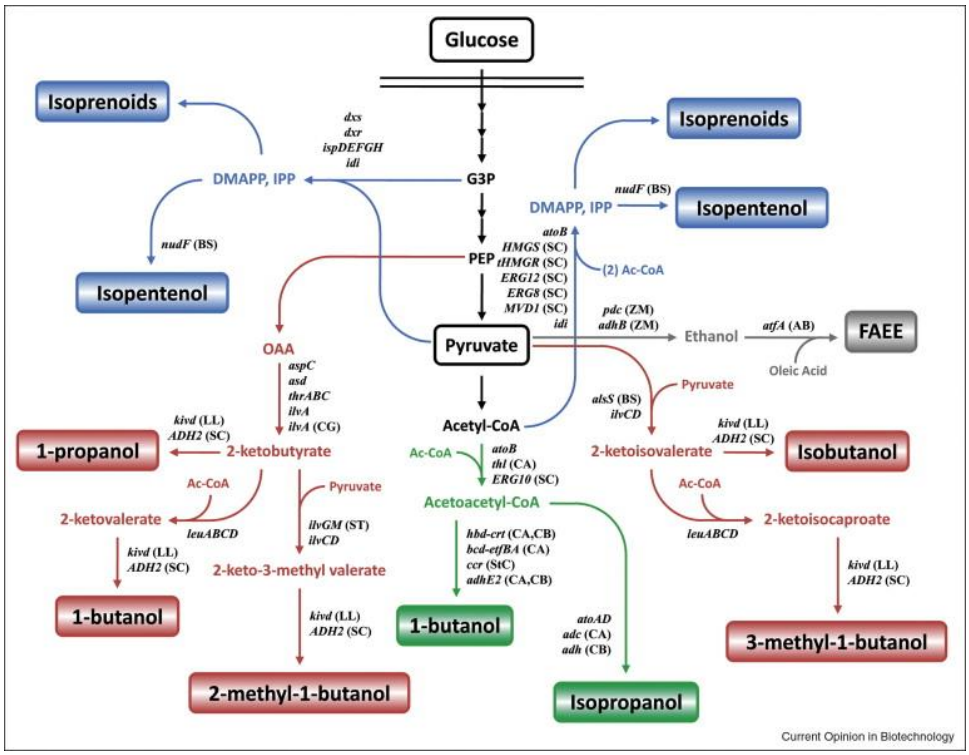


Figure 5 - Metabolic pathways for the production of biofuels. The metabolic diagram for biofuels derived from isoprenoids (blue), fatty acids (gray), amino acid biosynthesis (red), and traditional fermentative pathways (green). All genes are from *E. coli* unless noted otherwise: AB (*Acinetobacter baylyi*), BS (*Bacillus subtilis*), CA (*Clostridium acetobutylicum*), CB (*Clostridium beijerinckii*), CG (*Corynebacterium glutamicum*), LL (*Lactococcus lactis*), SC (*Saccharomyces cerevisiae*), ST (*Salmonella typhimurium*), StC (*Streptomyces collinus*), ZM (*Zymomonas mobilis*). Metabolite abbreviations: Acetyl-CoA & Ac-CoA (acetyl-coenzyme A), DMAPP (dimethylallyl diphosphate), G3P (glyceraldehyde-3-phosphate), IPP (isopentyl diphosphate), OAA (oxaloacetate), PEP (phosphoenolpyruvate) [68].

1.6 Metabolic pathways for isobutanol production

One of the most promising transport fuels is isobutanol. Isobutanol has a low hydroscopicity, vapour pressure, and corrosivity. It can be blended with gasoline in existing engines and also possess a high energy density [69].

A variety micro-organisms possess all enzymes endogenously for the production of biofuel. A key example are *Clostridia* species that endogenously produce butanol through their native acetone, ethanol, butanol pathway anaerobically during

solventagenesis [70]. Methanotrophs do not possess native pathways for butanol production though it has been seen extensively in the literature that native 2-keto acid pathway for valine biosynthesis, common in most organisms including methanotrophs, can be modified to create isobutanol (seen in Figure 5). As seen in Figure 6, using genomic data curated by KEGG, it can be seen that 2-ketoisovalerate (2-ketoic acid/2-oxoisovalerate) is produced for valine biosynthesis though does not possess either step to isobutyraldehyde or isobutanol.

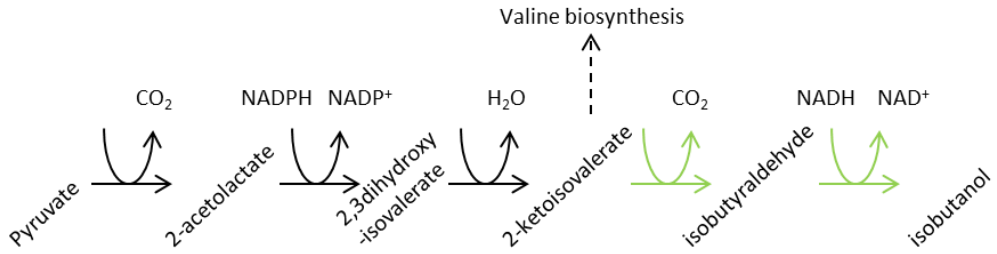


Figure 6 - Modified valine biosynthesis pathway for the production of isobutanol. Native reactions (black), heterologous reactions (green).

The enzymes that catalyse the conversion of pyruvate from the central metabolism to isobutanol in sequential order are acetolactate synthase, ketol-acid reductoisomerase, dihydroxy-acid dehydratase, keto-acid decarboxylase and alcohol dehydrogenase. These enzymes are often introduced and overexpressed from a variety of organisms to produce isobutanol. A collection of examples of isobutanol production via this approach can be seen in Table 3.

Table 3 - Summary of microbial production of isobutanol via engineered keto-acid pathway. Table adapted from [81]. Organisms include: *Escherichia coli, Clostridium thermocellum, Saccharomyces cerevisiae, Ralstonia eutropha, Clostridium cellulolyticum, Geobacillus thermoglucosidasius, Synechoccus elongates* and *Methylococcus capsulatus*.

Organism	Substrate	Gene overexpressed	Strain engineering	Titer	Fermentation reactor	Reference
E. coli	Glucose	*alsS, ilvCD, kivd, AdhA*	Δ*adhE*, Δ*ldhA*, Δ*frdBC*,Δ*fnr*, Δ*pta*, Δ*pflB*	50 g L^{-1}	Bioreactor, *In situ* product removal	[71]
E. coli	Glucose	*alsS, ilvCD, kivd, AdhA*	Δ*adhE*, Δ*ldhA*, Δ*frdBC*, Δ*fnr*, Δ*pta*, Δ*pflB*	22 g L^{-1}	Flask	[72]
C. thermocellum	Cellulose	*ilvBN, ilvCD, kivd*	Δ*hpt*	5.4 g L^{-1}	Tube	[73]
S. cerevisiae	Glucose	*ILV2, ILV5c, ILV3c, ILV2c, sMAE1, kivd, ADH6*	*lpd1*Δ	1.62 g L^{-1}	Flask	[74]
R. eutropha	CO_2	*alsS, ilvCD, kivd, yqhD*	Δ*phaC1AB1*	0.846 g L^{-1}	Bioreactor	[75]
C. cellulolyticum	Cellulose	*alsS, ilvCD, kivd, yqhD*	–	0.66 g L^{-1}	Tube	[76]
S. cerevisiae	Glucose	*Aro10, ILV2, ILV5, ILV3, adhA*	–	0.635 g L^{-1}	Tube	[77]
G. thermoglucosidasius	Cellobiose	*alsS, ilvC, Kivd*	–	0.6 g L^{-1}	Tube	[78]
***S. elongates* PCC7942**	CO_2	*alsS ilvCD, kivd, yqhD*	–	0.450 g L^{-1}	Bottle	[79]
S. cerevisiae	Glucose	*ILV2, ILV5, ILV3, kivd*	–	0.151 g L^{-1}	Flask	[80]
***M. capsulatus* (Bath)**	CH_4	*ilvK, ilvC, ilvD*, MCA0996, ADH6	–	0.001 g L^{-1}	Biorector	[65]

As seen in Table 3 *M. capsulatus* yields of isobutanol are significantly lower than all the other examples. A possibility of the poor relative performance lies with the choice of enzymes. Optimal flux through the engineered pathway requires balanced flux through each step, enzymes with: poor kinetics, instability at the hosts optimum growth temperature, or feedback inhibition can cause low yield and accumulation of toxic intermediates [82].

1.7 Specific Research Aims

The overall goal of this research will be to obtain and engineer methanotrophic bacteria for the production of a drop-in liquid fuel to adress the scalability issues associated with current GTL technologies. This overall goal can be broken down into the following sub-sections:

Isolation of methanotrophic bacteria – A methanotrophic host chassis for the heterologous pathway will need to be isolated from the environment. This will avoid IP issues and will open the possibility of obtaining a novel or better suited strain.

Characterisation of methanotrophic bacteria – Main phenotypic and genotypic characteristics are to be assessed for strain identification.

Genetic tractability of host – Genetic tractability will need to be assessed as a basis for pathway expressions.

Tools for pathway expression- Essential tools for pathway expression e.g. promoters and replicons will need to be assessed.

Expression of isobutanol biosynthesis metabolic pathway – Metabolic engineering of isobutanol biosynthetic pathway within a methanotrophic host.

2 *Materials and Methods*

2.1 Chemicals

All chemicals purchased from Sigma-Aldrich or Fisher Scientific unless stated otherwise.

2.2 Methanotrophic Organisms

Table 4 details a list of methanotrophic organisms used within this study. The strains include both newly isolated environmental strains and established strains from culture collections.

Table 4 - Methanotrophic strains used in this study.

Strain	**Description**	**Reference**
Methylococcus capsulatus (Texas)	*Methylococcus capsulatus* (Texas) (ATCC 19069)	
Methylococcus capsulatus (Bath)	Kindly donated by Dr Andrew Crombie	[28]
Methylocystis parvus	NCIMB 11129	
Isolate 01	Methanotroph isolated from Wollaton park lake	This study
Isolate 03	Methanotroph isolated from University of Nottingham lake	This study
Isolate 3*	Methanotroph isolated from Moseley bog	This study
Isolate 6	Methanotroph isolated from University of Nottingham lake	This study
Isolate 10	Methanotroph isolated from cow manure	This study
Isolate 12	Methanotroph isolated from Moseley Bog	This study
Isolate 14	Methanotroph isolated from the Roman Bath in Bath	This study

2.3 Methanotroph cultivation media

NMS or dilute nitrate mineral salts (dNMS) were used for routine cultivation of methanotrophs. Other media such as AMS, M2 and MNMS were used for methanotroph isolation experiments. The phosphate stock and vitamin stocks were made at 10X concentration and all stocks were stored at 4°C and protected from light. All media were made by adding salts to dH_2O along with sodium molybdenate and trace element solutions. Solid agar medium was made with 15 g L^{-1} Bacto agar unless stated otherwise. After autoclaving the media was allowed to cool down to near room temperature before adding 0.22 µm filter sterilised phosphate and vitamin stock. This media contains no added copper, for "added copper" media 1 ml L^{-1} of 0.22 µm filter sterilised 10 mM $CuCl_2$ was added to a final concentration of 10 µM. The composition of each media used are as follows [83].

NMS medium is used to support the growth of neutrophiles from freshwater environments [28] and is composed of:

- 1 g/L **$MgSO_4.7H_2O$**
- 0.05 g/L **KNO_3**
- 1 g/L **$CaCl_2.H_2O$**
- 0.5 ml/L **$Na_2MoO_4.4H_2O$ solution** ($Na_2MoO_4.H_2O$, 0.01 g/L)
- 1 ml/L **Trace element solution (1X)** ($FeSO_4.7H_20$, 500 mg/L; $ZnSO_4.7H_2O$, 400 mg/L; $MnCl_2.7H_2O$, 20 mg/L; H_2BO_3 (boric acid), 15 mg/L; $CoCl_2.6H_2O$, 50 mg/L; $NiCl_2.6H_2O$, 10 mg/L; EDTA, 250 mg/L)

- 0.1ml/L **3.8% (w/v) Fe-EDTA solution** ($FeSO_4$, 6.95 mg/L; Na_2EDTA, 9.3 mg/L)
- 10 ml/L **Phosphate stock solution (1X) pH 6.8** (KH_2PO_4, 26 g/L; Na_2HPO_4, 62 g/L)
- 10 ml/L **Vitamin stock (1X)** (Biotin, 2 mg/L; Folic acid, 2 mg/L; Thiamine HCl, 5 mg/L; Ca pantothenate, 5 mg/L; Vitamin B12, 1 mg/L; Riboflavin, 5 mg/L; Nicotinamide, 5 mg/L)

dNMS medium is a five times dilute version of NMS medium which more closely represents fresh water streams and lakes and is composed of:

- 0.2 g/L **$MgSO_4.7H_2O$**
- 0.2 g/L **KNO_3**
- 0.04 g/L **$CaCl_2.H_2O$**
- 0.02 ml/L **3.8% (w/v) Fe-EDTA solution** ($FeSO_4$, 6.95 mg/L; Na_2EDTA, 9.3 mg/L)
- 0.1ml/L **$Na_2MoO_4.4H_2O$ solution** ($Na_2MoO_4.4H_2O$, 0.01 g L)
- 0.2 ml/L **Trace element solution (1X)** ($FeSO_4.7H_20$, 500 mg/L; $ZnSO_4.7H_2O$, 400 mg/L; $MnCl_2.7H_2O$, 20 mg/L; H_2BO_3 (boric acid), 15 mg/L; $CoCl_2.6H_2O$, 50 mg/L; $NiCl_2.6H_2O$, 10 mg/L; EDTA, 250 mg/L)
- 2 ml/L **Phosphate stock solution (1X) pH 6.8** (KH_2PO_4, 26 g/L; Na_2HPO_4, 62 g/L)

- 2 ml/L-1 **Vitamin stock (1X)** (Biotin, 2 mg/L; Folic acid, 2 mg/L; Thiamine HCl, 5 mg/L; Ca pantothenate, 5 mg/L; Vitamin B12, 1 mg/L; Riboflavin, 5 mg/L; Nicotinamide, 5 mg/L)

AMS medium is similar to NMS medium with the nitrate source KNO_3 replaced with NH_4Cl and is composed of [28]:

- 1 g/L **$MgSO_4.7H_2O$**
- 0.5 g/L **NH_4Cl**
- 0.2 g/L **$CaCl_2.H_2O$**
- 0.1 ml/L **3.8% (w/v) Fe-EDTA solution** ($FeSO_4$, 6.95 mg/L; Na_2EDTA, 9.3 mg/L)
- 0.5 ml/L **$Na_2MoO_4.4H_2O$ solution** ($Na_2MoO_4.4H_2O$, 0.01 g/L)
- 1 ml/L **Trace element solution (1X)** ($FeSO_4.7H_20$, 500 mg/L; $ZnSO_4.7H_2O$, 400 mg/L; $MnCl2.7H_2O$, 20 mg/L; H_2BO_3 (boric acid), 15 mg/L; $CoCl_2.6H_2O$, 50 mg/L; $NiCl_2.6H_2O$, 10 mg/L; EDTA, 250 mg/L)
- 10 ml/L **Phosphate stock solution (1X) pH 6.8** (KH_2PO_4, 26 g/L; Na_2HPO_4, 62 g/L)
- 10 ml/L **Vitamin stock (1X)** (Biotin, 2 mg/L; Folic acid, 2 mg/L; Thiamine HCl, 5 mg/L; Ca pantothenate, 5 mg/L; Vitamin B12, 1 mg/L; Riboflavin, 5 mg/L; Nicotinamide, 5 mg/L)

M2 medium is a low salt medium used to grow acidophiles from ombotrophic wetlands and is composed of [84]:

- 0.02 g/L **$MgSO_4.7H_2O$**
- 0.2 g g/L **KNO_3**
- 0.004 g/L **$CaCl.H_2O$**
- 2 µL/L **Trace element solution (1X)** (EDTA, 5 g/L; $FeSO_4.7H_20$, 2 g/L; $ZnSO_4.7H_2O$, 0.1 g/L; $MnCl_2.7H_2O$, 0.03 g/L; $CoCl_2.6H_2O$, 0.2 g/L;$CuCl_2$, 0.1 g/L; $NiCl_2.6H_2O$, 0.02 g/L; Na_2MoO_4, 0.03 g/L)
- 250 µL/L **Phosphate stock solution (1X) pH 3-6** (0.04 g/L KH_2PO_4)

MNMS is a modified NMS medium shown to improve the number of culturable methanotrophs from freshwater sediment by the most probable number method. This media is composed of [85]:

- 0.1g/L **NaCl**
- 0.04 g/L **$MgCl_2.6H_2O$**
- 0.05 g/L **KCl**
- 0.015 g/L **$CaCl_2.H_2O$**
- 0.016 g/L **Na_2SO_4**
- 0.005 g/L **KNO_3**

- 1 ml/L **Trace element solution SL 10** (7.7M HCl, 10 ml/L with the addition of $FeCl_2$, 1.5 g/L; $CoCl_2$, 0.006 g/L; $MnCl_2$, 0.036 g/L; $ZnCl_2$, 0.024 g/L;H_3BO_3, 0.002 g/L; Na_2MoO_4, 0.19 g/L; $NiCl_2$, 0.1 g/L; $CuCl_2$, 0.07 g/L)
- 0.81 ml/L **Phosphate stock solution (1X) pH 7.2** (KH_2PO_4, 26 g/L; Na_2HPO_4, 62 g/L)

2.4 Methods of cultivation

Multiple species of methanotroph are cultivated in this study, as described above, all strains including *Methylococcus capsulatus* (Bath), *Methylocystis parvus*, isolate 01, isolate 03, isolate 3*, isolate 6, isolate 10, isolate 12 and isolate 14 were cultivated on NMS or dNMS. *M. capsulatus* (Bath) and isolate 14 were cultivated at 45°C, isolate 10 at 37°C and all other strains including isolate 01, isolate 03, isolate 3*, isolate 6, isolate 12 and *M. parvus* were cultivated at 30°C. Agitation in liquid culture was always 200 rpm. Research grade methane (BOC) was used throughout from compressed gas cylinders and was pre filtered using a 0.22 µm filter before use

2.4.1 *Serum bottles*

Liquid cultures were cultivated in pre-autoclaved clear glass serum bottles sealed with a rubber butyl stopper and clamped in place with an aluminium crimp. Liquid to headspace ratios were kept to approximately 1:5. Methane is either added to a 1:1 ratio by removing half of the air headspace and replacing it with methane. Alternatively, methane gas can be over pressured by adding methane on top of the existing headspace without removing any of the air from the headspace. Methane gas

was measured by collecting in a syringe and allowing to reach atmospheric pressure. The valve seen in Figure 7 is then turn and methane gas is dispelled from the syringe through a 0.22 µm filter through the needle into the serum bottle.

Figure 7 – Methane gas inlet pipe attached to syringe and 0.22 µm filter used for dispensing methane gas into cultures.

2.4.2 *Agar plates*

Inoculated plates were cultivated in Anaerocult® anaerobic jar by sparging with methane to ~1:1 methane:air. Headspaces were routinely refreshed every 3 days and restreaked every 2-3 weeks. To prevent contamination anaerobic jars were routinely sterilised with 70% industrially methylated spirits and Distel disinfectant.

2.4.3 *Agar slopes*

Agar slopes were created by allowing molten agar to set within a serum bottle at an angle under aseptic conditions followed by subsequent sealing and addition of methane as previously described for serum bottles.

2.4.4 *Multi-well plate cultivation*

As seen in Figure 8 the gas tight box (Enzyscreen®) was used to cultivate 96/48/24 well plates containing inoculated media. The multiwall plates were stacked and clamped in place. Methane was introduced through sealable valve and a 1 bar seal was left in to prevent the build up of pressure. The gas tight box was screwed in place to the incubator shelf so agitation at 200 rpm could be used.

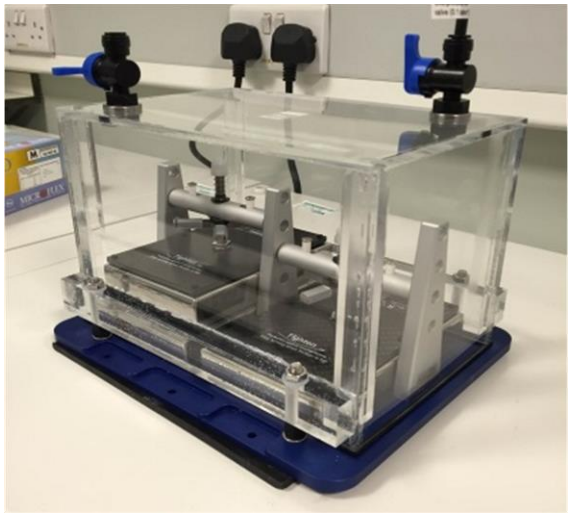


Figure 8 – Gas tight box (Enzyscreen®) containing multiwall plates held in place by screwable clamps.

2.4.5 *Maintenance of strains*

Liquid cultures were stocked in 10% DMSO or glycerol stocks in Cryobank^{TM} stocks and stored at -80°C. Revival of stocks was performed on solid NMS/dNMS agar by

restreaking from the stock and culturing in a methane atmosphere. Stocks had the headspace gas mixture refreshed every 3 days and plates were restreaked every 2-3 weeks. Purity was checked by streaking onto rich media such as LB or TSA agar.

2.5 Transformation of methanotrophs via conjugation

To establish genetic transfer into the methanotrophic isolates conjugation was performed using *E. coli* S17-1 conjugal donor strain (which has chromosomally integrated RP4 for conjugation) containing the desired plasmid. Isolates were cultivated to an OD600 of 0.3-0.4 as previously described. *E. coli* S17-1 harbouring respective plasmids was cultured overnight in 50 µg/ml kanamycin over-night and sub-cultured the day of transformation to OD0.05 then cultured to an OD600 of 1.0. The donor *E. coli* culture was then centrifuged (6000xg, 4°C for 10 mins) and washed three times with dNMS/NMS.

Methanotrophic isolates cultured to and OD600 of 0.4-0.8 centrifuged and re-suspended to an OD600 of 1.0/2.0. Cultures were then mixed at a donor:recipient of 1:1 using 500 µl of donor and 500 µl of recipient. Of the donor recipient mix 50 µL was pipetted on 4 different corners of a mating plate consisting of dNMS/NMS agar with 0.05% Yeast extract (though 0.02% proteose peptone, 20% LB in dNMS agar were used to supplement *E. coli* with less success) and incubated for 48 hours at 30°C (Isolate 6) and 37°C (Isolate 14) in 1:1 methane:air ratio. Cells were extracted by pipetting 200 µL of dNMS onto mating plates, scraping contents, and transferring to 2 mL Eppendorf tubes. Of the cell suspension extracted 100 µL was transferred onto

selective dNMS/NMS plates with 25 mg ml^{-1}naladixic acid and 15 mg ml^{-1} and 30 mg ml^{-1} kanamycin (for isolate 14 and 6 respectively) and incubated at 30°C (Isolate 6) and 45°C (Isolate 14) for 5-10 days in a methane air atmosphere. Transformant colonies were patch plated by restreaking or suspending in NMS and spotting on selective agar with further incubation. Colony PCR was used to confirm transformation.

2.6 Phase contrast microscopy

Phase contrast microscopy was performed by extracting 1 µl of culture onto a microscope slide and covered with a cover slip. A Nikon Eclipse C phase contrast microscopy was used to view the slides and images were captured on the inbuilt camera. For x100 magnification oil immersion was used.

2.7 Molecular Cloning

2.7.1 *Culturing E. coli*

All strains of *E. coli* were cultured in Luria Bertani (LB) medium composed of 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl. Antibiotics to select for plasmids were added at a concentration of: Kanamycin, 50 µg/ml; gentamycin, 10 µg/ml; chloramphenicol, 20 µg/ml; erythromycin, 500 µg/ml; ampicillin; 100 µg/ml. Cultures were incubated at 37°C at 200 rpm. Cryobank™ glycerol stocks were used to store the strains at -80°C.

2.7.2 *Monitoring OD600*

Growth of bacteria was assessed using absorbance at 600 nm (OD600), unless stated otherwise, using a ThermoFisher Scientific Biomate 3S UV-Visible Spectrophotometer. Of the sample 1 ml was transferred to a cuvette with appropriate dilutions to within the spectrophotometers linear range and the absorbance was recorded at a wavelength of 600 nm against a blank containing only the medium.

2.7.3 *Plasmid extraction from E. coli*

Plasmid extraction mini-prep kits were used for obtaining purified plasmid from host *E. coli* cells. Commercially available plasmid extraction kits were used including: QIAprep Spin Miniprep Kit (Qiagen), Monarch® Plasmid Miniprep (NEB) Kit and GenElute^{TM} plasmid Miniprep Kit (Sigma). DNA was extracted to manufacturers' protocol.

2.7.4 *Quantification and storage of DNA*

Purified DNA was eluted in nuclease free water and quantified using a SimpliNano Spectrophotometer. Nuclease free water was used as a blank and the detector was cleaned between samples. All purified DNA was stored at -20°C.

2.7.5 *Preparation and transformation of chemically competent E. coli*

Chemically competent cells were prepared by inoculating *E. coli* S17-1/DH5α/TOP10 into 10 ml of LB media in a 50 ml falcon tube using a sterile loop and incubated at 37°C at 200 rpm overnight. The 0.5 ml of the overnight culture was then sub-cultured into 50 ml of LB in an Erlenmeyer flask and cultured as previously described to

between 0.5 and 0.7 OD600. At this point cultures are centrifuged at 8000 rpm for 5 minutes in falcon tubes. The cell pellet was resuspended in ice-cold 30mM $CaCl_2$. Of the resuspended pellet 1.5 ml was pelleted and resuspended in 0.5 ml ice-cold 30mM $CaCl_2$ and aliquitted into 50 µl in an Eppendorf tube for subsequent heatshock transformation or stored at -80°C.

Transformation via heatshock was performed by thawing chemically competent cells on ice and adding 1-5 µl of DNA solution and gently swirling. The tubes were then incubated on ice for 30 minutes then heat shocked in a 42°C water bath for 45 seconds then back on ice for 2 minutes. Cells were then recovered in 0.6 ml of SOC medium for 45 minutes with 250 rpm at 37°C. The recovered media was then plates onto selective LB plates using 100 and 400 µl followed by 37°C incubation overnight.

2.7.6 *Preparation and transformation of electrocompetant E. coli*

E. coli strains were inoculated into 10 ml of LB media overnight. The overnight culture was sub-cultured 1 ml into 100 ml LB medium and further incubated to an OD600 of 0.5-1.0. The culture was then incubated on ice for 15 minutes and centrifuged at 4°C at 4000 g for 15 minutes. The pellet was then resuspended in 100 ml dH_20 then further pelleted and resuspended in 2 ml 10% glycerol (pre-chilled). The 2 ml solution was then pelleted and resuspended in 300 µl 10% glycerol and aliquoted into 50 µl in 1.5 ml Eppendorf tubes then stored at -80°C.

E. coli was transformed by adding 1-2 µl of DNA solution (ligation mixtures were dialysed by putting 5 µl on dialysis paper in a petri dish of dH_20 for 1 hour) into Eppendorf with 50 µl electrocompetant cells. The DNA cell mixture is then transferred to a pre-chilled 2mm electrocuvette and pulsed on the EcoR2 programme. Immediately 950 µl of SOC recovery medium was added and transferred to an Eppendorf for a 1 hour recovery at 37°C with 200 rpm agitation. Once recovered 100 µl of the solution was spread on a selective LB agar plate and incubated at 37°C overnight.

2.7.7 *HiFi DNA Assembly*

DNA assembly of multiple fragments was performed using NEBuilder® HiFi DNA Assembly Master Mix (NEB). Primers were created using NEBuilder online assembly tool or the online Gibson assembly tool by Benchling. Primers were designed with 20 bp of homology and 20 bp of overlap and were synthesised by Sigma-Aldrich. The 2-3 fragment assembly used a 1:2 vector:insert molar ratio with 50 ng of vector, respective insert(s), 10 µl of NEBuilder® HiFi DNA Assembly Master Mix (NEB) and then made to 20 µl with nuclease free water. Assemblies with 4-6 fragments used a 1:1 vector:insert molar ratio with 50 ng of vector.

2.7.8 *DNA restriction digestion*

Restriction digestion of DNA fragments were performed using either NEB restriction enzymes or ThermoFisher FastDigest. NEB restriction enzyme digestions were performed by creating a digestion mixture of 1 µg of DNA, 5 µl 10x NEBuffer, 1 µl

of each restriction enzyme and made to 50 µl with nuclease free water. Digest mixture was incubated at 1 hour at 37°C (dependant on enzyme) in a water bath.

Thermofisher Fast Digest was performed using 1 µg of DNA, 2 µl of 10x FastDigest® Green buffer, 1 µl of each restriction enzyme and then made to 20 µl using nuclease free water, briefly centrifuged and incubated at 37°C in a water bath for up to 1 hour.

2.7.9 *DNA ligation*

DNA ligations were performed using NEB T4 ligase. Reactions were set up typically with a molar ratio of 1:3 vector:insert using NEB BioCalculator or 75 ng of insert and 25 ng of vector. In addition to the DNA, 2 µl of T4 ligase buffer (NEB) and 1 µl of T4 DNA Ligase (NEB) were added to the mix and the solution was made to 20 µl with NFW then briefly centrifuged. The reaction mix was then incubated at 16°C overnight or at room temperature for 2 hours. The reaction was then heat inactivated at 65°C for 10 minutes then chilled on ice before transformation.

2.7.10 *Polymerase chain reaction*

PCR reactions were performed on a Tproffesional TrioThermocycler. For PCR reactions purified DNA or colony picks were used as a template. For colony picks colonies were picked using a sterile loop and suspended in 10-20 µl of nuclease free water and boiled at 98°C for 10 minutes. The boilate was used as a template.

2.7.10.1 PCR cloning

All DNA oligos were synthesised by Sigma-Aldrich. Polymerase chain reaction (PCR) for cloning or sequencing was performed using Q5® High-Fidelity DNA Polymerase 2X Master Mix (NEB). A 25 µl reaction mix contained 12.5 µl of Q5 High-Fidelity 2X Master Mix, 10 µM of forward and reverse primers, template DNA and made up to 25 µl with nuclease free water. Thermocycler parameters are described below.

To insert restriction sites into the amplified fragments the sequence was added onto the 5' end of the oligo with a 6 bp leader sequence to allow for restriction enzyme binding.

2.7.10.2 Colony PCR

Colony PCR for detection of plasmids or genes not to be sequenced were amplified using DreamTaq Green PCR Master Mix (2x) (ThermoFisher). For a 25 µl reaction mix the reaction contained 12.5 µl of DreamTaq Green PCR Master Mix (2x), 10 µM of forward and reverse primers, template DNA and made up to 25 µl with nuclease free water. Thermocycler parameters are described below.

2.7.10.3 Thermocycler parameters

Thermocycler parameters were used based on the manufacturers' guidelines including extension, denaturation, and annealing temperature/times. Annealing temperatures were calculated using NEB or ThermoFisher Tm online calculator. Typical thermocycler parameter settings were used in addition to touchdown PCR as seen below:

Table 5 - PCR thermocycler parameters for Q5 High-Fidelity 2X Master Mix and DreamTaq Green PCR Master Mix (2x). *, Annealing temperature calculated by online tools described above. **, 1 minute for 2 kb 1min/kb for longer fragments.

		Q5		DreamTaq	
		Time	Temp (°C)	Time	Temp (°C)
	Initial denature	30s	98	1-3m	95
Amplification (x30 cycles)	Denature	5-10s	98	30s	95
	Anneal	10-30s	*	30s	*
	Extension	20-30s/kb	72	**	72
	Final Extension	2m	72	5m	72
	Hold	Hold	4	Hold	4

Table 6 - Touchdown PCR thermocycler parameters for Q5 High-Fidelity 2X Master Mix and DreamTaq Green PCR Master Mix (2x). *, Annealing temperature was 5-10° above the annealing temperature so the annealing temperature would reduce past the ideal annealing temperature. **, 1 minute for 2 kb 1min/kb for longer fragments.

		Q5		Dreamtaq	
		Time	Temp (°C)	Time	Temp (°C)
	Initial denature	30s	98	1-3m	95
Touchdown (x15 cycle)	Denature	5-10s	98	30s	95
	Anneal	10-30s	*	30s	*
	Extension	20-30s/kb	72	**	72
Amplification (x20 cycles)	Denature	5-10s	98	30s	95
	Anneal	10-30s	60	30s	60
	Extension	20-30s/kb	72	**	72
	Final extension	2m	72	5m	72
	Hold	Hold	4	Hold	4

2.7.11 *Agarose gel electrophoresis*

Agarose gel electrophoresis was performed on plasmid digests and PCR products in a 1% agarose gel made using agarose and TAE buffer and dissolved in the microwave. Molten samples were poured into a casting tray where 10 µl of SYBR™ Safe DNA Gel Stain was added per 100 ml of molten agarose solution. Combs were then added to the tray and then left to solidify to room temperature. DNA samples were prepared by adding Gel Loading Dye Blue (6x) (NEB) to 1x concentration (if dye is not already present such as in DreamTaq Green PCR Master Mix (2x)). Samples were then loaded (typically 10-30 µl) along with a separate lane containing Quick-Load® 1 kb DNA Ladder (NEB). Agarose gels were then loaded into a Bio-Rad electrophoresis system with a PowerPacTM Universal Power Supply (Bio-Rad). The gel was run for approximately 45 minutes at 100 V with fixed A.

2.7.12 *Agarose gel Imaging*

Agarose gels contiaining DNA were imaged using a Bio-Rad Molecular Imager GelDoc XR+ Imaging System running Image Lab™ software.

2.7.13 *Purification for agarose gels*

DNA was excised from agarose gels using a scalpel and transferred to an Eppendorf tube. The DNA was purified using a variety of commercially available kits including: Zymoclean™ Gel DNA Recovery, Qaiquick Gel Extraction Kit, Monarch ® DNA Gel Extraction Kit (NEB). All manufacturers' protocol was followed.

2.7.14 *DNA sequencing*

DNA Sanger sequencing was performed by Sourcebiosciences' Overnight Service™ and Eurofins Genomics TubeSeq Service.

3 *Isolation of Methanotrophic Bacteria*

3.1 Chapter Introduction

The first step in developing the proposed methane to fuel biocatalyst is to obtain a methanotrophic host. The benefits of isolating an environmental strain of methanotroph include: potential beneficial characteristics for isobutanol production, reduce risk of IP infringement and the possibility of isolating a novel strain.

As reviewed previously, classic methanotroph isolation relies on the selective enrichment of environmental samples containing methanotrophs to increase the relative proportion of methanotrophs to non-methanotrophs. This is typically done by inoculating environmental sample into NMS or dNMS liquid medium in a methane atmosphere. Following this extinction dilution is used to dilute the enriched culture to 0 colony forming units (CFU) so that the dilution previous has one, or very few cells which form distinct colonies on solid media which represent growth from a single bacterium.

In this chapter extinction dilution techniques are used in enrichment isolation 1-3 (adapted from [41]) and a miniaturised extinction dilution adapted from [38] is employed in enrichment isolation 4 and 5. Both methods are outlined in 1.3.1.

Furthermore flow cytometry is assessed as a potential tool for isolation of methanotrophic bacteria. The methods described above yield pure cultures by culturing up one or very few cells obtained from continuous dilution of colonies. One method that can rapidly obtain single cells is flow cytometry (FC). FC takes cell suspensions and hydrodynamically focuses cells into a single row so they can be analysed as individual events. These events can be analysed based on optical scatter

can be subsequently sorted into collection tubes or wells using an electric field generated by deflection plates [86]. It is envisaged that FC cell sorting could be used for isolation of methanotrophic pure cultures from an enriched consortium.

3.2 Strains and Plasmids

The organisms obtained from culture collections and the environmental isolate methanotrophs used in this chapter are detailed within section 2.2.

3.3 Primers

The following primers were used for the detection and identification of methanotrophic organisms.

Table 7 - Primers used in this chapter.

Name	**Sequence (5' to 3')**	**Gene/Description**	**Reference**
pMMO (A189f/A682r)	F - GGNGACTGGGACTTCTGG R - GAASGCNGAGAAGAASGC	*pmoA* sub-unit of pMMO of proteobacterial methanotrophs	[87]
pMMO (A189f/A650r)	F - GGNGACTGGGACTTCTGG R - ACGTCCTTACCGAAGGT	*pmoA* sub-unit of pMMO of proteobacterial methanotrophs	[88]
pMMO (A189f/MB661r)	F - GGNGACTGGGACTTCTGG R - CCGGMGCAACGTCYTTAC C	*pmoA* sub-unit of pMMO of proteobacterial methanotrophs	[89]
sMMO (166f/1401R)	F - ACCAAGGARCARTTCAAG ' R - CCGATCCAGATDCCRCCC CA	mmoX (mmoXA/mmoXD) sub-unit of sMMO	[41]
16S rRNA (u199f/U1391r)	F - AGYGGCGNACGGGTGAGT AA R - GACGGGCGGTGWGTRCA	16S ribosomal RNA sequence	This Study

3.4 Methods Specific to this Chapter

The two key methods of isolation utilised in this study, dilution plating isolation and miniaturised extinction dilution, can be seen in the flow diagram in Figure 9. For clarity this figure provides an overview of the workflow, detail for each individual experiment is described below.

The final two steps of both processes which use passaging from liquid to solid media are not included in the referenced methods though were additions made to the protocols.

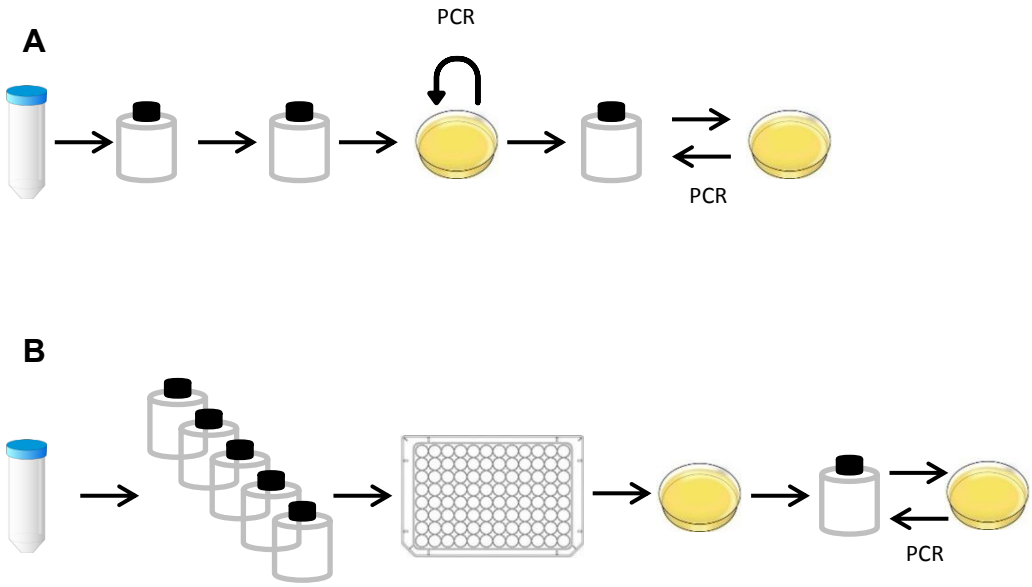


Figure 9 - (A) Traditional dilution plating isolation that follows the steps from left to right: environmental sampling, initial enrichment, sub-cultured enrichment, PCR guided re-streaking with serial dilution of single colonies, single colony inoculation into liquid then finally cycling liquid to solid using PCR to select methanotrophic colonies. (B) Miniaturised extinction dilution that follows the steps from left to right: environmental sampling, serial dilution enrichment, subculture of most dilute grown sample and serial dilution onto a 96 well plate, serial dilution to solid media then finally cycling liquid to solid using PCR to select methanotrophic colonies.

3.4.1 *Enrichment Isolation 1 (Dilution Plating)*

3.4.1.1 Samples

Environmental samples were obtained from Sutton Bonnington campus, University of Nottingham, Leicestershire, LE12 5RD. On 27/11/14 the following samples were obtained: Cow slurry from the surface of the tank (SS), cow slurry from the bottom of the slurry tank (BS), fresh cow manure (CMF), non-fresh cow manure sample (CMO), soil taken from an anaerobic digester (AD) and food digestate from an anaerobic digester (FS).

3.4.1.2 Enrichment Isolation method

Based off a protocol by Auman et al [41], 1 ml of each liquid sample was inoculated into 10 ml of dNMS media in 60 ml serum bottles. Solid samples were prepared by adding approximately 5 ml of the sample to 10 ml of sterile water, vortexed until homogenous then centrifugation at 1000 rpm for 1 minute. Of the supernatant 1 ml was inoculated into 10 ml of dNMS media in 60 ml serum bottles [34]. For each individual environmental sample the addition of $CuSO_4$ to the media and methane:air within the headspace were varied. Four separate enrichment conditions were used: 50:50 (methane:air) with 10µM $CuSO_4$, 50:50 (methane:air) no $CuSO_4$, 80:20 (methane:air) with 10µM $CuSO_4$, and 80:20 (methane:Air) without $CuSO_4$. In addition two control samples were made replicating the test sample but with replacing the environmental sample with 1 ml of sterile water to ensure contamination was not coming from liquid handling. Cultures were incubated at 200 rpm for 30°C for 4 days. Enrichment cultures were sub-cultured into fresh dNMS media, $CuSO_4$ and methane:air ratios kept constant and incubated at 200 rpm at 30°C for a further 4 days. Series dilutions of each sample were made (neat, 10^{-1}, 10^{-2}, 10^{-3}), 100 µl of each dilutions were transferred to dNMS agar slopes consisting of 20 ml agar within 155 ml

serum bottles. All agar slopes contained 10 µM $CuSO_4$ with 50:50 (methane:air) at 30°C.

Selected agar cultures from all sample types were assessed for growth and single colonies were picked and analysed using colony PCR using 16S rRNA primers (u199f/U139r) and pMMO primers (A189f/A682r and A189f/650r). Relevant PCR products extracted and sequenced.

Colonies suggesting the presence of the pMMO gene were serially diluted in PBS and re-streaked onto dNMS agar plates containing 10 µM $CuSO_4$. The plates were exposed to (methane:air) 50:50 by putting in an Anaerobic Oxoid jar with an adapted rubber stopper in the lid where the headspace would be adjusted using a syringe . Picking colonies, PCR screening, and subsequent re-streaking was cycled many times.

Single colonies were screened using PCR to assess for pMMO containing colonies and were inoculated into liquid media, once growth was viewed samples were serially diluted onto solid media and single colonies were screened using PCR again. The media used for this was dNMS containing 10 µM $CuSO_4$ but with no added vitamin solution with 50:50 methane:air ratios. In addition to this the sample originating from fresh cow manure had its incubation temperature raised to 42°C. This passaging was done to purity. Purity was continuously monitored through characterisation which included: No growth on rich media (TSA, 10% TSA, LB, 10% LB), phase contrast microscopy, 16S rRNA sequence chromatogram and colony morphology.

3.4.1.3 Alternative Carbon Source Supplementation for Isolation

Three distinct dNMS Bacto agar (1.5%) plates were used that contained: Yeast extract (0.025%) and methanol (0.025%), yeast extract (0.025%) and a control with neither yeast extract nor methanol. Series dilutions of previously identified positive

methanotrophic colonies were serially diluted in PBS (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) and re-streaked. The plates were placed in an adapted anaerobic Oxoid jar with the headspace at (methane:air) 50:50 and incubated at 30°C and also 37°C.

3.4.2 *Enrichment Isolation 2 (Dilution Plating)*

3.4.2.1 Environmental Samples

A lake sediment sample was obtained from The University of Nottingham, University Park Campus, Nottingham, NG72RD on 17/03/15.

3.4.2.2 Enrichment Isolation Method

The environmental sample was prepared by briefly vortexing then centrifugation at 1000 rpm for 1 minute. 10 µl of the supernatant was inoculated into 10 ml of dNMS media within a 60 ml serum bottle supplemented with 10µM $CuSO_4$ with vitamin concentration ranging from x0, x0.0001, x0.001, x0.001, x0.01 and x0.1 in duplicate. Headspace adjusted to air:CH_4:CO_2 (76:20:4) and incubated at 150 rpm at 30°C until turbidity observed.

To assess the ability of different media to support enrichment growth 10 µl of the supernatant was inoculated into 10 ml of a range of media including NMS (supports neutrophils from freshwater), AMS (NMS with ammonium as the nitrogen source), M2 (low salt for acidophiles), MNMS (optimised NMS for cultivation of freshwater methanotrophs) and dNMS (0.2x concentrate NMS) media within a 60 ml serum bottle supplemented with 10µM $CuSO_4$. Each was made in duplicate. Headspace adjusted to air:CH_4:CO_2 (76:20:4) and incubated at 150 rpm at 30°C until turbidity observed. Three controls were used:

1. Environmental sample spiked with 1µl of methanotroph positive inoculum with methane conditions similar to test samples. Methanotrophic

inoculum made by suspending 1 colony of *M. capsulatus* in 20µl of sterile water and diluting to 10^{-3}. Cultures done in duplicate at 45°C 150 rpm.

2. Environmental sample spiked with 1µl of methanotroph positive inoculum without being enriched with methane. Cultures done in duplicate at 45°C 150 rpm.

3. One microliter of methanotroph negative inoculum with no environmental sample added and methane conditions similar to test samples. Non-methanotrophic (confirmed with PCR) inoculum by suspending 1 colony of non-methanotrophic contaminant from previous isolation in 20µl of sterile water and diluting to 10^{-3}. Cultures done in duplicate at 30°C 150 rpm. All the cultures had the headspace fixed to air:CH_4:CO_2 (76:20:4) and were incubated at 150 rpm at 30°C for 15 days.

Samples indicating growth by turbidity after the 15 days incubation included one of the two replicates for the dNMS media and AMS media. For the vitamin variants one of the two duplicates showed growth in: x0, x0.001, x0.01 and x0.1. These samples and all controls were passaged into fresh media in duplicate by transferring 10µl of the culture into identical conditions. Similar to the first step the headspace fixed to air:CH_4:CO_2 (76:20:4) and other appropriate conditions kept constant. The samples were incubated at 30°C at 150 rpm for 5 days.

After incubation cultures that showed growth were dNMS, AMS and x0, x0.001, x0.1. A serial dilution was made (neat, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) and 1 µl was streaked onto dNMS agar plates (1.5% Bacto Agar) and incubated in Oxoid jar fixing the headspace to air:CH_4:CO_2 (76:20:4) with appropriate conditions, and left until colonies formed (5 days).

A distinct colony from the most diluted plate showing growth was picked using a sterile loop. Colony PCR was performed as previously described using sMMO, pMMO, and Universal 16S rRNA primers.

After 5 days, single colonies picked using a sterile loop and added to 15 µl of nuclease free water and Using the inoculated nuclease free water sample PCR is performed with a range of primers including pMMO (A189f/A682r, A189f/A650r, A189f/MB661r), sMMO (166f/1401R) and 16S rRNA (u199f/U1391r) of which relevant products were sequenced. pMMO possessing colonies re-streaked onto dNMS agar and Incubated in Oxoid jar fixing the headspace to air:CH_4:CO_2 (76:20:4) with appropriate conditions. This PCR screening and re-streaking was cycled in excess of 10 times without purity being obtained.

As purity was not initially achieved, single colonies were screened using PCR to assess for pMMO and sMMO and were inoculated into liquid media, once growth was viewed samples were serially diluted onto solid media and single colonies were screened using PCR again. The media used for this was dNMS containing 10 µM $CuSO_4$ but with no added vitamin solution with 50:50 methane:air ratios. This was cycled to purity.

Purity was continuously monitored through characterisation which included: No growth on rich media (TSA, 10% TSA, LB, 10% LB), phase contrast microscopy, 16S rRNA sequence chromatogram, colony morphology and growth on alternative carbon substrate.

3.4.3 *Enrichment Isolation 3 (Dilution Plating)*

3.4.3.1 Environmental Samples

Environmental samples were taken from Wollaton Park, Nottingham, NG8 2AE on 23/03/14. Samples included: Lake stream sediment, shallow lake sediment, lake stream sediment, reed bed sediment and moss sample.

3.4.3.2 Enrichment Isolation

This enrichment isolation followed similar protocol to enrichment isolation 2. In this method vitamin concentration and media were varied during the enrichment phase. For this only the shallow lake sediment sample was used so a comparison could be drawn. In addition, the following adjustments were made:

- Centrifugation on the environmental sample was replaced with letting the sample settle for 10 minutes
- Sample inoculated into cultures and sub-cultured through each step was increased from 10 µl to 100 µl
- A 10^{-2} dilution of positive methanotroph inoculum was used for the control instead of a 10^{-3} dilution
- All samples and controls incubated at 30°C

The shallow lake sediment, stream sediment, reed bed sediment and moss samples underwent enrichment isolation. Firstly, the moss sample was prepared by being cut into smaller moss fragments and vortexed for 30 seconds. The other samples were briefly vortexed and left to settle for 10 minutes. For each sample 100 µl was inoculated into 10 ml of dNMS media within a 60 ml serum bottle supplemented with 10µM $CuSO_4$. Headspace adjusted to air:CH_4:CO_2 (76:20:4) and incubated at 150 rpm at 30°C until turbidity observed for 16 days.

After 16 days, growth was seen in the dNMS sample and x0.001 vitamin sample. In addition to this turbidity was observed in all environmental samples (shallow lake

sediment, lake stream sediment, reed bed sediment and moss samples). These along with all controls were sub cultured into fresh media as described in enrichment isolation 2.

After enrichment all but dNMS sample showed turbidity. All the samples indicating growth along with all controls were serially diluted, neat to 10^{-5}, and streaked as previously described in enrichment 2.

Distinct colonies from the most diluted plate showing growth was picked using a sterile loop. Colony PCR was performed as previously described using pMMO (A189f/A682r, A189f/A650r, A189f/MB661r), sMMO (166f/1401R) and 16S rRNA (u199f/U1391r).

Relevant products were sequenced. Methanotroph and heterotrophs were both identified during screening though no pure cultures could be obtained.

Following this approach single colonies were screened using PCR to assess for pMMO and sMMO and were inoculated into liquid media, once growth was viewed samples were serially diluted onto solid media and single colonies were screened using PCR again. This was cycled to purity. The media used for this was dNMS containing 10 µM $CuSO_4$ but with no added vitamin solution with 50:50 methane:air ratios.. Passaging was done to purity which was assessed through: No growth on rich media (TSA, 10% TSA, LB, 10% LB), phase contrast microscopy, 16S rRNA sequence chromatogram, colony morphology and growth on alternative carbon substrate.

3.4.4 *Enrichment Isolation 4 (Miniaturised extinction dilution isolation)*

The miniaturised extinction dilution method was adapted form [38] discussed above.

3.4.4.1 Environmental samples

Water sediment samples were obtained from: Roman Bath (Bath, BA1 1LZ), River Severn, Slimbridge Wetlands (GL2 7BT), Moseley Bog (B13 OAP) and Nottingham landfill (NG24 3JJ).

Samples were homogenised in 27 ml of dNMS containing 10 µM $CuSO_4$ and a dilution series up to 10^{-7} in similar media within serum bottles and incubated at 30 and 45°C at 150 rpm with a 2:8 methane:air for up to 5 weeks. Growth from the highest dilution was transferred to a 96 well plate containing dNMS containing 10 µM $CuSO_4$ and a further serial dilution was performed up to 10^{-7} which was they incubated at similar conditions to the first incubation for 2 weeks. Growth was measured by change in OD600 and 2 µl of each dilution was plated and spread on 3% Bacto and 3% gellan gum dNMS containing 10 µM $CuSO_4$ agar and further incubated, this initially yielded no pure methanotrophic cultures.

Single colonies were screened using PCR to assess for pMMO (A189f/A682r, A189f/A650r, A189f/MB661r) and sMMO (166f/1401R) and were inoculated into liquid media, once growth was viewed samples were serially diluted onto solid media and single colonies were screened using PCR again. The media used for this was dNMS containing 10 µM $CuSO_4$ but with no added vitamin solution with 50:50 methane:air ratios. Purity was checked as previously described. This was cycled to purity.

3.4.5 *Enrichment Isolation 5 (Miniaturised extinction dilution isolation)*

3.4.5.1 Environmental samples

Environmental samples were taken from the hydrothermal spring in the Roman Bath in the King Spring (Bath BA1 1LZ). The sample contained surface water from the spring along with plant like vegetation.

3.4.5.2 Enrichment Isolation

Of the sample described above 2 ml of the water was transferred into 35 ml PBS. Following this 200 µl of PBS sample was transferred into 10 ml of dNMS media with the addition of 10 µM $CuSO_4$ and with vitamin stock taken out the media. This was incubated at 45°C at 200 rpm with a methane:air ratio of 1:1 for 12 days. Of the resulting culture 10 µl was inoculated into 96 well plates and serially diluted to 10^{-7}. After 13 days of incubation 5 µl of each dilution were streaked onto dNMS agar, the colonies forming on the highest dilution were passaged from the same liquid to solid media using serial dilutions and using colony PCR of pMMO and sMMO to ascertain methanotrophic colonies as previously described. Purity was checked as previously described. Purity was confirmed without further screening and purity was checked as previously described.

3.4.6 *Flow cytometry methanotroph isolation*

Isolation of pure cultures of methanotrophic bacteria from heterogeneous samples was attempted using a BD FACS Canto II flow cytometer to sort individual cells into a 96 well plate and enrich with methane. In addition to random sorting of individual cells pure cultures were cultured and used as references to refine the forward scatter and side scatter sorting parameters to narrow the sorting criteria. Organisms used as reference included *M. capsulatus* (Bath) (cultured in 10 ml dNMS media for 7 days, in a 1:1 methane:air headspace, in 45°C at 200 rpm) and *Escherichia coli* (cultured in 10 ml LB, for 24 hours, in 37°C at 200 rpm).

To test the effect of the process on viability of single cells *E. coli* was sorted into 96 wells, a range of single wells containing 0, 1, 10^1, 10^2 up to 10^5 cells. These cells were sorted into a 96 well plate containing 200 µl of LB. In addition to this 8 LB media only wells were present to check if the process was sufficiently aseptic.

As a positive control, single cell sorting of a monoculture of *M. capsulatus* (Bath) was sorted into 14 wells, in addition to wells containing 0, 1, 10^1 up to 10^5 cells. Cells were sorted into 200 µl of dNMS containing 10µM $CuSO_4$.

Liquid samples enriched from the Roman Bath (See enrichment isolation 4) and similarly enriched 30°C environmental sample was used for sorting. Single cells from an *M. capsulatus* (Bath) pure culture was sorted to attain forward and side scatter parameters to use as sorting criteria for the Roman Bath sample. A maximum range was obtained and single cells from the environmental enrichment sample were sorted into individual wells on a 96 well plate containing 200 µl of dNMS containing 10µM $CuSO_4$. Sorting was also repeated removing the sorting criteria to obtain a variety of morphologies. Furthermore 30°C Enrichment sample was sorted using no sorting criteria in a similar manner.

Plates containing *E. coli* were incubated overnight at 37°C at 200 rpm. Plates containing *M. capsulatus* (Bath) or 45°C sorted mixed cultures were incubated in a gas tight box in approximately 1:1 methane:air at 45°C. Environmental samples enriched at 30°C were cultured in a similar manner though at 30°C. Growth assessed every 2 weeks.

3.5 Results

3.5.1 *Enrichment isolation*

3.5.1.1 Enrichment isolation 1

Following methane enrichment passages in liquid media and transfer to solid media, 16S rRNA gene PCR products obtained from single colonies were sequenced. Using BLAST (NCBI) homology search to ascertain the identity of the colony, it was seen that no methanotrophic bacteria were identified (as seen in Table 24 in supplementary data).

As seen in Table 25 (in the supplementary data) further re-streaks were assessed using 16S rRNA sequencing and colony PCR for pMMO and sMMO. No 16S rRNA gene sequencing suggests methanotrophic colonies though the presence of pMMO and sMMO suggest the presence of MMO possessing methanotrophs within the colony.

Continued re-streaks and screening of potential methanotrophic colonies in conjunction with 16S rRNA and pMMO sequencing eventually led to the identification of methanotrophic species seen in Table 26. Colonies originating from a surface slurry sample, using pMMO sequencing were identified to contain a pMMO sequence closely related to *Methylococcus capsulatus* pMMO and *Methylocystis* SC2. Sequences from 16S rRNA often contradicted the pMMO for example *Niastella koreensis* (a non-menthanotrophic organism unlikely to contain pMMO) giving a pMMO identification for *Methylocystis* sp.SC2 this likely occurred as a result of a mixed colony containing methanotrophs and contaminating heterotrophs. Other colonies where both the 16S rRNA and pMMO sequence would corroborate, as seen in sample SS1 condition A in Table 26, would be discovered to be a mixed colony by purity tests.

To reduce possible sources of carbon supporting growth of non-methanotrophic species such as the vitamin solution and the agar within the solid dNMS media,

screened colonies were passaged through solid to liquid dNMS with no added vitamins all containing copper to promote methanotroph growth.

In addition as the samples originating from cow manure had there incubation temperatures increased to 37 and 42°C of which the 42°C cultures yielded a pure culture belonging to the *Methylocaldum* genus with a 16S rRNA sequence that has a 99% query cover and identity to that of the *Methylocaldum gracile* strain KAR5Ro7. This isolate was given the identification "isolate 10".

Alternative carbon source isolation method – To reduce heterotrophic cross-feeding of contaminant species with target methanotrophs dNMS was supplemented with combinations of yeast extract and methanol. Supplementing cultures with alternative carbon source did not break the syntrophic bond with the contaminants and as a result no pure methanotrophic cultures were obtained.

3.5.1.2 Enrichment isolation 2

Decreasing the initial inoculation volume from 1 ml to 10 µl, improved the reduction in visible organic matter contamination that provides a carbon and energy source for the growth of contaminants.

On suspecting carbon from the vitamin stock may play a role in supporting the growth of non-methanotrophic species during the enrichment isolation, vitamin stock concentration within the media was varied. All enrichments from 0x-0.1x supported growth with the exception of 0.0001x.

A variety of media were used to improve the effective enrichment of the environmental samples. NMS media is the typical media for cultivation of Methanotrophs, dNMS in a 0.2x concentrated version of NMS suitable for freshwater

methanotrophs as it contains low concentrations of salts. AMS is similar to NMS though its nitrogen source is ammonium instead of nitrate (ammonium chloride instead of potassium nitrate). M2 is a medium has a slightly lower concentration of $MgSO_4$ and $CaCl_2$ than dNMS though a much higher KNO_3 (higher than NMS). Furthermore M2 contains a very low concentration of phosphate stock and also no vitamin stock which is used freshwater wetlands and mildly acidic soils. MNMS is a media with a different source of sulphate and contains magnesium chloride. The concentration of its components is typically lower than NMS though it has shown to yield higher MPNs [85]. It was seen that dNMS and AMS were able to support growth of the enrichment sample were as the others did not.

The control including environmental sample inoculated with *M. capsulatus* (Texas) with methane was used to verify if an organism with growth characteristics that would be considered easily selectable (thermotolerant growth from a mesophilic consortia) could be achieved. This could not be achieved as no initial growth was seen.

M. capsulatus (Texas) culture with environmental sample without methane was used to see if growth would be observed without methane indicating that growth seen was as a result of contaminating carbon source. Growth was not observed suggesting that turbidity was seen as a result of methanotrophic growth.

The final control was no environmental sample or *M. capsulatus* (Texas) culture. This was used to see if contamination in the inoculation process could grow on the dNMS media. As expected this did not grow.

Initial colonies scanned via PCR using pMMO (A189f/A682r, A189f/A650r, A189f/MB661r) and sMMO (166f/1401R) showed no sign of methanotrophic organisms. Further incubation led to the appearance of new colonies containing MMO

that were serially diluted and re-streaked. This suggests a layer of heterotrophs may have been growing above the methanotroph colony. This was repeated multiple times with colonies remaining contaminated with non-methanotrophic species, similarly to enrichment isolation 1 colonies were serially diluted and passaged from liquid to solid dNMS with no vitamins with the addition of 10µM $CuSO_4$. This led to the isolation of two *Methylocystis* species given the I.D Isolate 03 and Isolate 6 which originated from the University of Nottingham lake sample.

3.5.1.3 Enrichment isolation 3

For the samples using the surface lake sediment for varying vitamin concentrations only one of the duplicates of 0.001x vitamin stock grew, the rest did not. Out of all the varying media including dNMS NMS AMS M2 and MNMS only one replicate of dNMS grew and were sub-cultured.

Of the controls the positive *M. capsulatus* (Texas) culture with methane grew as expected though similar to isolation two the environmental sample + *M. capsulatus* (Texas) with no methane and also the non-methanotrophic contaminant with environmental sample with no added methane did not grow.

All other environmental samples including reed bed, lake stream sediment, moss and shallow lake sediment grew and were sub cultured for further enrichment.

On plating dilutions of enrichments samples including controls, typical contamination was observed. The *M. capsulatus* (Texas) control with methane was screened using pMMO, sMMO and 16S rRNA analysis and a successful identification of the organism was achieved. This was surprising as it was achieved 15°C below its optimum temperature of 45°C. Uniform cocci morphology under phase contrast was observed which suggests possible purity.

Similar to the other enrichment isolations passaging from liquid to solid dNMS containing 10µM $CuSO_4$ was done to purity in which a representative of the Methylocystis genus was isolated from surface lake sediment from Wollaton park. This organism was given the I.D isolate 01

3.5.2 *Miniaturised extinction dilution methanotroph isolation*

3.5.2.1 Enrichment Isolation 4 (Miniaturised extinction dilution)

Using the protocol adapted from [38] initially no methanotrophic pure cultures were obtained. On subsequent passaging from liquid to solid media with copper and without vitamins using PCR to confirm the presence of an MMO gene pure cultures were obtained. The I.D. given to these isolates were 3* and 12 which 3* Moseley Bog samples and 12 from Nottingham landfill all of which were isolated from 30°C incubations.

3.5.2.2 Enrichment Isolation 4 (Miniaturised extinction dilution)

This enrichment isolation produced a pure culture of methanotroph on initial plating that can grow at 45°C from the hydrothermal water of the Roman Baths in Bath. This isolate was a member of the *Methylococcus* genus and was given the I.D isolate 14.

3.5.3 *Flow cytometry methanotroph isolation*

Flow cytometry methanotroph isolation yielded no isolates. No cells that were individually sorted into wells from enriched environmental samples showed evidence of growth. This was also seen in the *M. capsulatus* positive control. Conversely 75% of 56 wells individually sorted *E. coli* cells produced a growing culture though the negative control containing LB only showed growth in 2 of 8 wells suggesting contamination during the sorting

3.5.4 *Overall isolation outcome*

As summarised in Table 8 a range taxonomically diverse isolates spanning three genera including *Methylocystis*, *Methylocaldum* and *Methylococcus* were isolated from a variety of ecosystems. Five *Methylocystis* cultures were isolated. One *Methylocaldum* species was isolated from a cow manure sample most likely due to the fact that the Methylocaldum genus are largely thermotolerant organisms [90] which reflects the conditions found in the ruminants gastrointestinal tract. The *Methylococcus* species was isolated from the Roman Baths which is the sample location where the type strain *Methylococcus capsulatus* (Bath) was isolated from.

Table 8 - Summary of the pure cultures of methanotrophic isolates obtained from a range of isolation techniques. University of Nottingham (UoN), Sutton Bonington (S.B).

	Isolate 01	**Isolate 03**	**Isolate 3***	**Isolate 6**	**Isolate 10**	**Isolate 12**	**Isolate 14**
NCBI BLAST	*Methylocystis*	*Methylocystis*	*Methylocystis*	*Methylocystis*	*Methylocaldum*	*Methylocystis*	*Methylococcus*
Isolation Location	Wollaton Park lake	UoN lake	Moseley bog	UoN lake	S.B Cow manure	Moseley bog	Roman bath
Isolation method	Extinction dilution 3	Extinction dilution 2	Miniaturised Extinction Dilution	Extinction dilution 2	Extinction dilution 1	Miniaturised Extinction Dilution	Extinction dilution 4

As seen in Figure 10 a variety of morphologies including pink, cream, white and brown were observed. Most noticeably purity could be observed when the colony produced had a clear edge surrounding the perimeter of the colony. Often contamination could be seen as a thin film of growth protruding out from the colony edge. In addition, colony morphologies of became uniform once pure.

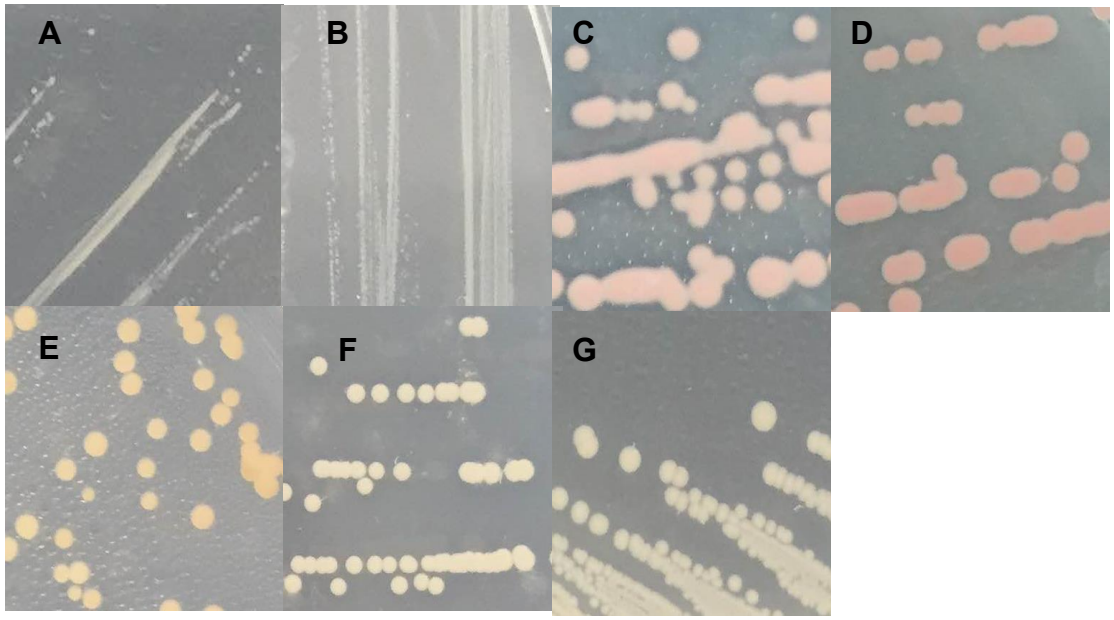


Figure 10 - Colony morphology of isolates obtained from the sum of isolation techniques. Isolate 01 (A), Isolate 03 (B), Isolate 3* (C), Isolate 6 (D), Isolate 10 (E), Isolate 12 (F), Isolate 14 (G).

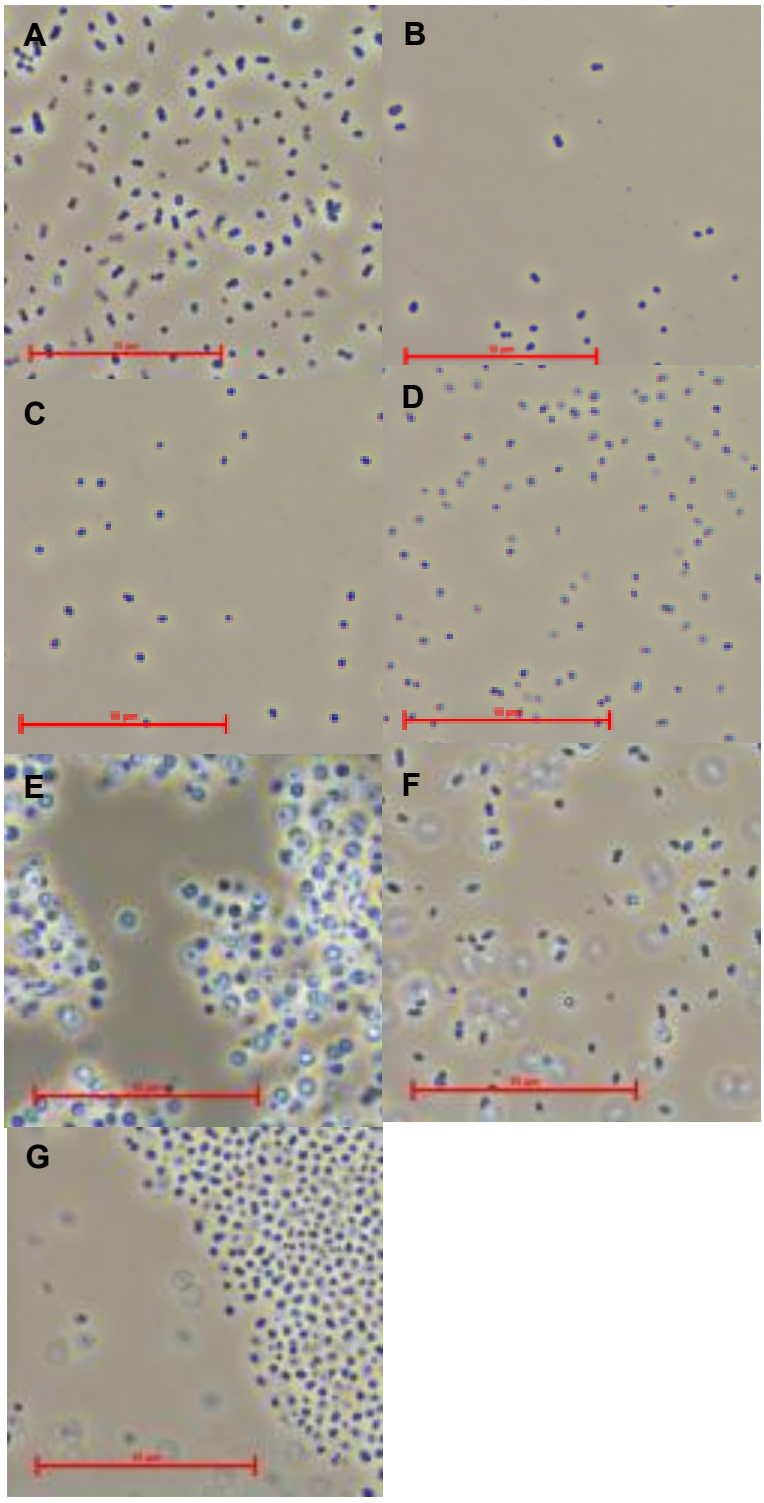


Figure 11 – Phase contrast microscopy (x100) of isolates obtained from the sum of isolation techniques. Isolate 01 (A), Isolate 03 (B), Isolate 3* (C), Isolate 6 (D), Isolate 10 (E), Isolate 12 (F), Isolate 14 (G).

3.6 Discussion

3.6.1 *Enrichment Isolation (Dilution Plating)*

Throughout the dilution plating experiments the protocols were initially unsuccessful and then slowly altered to the point of achieving pure cultures. It was seen that the most reliable media to ensure the successful enrichment of methanotrophs was dNMS, as dNMS and AMS were the only media to facilitate growth of organisms from lake sediment samples and dNMS was the only media to support growth from the Wollaton Park lake sediment. NMS may have not been successful due to the high salt concentrations causing osmotic pressure on the cells though contrary to this point MNMS, which has lowered salt in order to be optimised for the specific growth of freshwater methanotrophs, also did not support growth. Interestingly controls such as *M. capsulatus* (Texas) spiked cultures containing no methane did not grow. It suggests that the growth seen in each enrichment is a result of methanotrophic growth though the heterotrophs are surviving on either by products of methanotrophic growth or by remaining in a dormant endospore state until on agar. Growth of contaminants was consistently seen on agar which may have been able to grow as a result of carbon source from either the carbohydrates found in Bacto agar, carbon from the vitamin stock, or carbon derived from methanotrophic metabolism.

PCR was an excellent tool to identify methanotrophic colonies. Sequencing 16S rRNA PCR amplifications alone was not suitable as initially performed as it was time consuming and often PCR sequencing was poor due to the fact multiple sequencing would be simultaneously extracted and sequenced as indicated by noisy multiple peak chromatograms. PCR amplification of pMMO and sMMO gave an instant identification and was a key tool to differ heterotrophs from methanotrophs. It was seen that heterotrophs would grow first within approximately the first few days and

methanotrophic colonies would appear after 5 days. This implicates that the contaminants were able to metabolise the agar. It was also observed that methanotrophic colonies that were contaminated had a thin film surrounding the colony, presumably heterotrophs that were syntrophically bound to the methanotrophs feeding of secreted methane derived carbon sources. Further additions to the protocol improved the techniques for example reducing the inoculation volume visibly reduced the amount of contaminating organic matter. In assessing for purity growth on diluted rich media proved most effective as in some cases presumed oligotrophs would grow on 1/10 diluted LB or TSA but not the non-diluted (Data not shown).

Enrichment isolation was based on a simple enrichment isolation method performed by Auman *et al* [41] that was previously discussed. The protocol varied methane, air, copper and nitrogen and yielded 11 isolates over 4 genera. For the isolation described in this study nitrogen was not used as a variable factor (outside of using AMS media) and only methane and copper were varied. Unlike the results seen by Auman *et al* pure cultures were not seen immediately after re-streaking following initial serial diluting onto solid media after the two liquid enrichment steps. Further PCR screening and re-streaking further did not yield any pure cultures. Following this a different approach was attempted. As shown by Tsubota *et al* [91] the presence of vitamins led to only heterotrophic contaminants to remaining on solid media after multiple re-streaks, removal of the vitamins had an inhibitory effect on heterotrophs and led to the isolation of a pure culture of *Methylothermus thermalis*. Therefore, an additional step was added to the dilution plating which was to remove vitamins from all media. In addition to this it was suspected that agarose was supporting the growth of heterotrophs so passages from liquid to solid were performed. This technique proved successful as multiple pure cultures were subsequently isolated when removing the

vitamins from the media along with serial dilution and passaging from solid to liquid media.

No conclusion can be made on the effectiveness of isolation regarding methane copper or vitamins as all supported growth though not enough pure cultures were isolated to compare conditions. It can be seen that dNMS supported growth more consistently that other media most likely due to the fact that is more closely resembled the conditions of the fresh water environments they were sampled form. Other methods such as adding alternative sources of carbon to break the synthrophy of the contaminants proved unsuccessful.

3.6.2 *Miniaturised extinction dilution isolation*

As previously discussed the miniaturized extinction culturing method devised by Hoefman *et al* [38] showed to isolate 22 pure cultures where as traditional dilution plating, performed in parallel, yielded no pure isolates. When the miniaturised extinction method was performed on a range of samples 16S rRNA analysis revealed no methanotrophic colonies which differed greatly from the results obtained by Hoefman *et al*. Deviations from the protocol, and therefore possible sources of error, include increasing the incubation temperature from 20°C to a more typical 30°C and varying the copper concentration from 0.8 µM Cu^{2+} to 10 µM $CuSO_4$. The lower temperature was chosen to more closely represent the environmental conditions of the sample and the increase in temperature may have given a selective advantage to the heterotrophs. The rationale to increase the copper concentration over the long 5 week period of growth was intended to further support methanotrophic growth. It is unlikely the increase in copper gave growth advantages to heterotrophs over methanotrophs. Eventually employing the method added onto the dilution plating method that evolved

passaging from liquid to solid with copper and removing vitamins eventually led to the isolation of pure cultures of methanotrophs.

3.6.3 *Flow cytometry isolation*

Flow cytometry (FC) is typically used as an analytical method that can measure light scattered and fluorescence emissions from individual cells that can be subsequently sorted. Isolation of pure cultures of microorganisms using FC is not regularly used though an example was seen where viable phytoplankton cultures were obtained after isolation by single cell sorting using FC [92].

The major problem with FC sorting is producing a viable cell after sorting. FC sorting causes physical stress to cells which includes changes in fluidic pressure, exposure to laser beam, electrostatic charges, and deflection through high voltage fields [92]. The positive control of *E. coli* single cell sorting was implemented to assess this and it was seen that 75% of wells produced growth even though it was clear that the process was susceptible to contamination (25% of un-inoculated wells showed growth). This suggests that *E. coli* can be sorted and remain viable. Unlike *E. coli* the methanotrophic positive control of single cell separated *M. capsulatus* (Bath) showed no growth in any wells, one suggestion for this could be that methanotrophic species are more susceptible to physiological damage due to the reliance on the membrane associated pMMO. Though it is more likely that the recovery medium is not effective enough to re-grow cells which was shown to play a role in viability of single cell sorted cultures in phytoplankton single cell sorting and culturing [92]. Using methanol as a growth substrate instead of methane could be a possible solution as methanol may be more bioavailable due to methane's low mass transfer and also circumvents the use of pMMO.

3.6.4 *Conclusion*

Overall the enrichment isolation based on the existing protocol did not initially yield pure cultures of methanotrophs, the additional steps of PCR screening combined with passaging to liquid and solid including copper and excluding vitamins in conjunction with extinction dilution proved to be a reliable and effective in isolating pure cultures of methanotrophs.

4 *Characterisation of Methanotrophic Bacteria*

4.1 Chapter Introduction

Taxonomy is the process of classifying and naming groups of organisms into taxa based on shared characteristics; these taxa are given ranks and as the ranks increase organisms are aggregated into groups. Modern hierarchal groups include: Domain, Kingdom, Phylum, Class, Order, Family, Genus and species. Organisms are named using binomial nomenclature which includes only the genus and species (e.g. *Methylococcus capsulatus*). Genera are typically based around groupings of characteristics broadly encompassed by phenotype, genotype and phylogeny. The term species when applied to bacteria that defines a distinct group of strains that have certain distinguishing features and that generally bear a close resemblance to one another in the more essential features [93]. Previously an often used *ad hoc* method of resolving species is 16S rRNA comparison using pairwise alignment in which 97% or lower homology should be seen to confirm a novel species [94]. This method in recent years has often been overlooked when characterising new species as often significant distinct phenotype, strongly suggesting a distinct species identification, have been seen with 16S rRNA pairwise alignments <97%. With the dramatic drop in price of sequencing since 1994, when the <97% rule was accepted by scientific consensus, a vast increase in the number of quality 16S rRNA sequences have emerged. As a result the *ad hoc* <97% rule has been reviewed. It was recently seen that using a variety of taxonomic clustering algorithms that a consensus was reached suggesting the optimal identity thresholds wascloser to ~99% for full-length sequences [95].

Guidelines to assessing the broad array of microbial traits for taxonomic purposes can be seen in [42] though as detailed a minimal standards are set for methanotrophs

specifically which have are described by [43] and have been previously reviewed. Following identification, nomenclature, the assignment of names to taxonomic groups is done according to International Code of Nomenclature of Bacteria.

Basic characterisation was performed in the previous chapter in regards to colony morphology and phase contrast microscopy. To taxonomically identify each isolate and to assess for novelty thorough characterisation is needed. Characterisation typically used to describe novel methanotrophic species provides important information for the use of an industrial strain. For example, knowing which metabolic pathways are present directs what pathways can be exploited, also what competing pathways are likely to exist, and certain methanotrophs naturally produce value added compounds of interest. Genes giving rise to competing pathways are often targeted for gene knockout, as a result genome sequencing and annotations are essential. Phenotypes often not assessed for general microbiological purposes that are essential for industrial strains include experiments such as tolerance to end products.

4.2 Strains and Plasmids

The organisms obtained from culture collections and the environmental isolate methanotrophs used in this chapter are detailed within section 2.2

4.3 Primers

The following primers are used to assess key genes within isolated methanotrophic organisms.

Table 9 - primers used in this chapter.

Name	Sequence (5' to 3')	Gene/Description
pMMO-2 (A189f/MB661r)	F - GGNGACTGGGACTTCTGG R - CCGGMGCAACGTCYTTACC	Amplifies the *pmoA* sub-unit of pMMO [89]
sMMO (534f/1393R)	F - ACCAAGGARCARTTCAAG' R - CCGATCCAGATDCCRCCCCA	Amplifies the *mmoX* sub-unit of sMMO *(mmoXA/mmoXD)* [96]
16S rRNA (u199f/U1391r)	F - AGYGGCGNACGGGTGAGTAA R - GACGGGCGGTGWGTRCA	Amplifies the sequence encoding 16S ribosomal subunit
Divergant pMMO (pmoA189f/pxmA634R)	F – GGNGACTGGGACTTCTGG R -CTATGATGCGCAGATATTCTGG	Amplifies a the *pxmA* sub-unit of the divergent pMMO known as *pXMO* [89]
Nitrogenase (F1/nifH439R)	F- TAYGGNAARGGNGGNATYGGNA ARTC R - GGCATNGCRAANCCDCCRCA	Amplifies the nitrogenase sub-unit *NifH* [97]
Methanol dehydrogenase (f1003/r1561)	F- GCGGCACCAACTGGGGCTGGT R - GGGCAGCATGAAGGGCTCCC	Amplifies the methanol dehydrogenase sub-unit *mxaF* [98]

4.4 Methods used in Chapter

4.4.1 *Transmission electron microscopy*

Isolates were cultivated as previously described. Cultures were centrifuged 5,000 rpm for 5 minutes and suspended in 0.1M cacodylate buffer overnight then re-suspended

again in in the same solution a further 3 times. Samples re-suspended in 1% aqueous Osmium Tetroxide and pipetted into beam tubes. Samples were then washed once for 1 min in distilled water then dehydrated using ethanol series of 50, 70, 90, 100%. The final dehydration step was done by re-suspending the pellets twice in 100% Propylene Oxide for 15 minutes then centrifuged and incubated at room temperature in 1:3 resin:propox mix for 4 hours. The resin propox mix was then removed and replaced with 1:1 resin and incubated at room temperature overnight. The 1:1 resin was removed and cells incubated in pure resin at room temperature for 2.5 hours pelleted, re-suspended and repeated. The samples were then incubated at 60°C for 48 hours. Embedded isolates were then sectioned using an ultramicrotome diamond cutter. These slices were placed onto a copper grid. Sections were stained by first washing the copper grids in drops of urinyl acetate for 5 minutes then washed in ethanol and then sterile water. Drops of lead citrate were applied to a petri dish as described above with the addition of sodium hydroxide pellets to remove carbon dioxide. The washed grids placed on the lead citrate for 5 minutes then washed in water. The fixed stained samples were viewed using a FEI Tecnai 12 Biotwin transmission electron microscope.

4.4.2 *Phylogenetic Analysis of 16S rRNA sequences.*

Isolates cultivated on solid dNMS were subject to colony PCR using primers U119f and U1391r, all PCR reactions were carried out using Q5® High-Fidelity 2X Master Mix in a 25 µl reaction. PCR products were visualised on agarose gel, purified using Zymoclean™ Gel DNA Recovery Kit and sequenced using Source Bioscience's Overnight Service™.

The nucleotide sequences were trimmed based on ambiguously labelled ends and the sequences chromatogram was viewed for noise which could imply a source of

contaminating organism present. Sequences were analysed against the NCBI database using BLAST search. To establish genetic evolutionary relationship 16S rRNA based phylogenetic trees were generated by the neighbour-joining method using Mega5 software against closely related species [99].

4.4.3 *PCR of key genes*

The presence of key genes in the isolates was confirmed with PCR using Q5® High-Fidelity 2X Master Mix in conjunction with primers targeting subunits within genes: particulate methane monooxygenase (*pmoA*), divergant sequence particulate methane monooxygenase (*pxmA*), soluble methane monooxygenase (*mmoX*), Nitrogenase (*NifH*) and methanol dehydrogenase (*mxaF*).

4.4.4 *Naphthalene assay*

The naphthalene assay was performed in a qualitative [100] and quantitative [101] manner to observe the presence of sMMO. In the qualitative experiment isolates were grown on dNMS agar, as previously described, for 11 days (no extra copper was added to the media). Plates were inverted and naphthalene crystals were sprinkled on the lid and the plates were returned to their respective incubation temperatures for 30 minutes. After incubation the plates were coated with 500 µl of 5 mg ml^{-1} of freshly prepared tetrazotized o-diansidine. The expression of sMMO was characterised by a purple-red colour forming in the colonies.

The qualitative assay was performed using a modified version of the naphthalene assay [101]. Liquid cultures of isolates with an OD600 of between 0.3 and 0.4 and triplicate samples of each had 3 ml aliquotted into falcon tubes with several flakes of naphthalene sprinkled in and closed. Cultures were incubated at their respective temperatures for 2 h at 200 rpm. The cell suspension was centrifuged for 5 minutes at

6300 x g. Of the supernatant 1.3 ml was added to a cuvette and 130 µl of freshly prepared 4.21 mM tetrazotized o-diansidine was added and immediately the OD528 was measured.

4.4.5 *Poly-3-hydroxybutyrate quantification.*

Cultures incubated for 2 weeks to induce starvation were freeze-dried and weighed. Poly-3-hydroxybutyrate was quantified using GC-MS by SBRC analytics team. Biomass hydrolysed and derivative and run on a GC/MS is an Agilent (6890N/5973) using an Agilent HP-5MS 30m x 0.25mm x 0.25um column. The run was at 60°C for the initial temp then held for 3 minutes, ramp to 100°C at 20°C/min, ramp to 300°C at 50°C/min and hold for 5 minutes. Total run time of 14 minutes. Injection was using a splitless injector at 300°C and detected by FID at 150°C. The carrier gas used was hydrogen.

4.4.6 *Carbon source utilisation*

Isolates were inoculated from stationary phase pre-cultures into 10 ml of dNMS in 60 ml serum bottles to give an initial OD600 of 0.02 with no added methane. Alternative carbon substrates were prepared, filter sterilised and added to a final concentration of 0.05% (w/v). These carbon sources included: Acetate, ethanol, pyruvate, glucose and lactate. From the same pre-cultures positive controls containing 1:1 methane:air and negative controls containing no added carbon source with only air in the headspace. The ΔOD600 was calculated by subtracting start OD600 of 0.02 with the final OD600 recorded to assess growth on the various substrates.

4.4.7 *Methanol Utilisation*

All isolates were inoculated into a gradient of methanol concentrations ranging from 0, 0.01, 0.1 and 1% within 10 ml dNMS in 60 ml serum bottles. OD600 was recorded after 15 days.

4.4.8 *Tolerance to salt*

Stationary phase pre-cultures of isolate 3*, 6, 10 and 12 were inoculated into a 24 well plate in 1ml of NMS media in triplicate with the following NaCl concentrations: 0, 0.25, 0.5, 0.75, 1 and 5% (w/v). Plates were sparged with methane and incubated at 30°C at 200 rpm in a gas tight box. Cultures had the gas headspace refreshed at 3 days and incubated for a further 4 days on which the OD600 was recorded. OD600 was measured by re-suspending cells with a 1 ml pipette and using a Tecan Infinite M1000 Pro with 1 point read per well.

4.4.9 *Nitrogen source utilisation*

Stationary phase pre-cultures of isolate 3*, 6, 10 and 12 were pelleted and re-suspended in nitrate free NMS. Isolates were inoculated into a 24 well plate in 1ml of media in triplicate with the following nitrate sources at 0.05% (w/v): Ammonium chloride, L-asparagine, L-aspartate, L-glutamine, L-lysine, L-ornithine, and putrescine. In addition to this standard NMS and nitrate free NMS were used as controls. Plates were sparged with methane and incubated at 30°C at 200 rpm in a gas tight box. Culture gas headspace was refreshed at 4 and 8 days and OD was recorded after 14 days. Absorbance was measured at OD600 was measured by re-suspending cells with a 1 ml pipette and using a Tecan Infinite M1000 Pro with 9 point reads per well with a 2500 µM border. Nitrate free negative controls that showed final OD600 growth of around 0.01-0.06 were subtracted from the end results to give the final change in OD600.

4.4.10 *Motility and morphology of isolate 10*

Isolate 10 inoculated to an OD600 of 0.05 in 10 ml NMS with 1:1 methane air headspace over pressured. Phase contrast microscopy performed, as previously described, at x100 magnification at 0, 24, 48, 72, 96, 120 hours.

4.4.11 *End product tolerance*

Pre-cultures of Isolate 14 and 6 grown to exponential phase in dNMS in 250 ml serum bottles with 100 ml methane over-pressured. Pre-culture for isolate 14 and 6 were sub-cultured into 60 ml serum bottles with 0, 0.025, 0.05, 0.1, 0.2 and 0.4% (v/v) isobutanol, isopropanol, and acetone in triplicate to and OD600 of 0.1 in 5 ml dNMS. Cultures over-pressured with 30 ml of methane and incubated at respective temperatures with 200 rpm agitation. The OD600 of each culture is then checked after 3 days.

4.5 Results

4.5.1 *Phenotypic Characterisation*

4.5.1.1 Transmission electron microscopy

Transmission electron microscopy revealed a diverse morphology, type I and II ICM, storage granules, exopolysaccharides and fimbriae. The TEM images of a range of isolates can be seen below.

Isolate 3* -

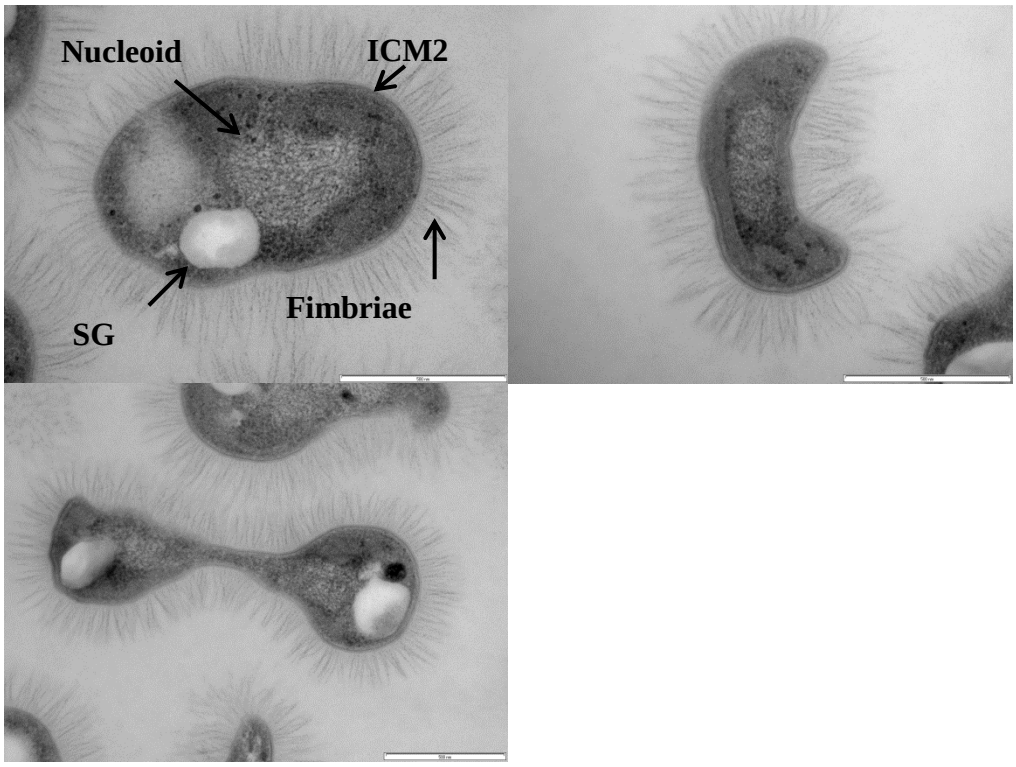


Figure 12 - Transmission electron microscopy of isolate 3*. SG, Storage Granules. ICM2, type II intracytoplasmic membrane. Bar, 500nm

Isolate 3* showed a polymorphic short rod morphology with a type II ICM that ran in parallel with the cell wall. In addition to this electron dense areas showed inclusion bodies of what is likely to be PHB. Furthermore, isolate 3* showed an extensive fimbriae attached to the cell surface spanning the entire membrane with a uniform length of approximately 140 and 210 nm in length.

Isolate 6 –

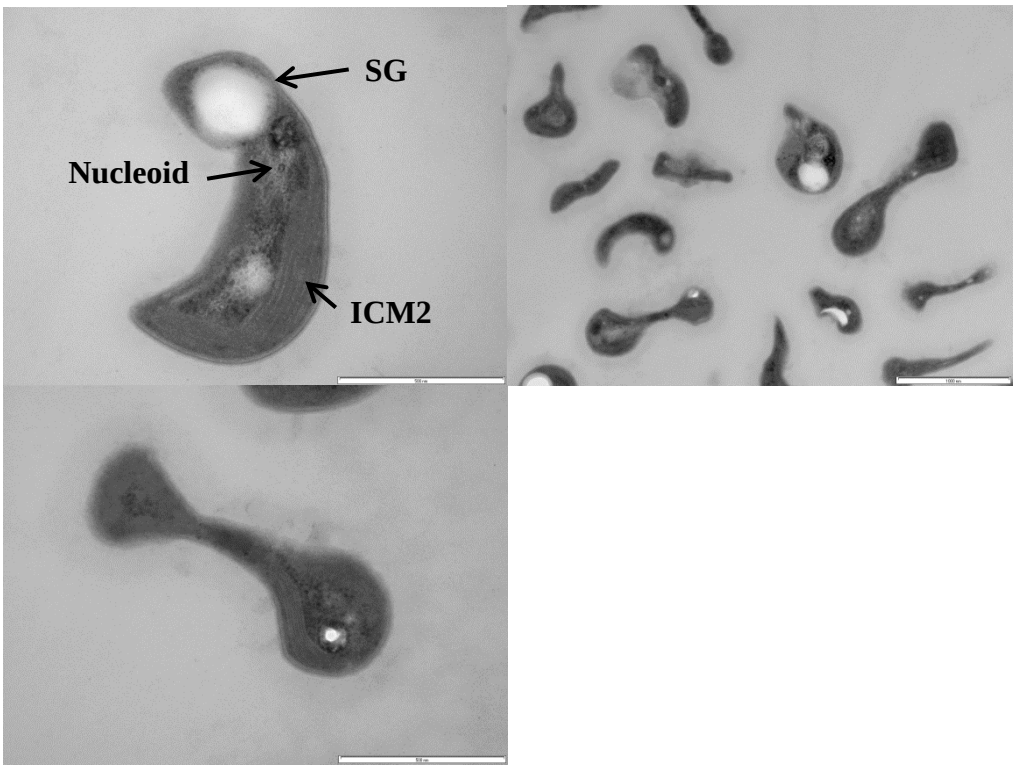


Figure 13 - Transmission electron microscopy of isolate 6. SG, Storage Granules. ICM2, type II intracytoplasmic membrane. Bar, 500 nm.

Isolate 6 showed at polymorphic morphology though often exhibited dumbbell morphologies possibly during cellular division. Suspected PHB granules and type II ICM membrane observed. No fimbriae were seen.

Isolate 10 -

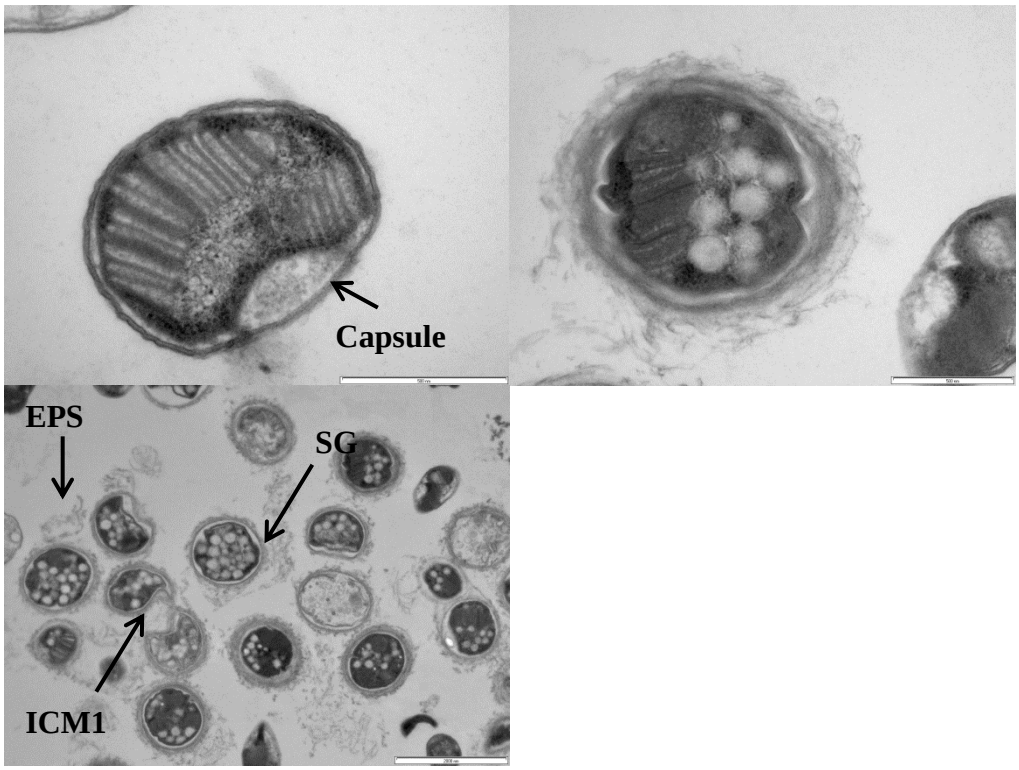


Figure 14 - Transmission electron microscopy of isolate 10. EPS, exopolysaccharide. SG, storage granule. ICM1, type I intracytoplasmic membrane. Bar, 500 nm

Isolate 10 had a regular cocci morphology and a type I ICM of bundled membranes running anti-parallel to the cell membrane. Electron dense areas show storage granules present which may contain glycogen. Cell capsules were observed surrounding the cells and also exopolysaccharides layer often surrounded the capsule.

Isolate 12 -

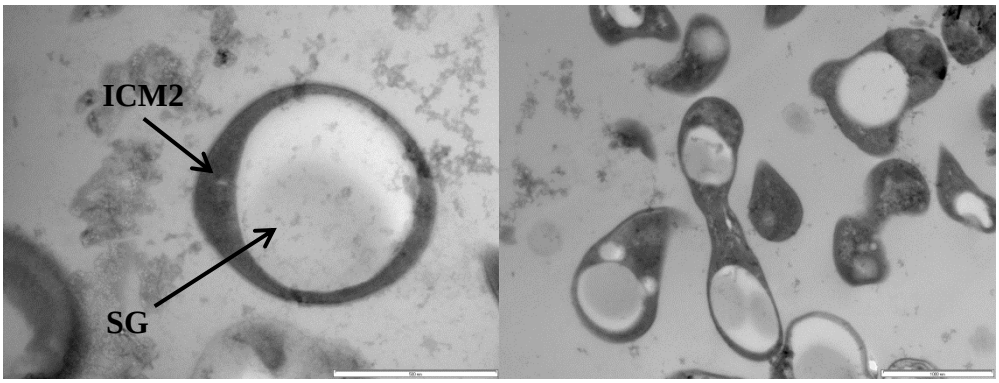


Figure 15 - Transmission electron microscopy of isolate 12. SG, Storage Granule. ICM2 type II intracytoplasmic membrane. Bar, 500 nm

Isolate 12 exhibited a rod/coccal morphology though extensive carbon storage granules possibly misshape the cells original morphology. Isolate 12 also exhibited a Type II ICM and no fimbriae were present.

Isolate 14 -

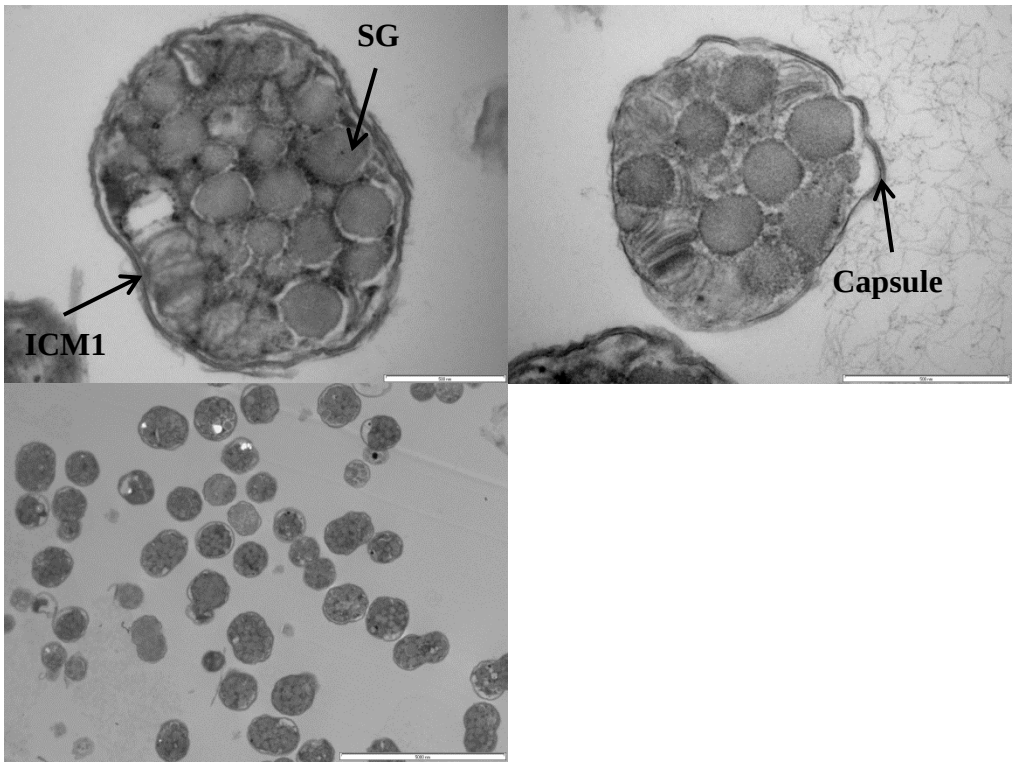


Figure 16 - Transmission electron microscopy of isolate 14. SG, Storage Granule. ICM1, Type I intracytoplasmic Membrane. Bar, 500 nm

Isolate 14 showed regular cocci morphology with a type I ICM. Carbon storage granules were seen within the cells and the cells also showed a surrounding capsule.

4.5.1.2 Phylogenetic analysis of 16S rRNA sequences

Figure 17 shows the phylogenetic relationship of the isolates to known representatives of each species, this is based on 16S rRNA sequence of around 1272 bp. Isolates were grouped into three phylogenetic trees based on the most closely related genus when searched for via NCBI BLAST.

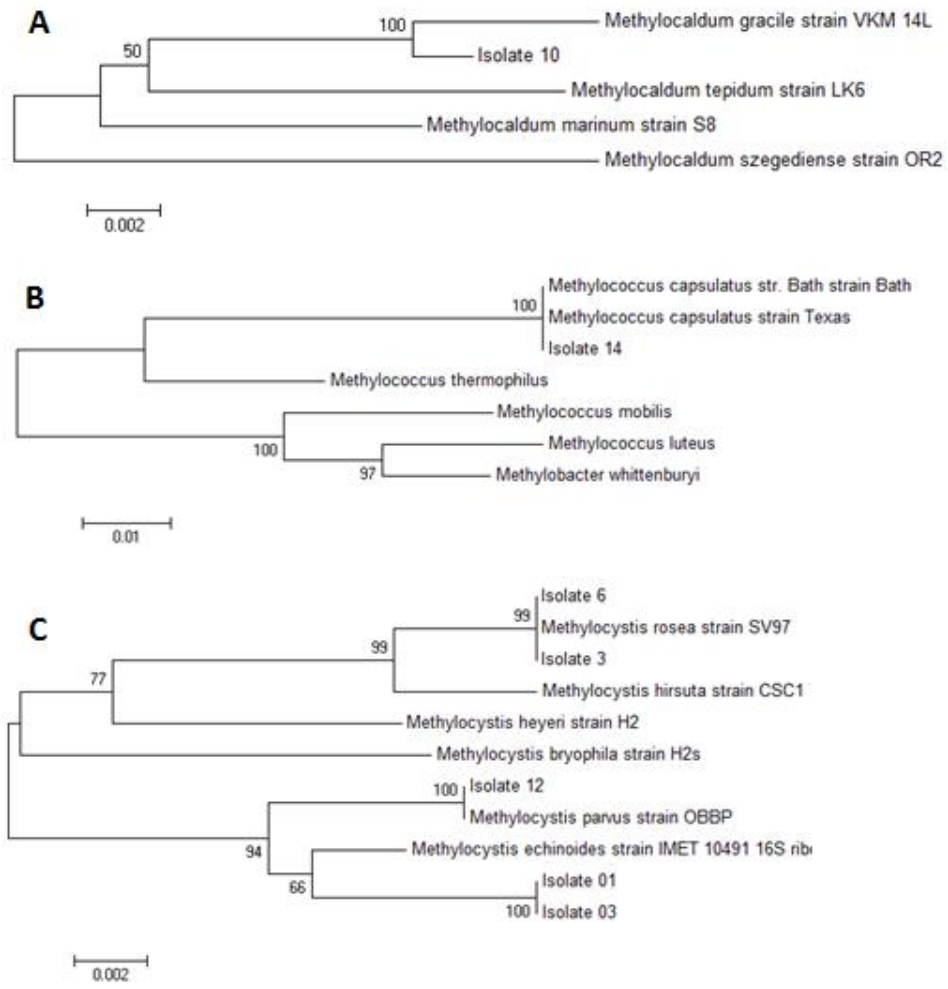


Figure 17 - Phylogenetic tree showing the relationship between isolates and the species within the genera as identified by BLAST search. Neighbour joining statistical method with bootstrap replications of 500 with a maximum composite likelihood model was used. Bar, shows the substitutions per nucleotide difference. Nodes, levels of bootstrap support (%) based on 500 resamples. (A) Isolate 10 within the *Methylocaldum* genus (B) Isolate 14 within the *Methylococcus* genus (C) Isolate 01, 03, 3*, 6 and 12 within the *Methylocystis* genus.

Figure 17A shows that Isolate 10 has a divergent 16S rRNA sequence that shares its closest common ancestor with *M.gracile*. Isolate 14 within the *Methylococcus* genus has identical 16S rRNA with the Bath and Texas strain of *M. capsulatus*. Similarly in the *Methylocystis* genus isolate 3 and 6 share identical 16S rRNA sequence with *M. rosea*, Isolate 12 has identical 16S rRNA with *M. parvus* OBBP. Whereas Isolate 01

and 03 have identical 16S rRNA sequences though these sequences are divergent from its closest related species *M.echnoides*.

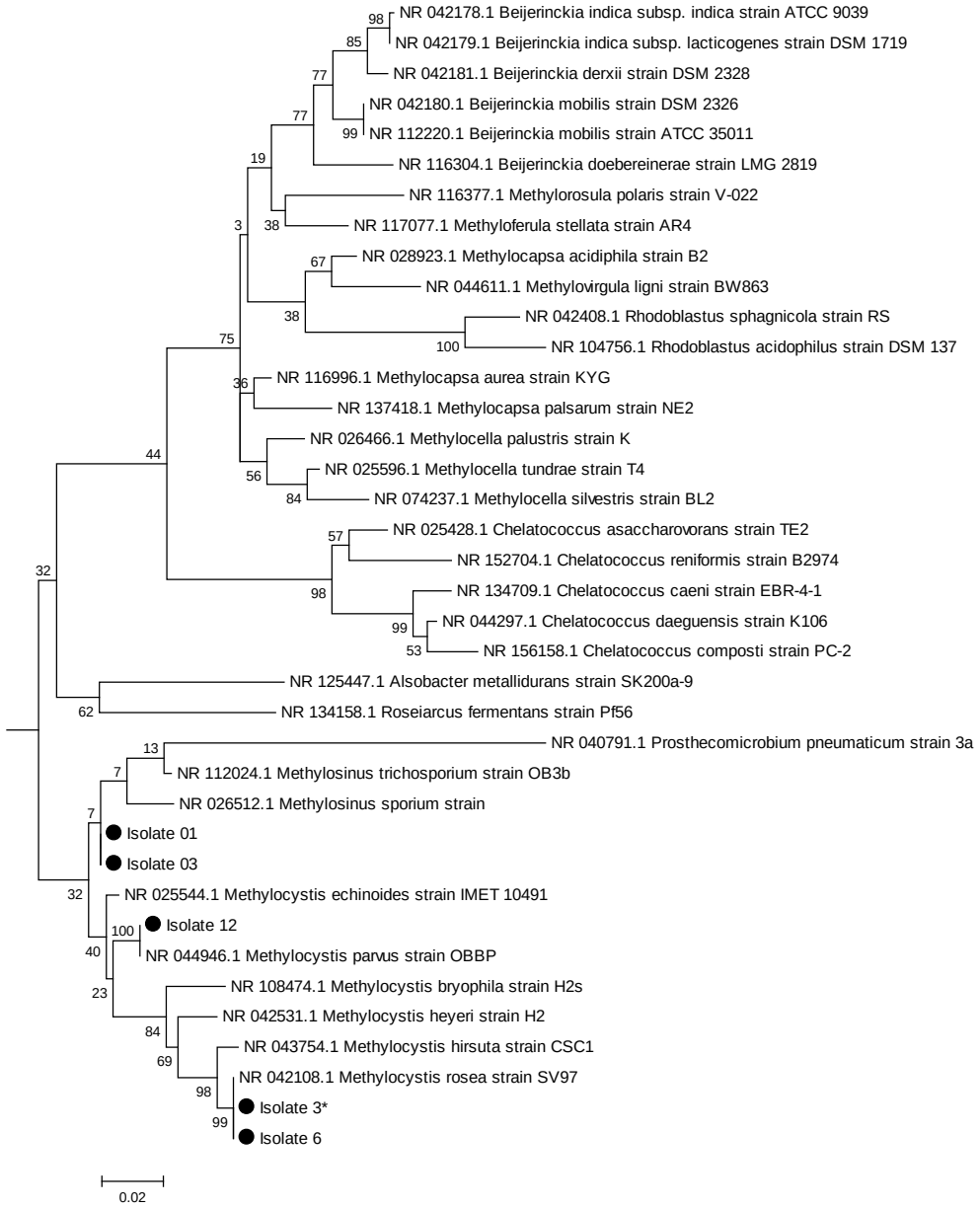


Figure 18 - Phylogenetic tree showing the relationship, based on 16S rRNA sequence, between Type II isolates and the species within the genera as identified by BLAST search. Neighbour joining statistical method with bootstrap replications of 500 with a maximum composite likelihood model was used. Bar, shows the substitutions per nucleotide difference. Nodes, levels of bootstrap support (%) based on 500 resamples. Black solid circles, identifies isolates. NR, BLAST NCBI accession number.

Figure 18 shows a phylogenetic comparison based on 16S rRNA sequences that includes closely related 16S rRNA sequences. This phylogenetic tree confirms the phylogenetic position of 3* and 6 though it can be seen that isolate 01 and 03 represent a divergent clade though the bootstrap support for this conclusion is 7%

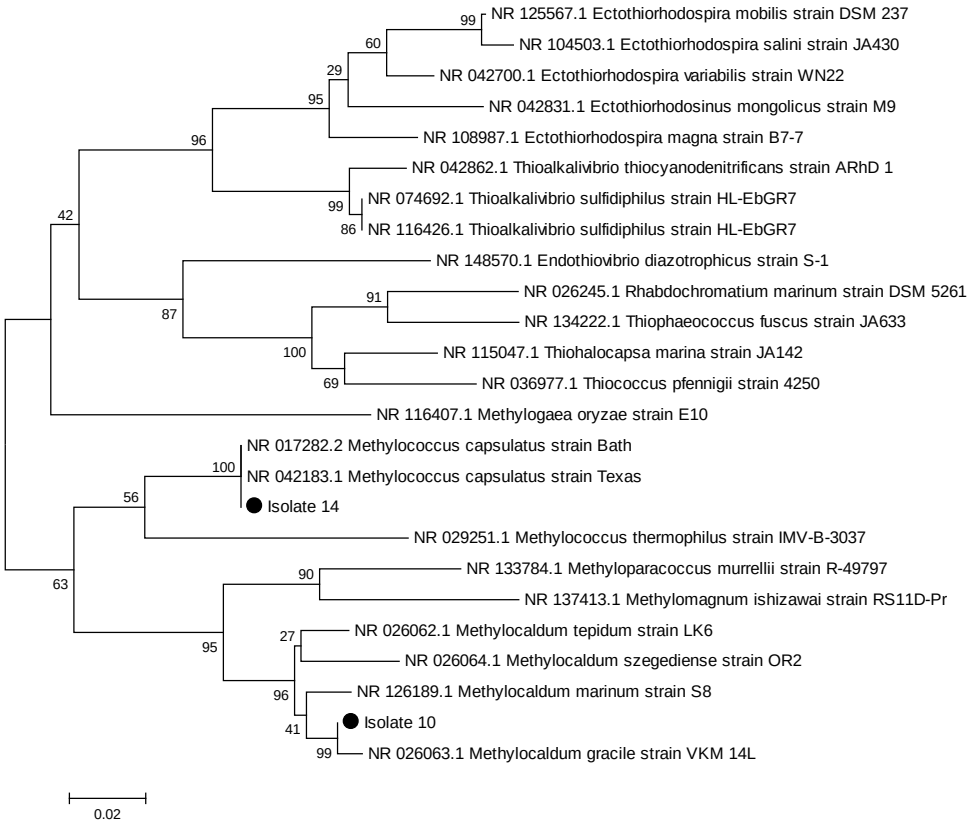


Figure 19 - Phylogenetic tree showing the relationship, based on 16S rRNA sequence, between Type I isolates and the species within the genera as identified by BLAST search. Neighbour joining statistical method with bootstrap replications of 500 with a maximum composite likelihood model was used. Bar, shows the substitutions per nucleotide difference. Nodes, levels of bootstrap support (%) based on 500 resamples. Black solid circles, identifies isolates. NR, BLAST NCBI accession number.

Phylogenetic comparison between type I isolates and the closely related species in Figure 19 further confirms isolate 14 close identity with *M. capsulatus Bath* and Texas strain. Furthermore isolate 10 is confirmed to be phylogenetically divergent from *M.gracile*.

4.5.1.3 Alignment of 16S rRNA sequence to closely related species

To quantify the homology of 16S rRNA sequences a comparative analysis multiple alignment was performed using Clustal Omega 2.1. Candidates for comparison were identified by previous phylogenetic analysis seen in Figure 19. Only isolates with divergent 16S rRNA sequences were used because non-divergent sequences would give 100% similarity.

Table 10 – Percent identity matrix of isolate 10 and closely related methanotrophic species based on 16S rRNA sequence (full sequence) homology by multiple sequence alignment. Statistics were generated using 16S data taken from the phylogenetic analysis and aligned using Clustal Omega 2.1. Values expressed as (%).

	Methylocaldum szegediense	***Methylocaldum tepedium***	***Methylocaldum gracile***	**Isolate 10**
Methylocaldum szegediense	100	97.05	97.65	97.72
Methylocaldum tepedium	97.05	100	97.92	97.99
Methylocaldum marinum	97.79	97.65	98.66	98.73
Methylocaldum gracile	97.65	97.92	100	99.93
Isolate 10	97.72	97.99	99.93	100

Table 10 shows isolate 10 is most closely related to *M.gracile* with a similarity of 99.93%.

Table 11 - Percent identity matrix of isolate 01, 03 and closely related methanotrophic species based on 16S rRNA sequence homology by multiple sequence alignment. Statistics were generated using 16S data taken from the phylogenetic analysis and aligned using Clustal Omega 2.1. Values expressed as (%).

	Methylosinus trichosporium	***Methylosinus sporium***	***Methylocystis parvus***	**Isolate 01**	**isolate 03**	***Methylocystis echinoides***
Methylosinus trichosporium	100.00	98.02	97.93	98.36	98.36	97.76
Methylosinus sporium	98.02	100.00	97.93	98.54	98.54	97.85
Methylocystis parvus	97.93	97.93	100.00	98.71	98.71	99.14
isolate 01	98.36	98.54	98.71	100.00	100.00	99.14
isolate 03	98.36	98.54	98.71	100.00	100.00	99.14
Methylocystis echinoides	97.76	97.85	99.14	99.14	99.14	100.00

Table 11 shows isolate 01 and 03 have identical 16S rRNA sequences that are most closely related to *Methylocystis echinoides* with a 99.14% homology.

4.5.1.4 PCR of essential genes

Isolates were subject to PCR targeting 16S rRNA, *pmoA*, *pxmA*, *mmoX*, *NifH* and *MxaF*. As seen in Figure 20 all 16S rRNA sequences were amplified and sequenced. All isolates contained *pmoA*, a sub-unit of pMMO and *mxaf*, a subunit of methanol dehydrogenase. Only isolate 14 was shown to possess the *mmoX* sub-unit from sMMO. Furthermore all isolates contained the nitrogenase subunit *nifH* and none contained the divergent pMMO sub-unit *pxmA* often found in the *Methylomonas*, *Methylobacter* and *Methylomicrobium* genus [102].

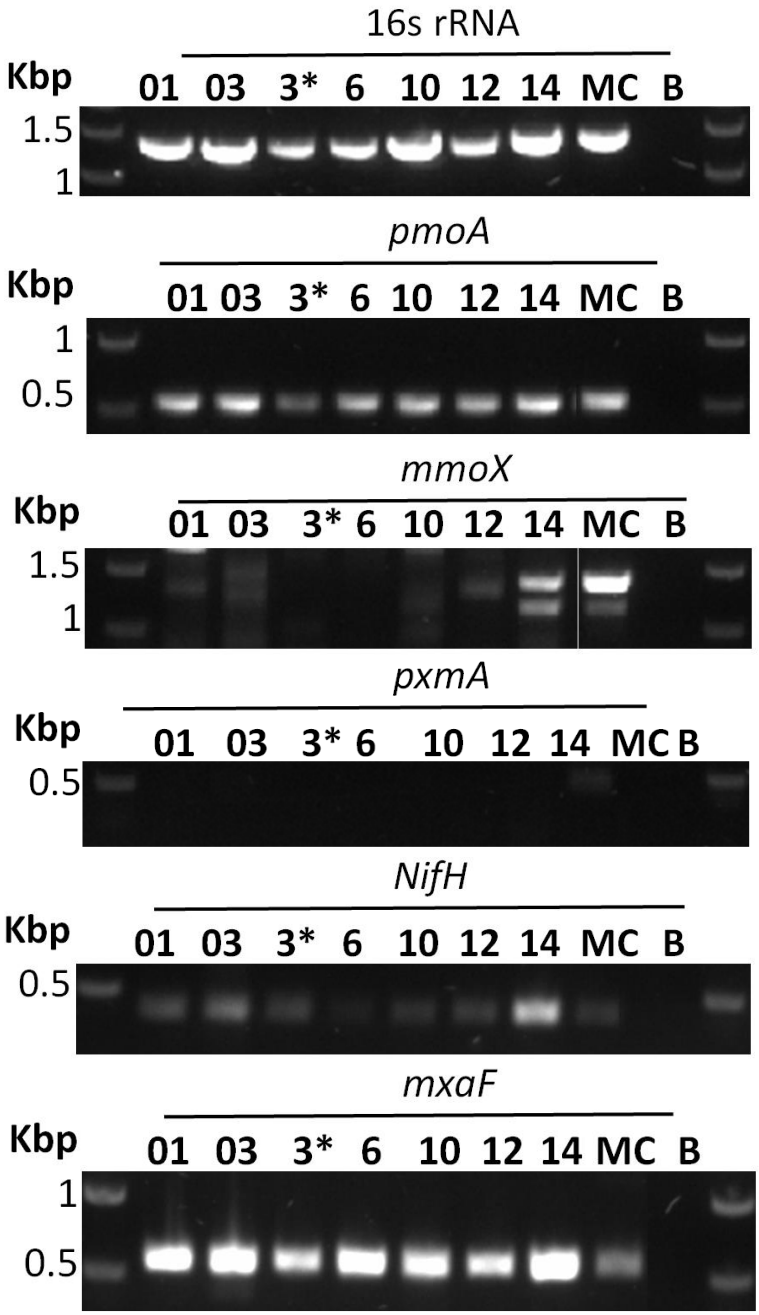


Figure 20 - PCR of essential genes in methanotrophic isolates. Each number above the lanes corresponds to the respective isolate being assessed. MC, *M. capsulatus* . B, Blank (PCR reaction with no template).

4.5.1.5 Naphthalene assay

The Naphthalene assay is a colorimetric assay for the detection of sMMO. Under the low copper to biomass ratios methanotrophs preferentially express the iron dependant sMMO over the Cu dependant pMMO. The catalysis naphthalene to naphthol via

sMMO can be detected by the o-diansidine in which a purple colour is viewed. This purple colouration can be quantified using absorbance at 550 nm.

The naphthalene assay was performed on agar plates using all the isolate and *M. capsulatus* (Texas) as a positive control as it contained sMMO. As seen in Figure 21 There was no significant colour change seen and only isolate 10 and isolate 03 showed a purple colour change. A possible explanation for the failure of the positive control *M. capsulatus* may be due to copper contamination on the glassware.

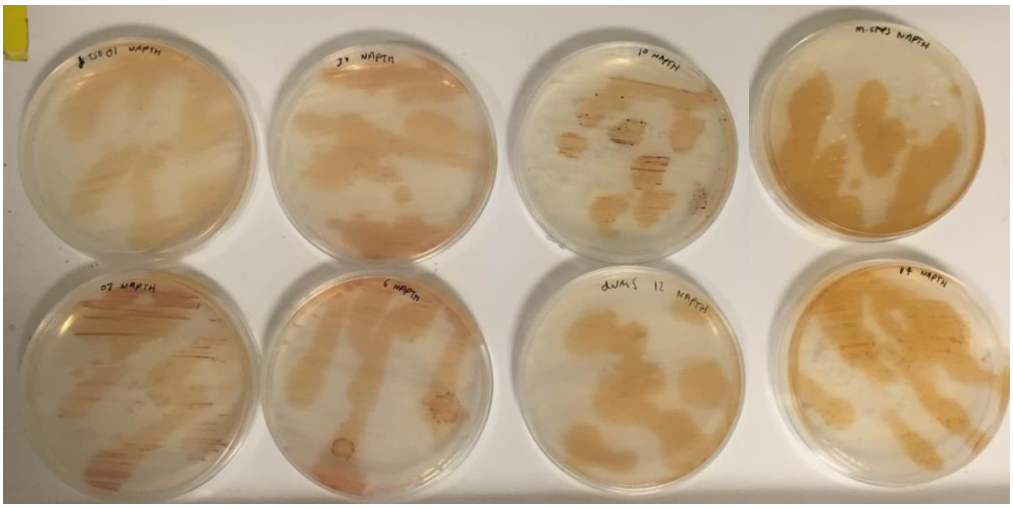


Figure 21 – Naphthalene assay on plates containing no copper using isolate 01, 03, 3*, 6, 10, 12, 14 and *M. capsulatus* (Texas).

Following the experiment described in Figure 21 the experiment was done quantitatively using a spectrophotomter and comparing plates with 0 and 10 µM $CuSO_4$. As seen in Figure 22 isolate 10 again showed an increase in absorbance at 550 nm in low copper conditions suggesting the presence of sMMO. The positive control *M. capsulatus* failed to grow during the experiment so was not included. *M. parvus* was used as a negative control as it does not possess sMMO.

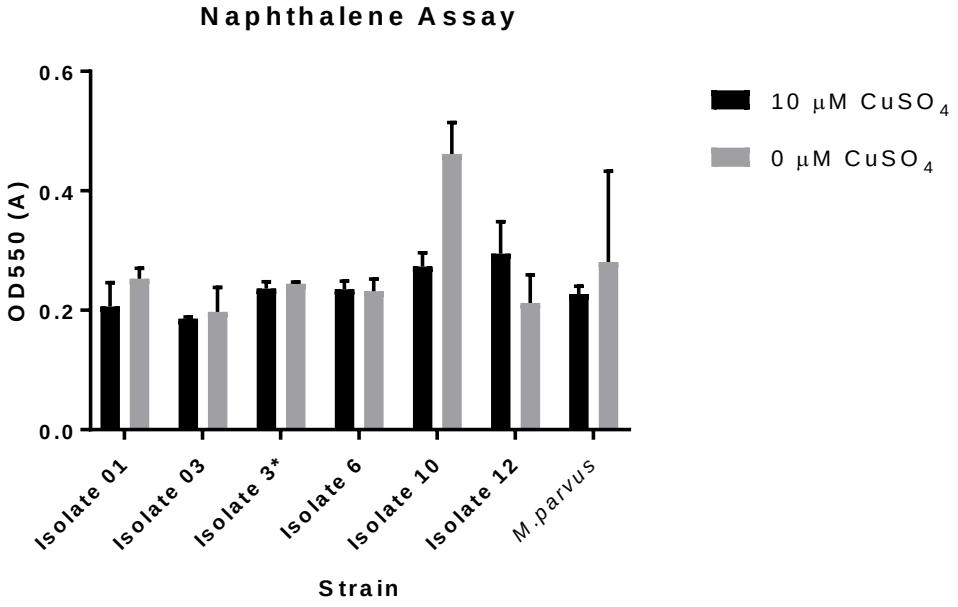


Figure 22 – Naphthalene assay for the detection of sMMO using isolate 01, 03, 3*, 6, 10, 12 and *M. parvus*.

After increasing the concentration of copper to 20 µM the experiment was repeated with isolate 3*, 6 and 10 and *M. capsulatus* (Bath) was used as a positive control and *M. parvus* as a negative control. Differing from the two pervious experiments isolate 10 did not exhibit any increase in absorbance in low copper concentrations and the positive control *M. capsulatus* (Bath) did not indicate the expression of sMMO under low copper conditions.

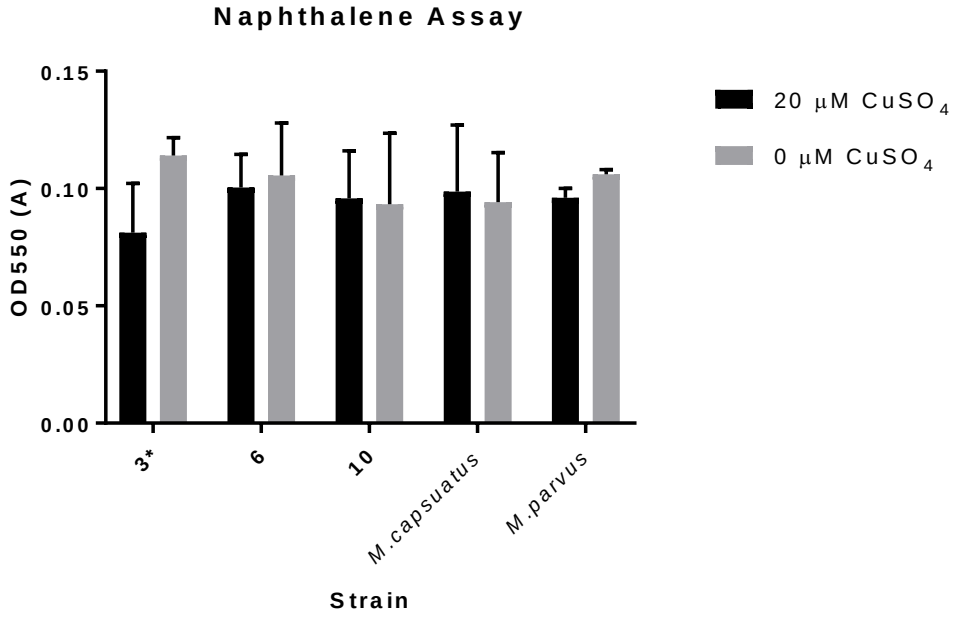


Figure 23 - Naphthalene assay for the detection of sMMO using isolate 3*, 6, 10, *M. capsulatus* and *M. parvus*.

4.5.1.6 Poly-3-hydroxybutyrate quantification

Quantification of PHB by GC-MS, as seen in Figure 24, that as typically seen in methanotrophs, all type I methanotrophs (isolate 10 and 14) did not contain PHB and all type II methanotrophs (isolate 01, 03, 3*, 6 and 12) did. Between Isolates 01, 03, 3* and 6 produced between 0.28 and 1.63% CDW in total dry weight of PHB. Isolate 12 produced significantly more PHB that the other type II methanotrophs at 4.17% CDW. This greater PHB accumulation in isolate 12 can be seen in the TEM as seen in Figure 15**.**

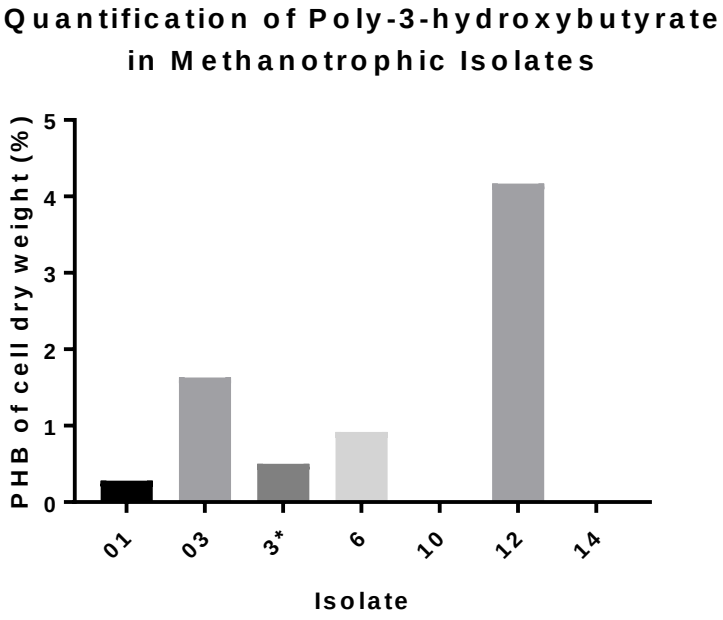


Figure 24 - Quantification of Poly-3-hydroxyburyrate in Methanotrophic isolates. Samples in singlicate.

4.5.1.7 Carbon source utilisation

The isolates ability to grow on alternative carbon sources was assessed on single and multi-carbon compounds. As seen in both Figure 25 and Figure 26 clear difference in OD600 was seen in all isolates grown on methane as this is the preferred carbon source of methanotrophs, this also shows the viability of the cells. No growth was seen in cultures grown in a headspace with only air showing that substrates from trace atmospheric methane or components of the media are not being used for growth. The isolates able to grow on ethanol include 01 and 3*; on acetate 01, 3*, 6 and 12; on pyruvate 01, 6 and 12; and no isolates could grow on glucose or lactate. All growth on substrates other than methane was trace growth not exceeding OD600 0.032 over 15 days.

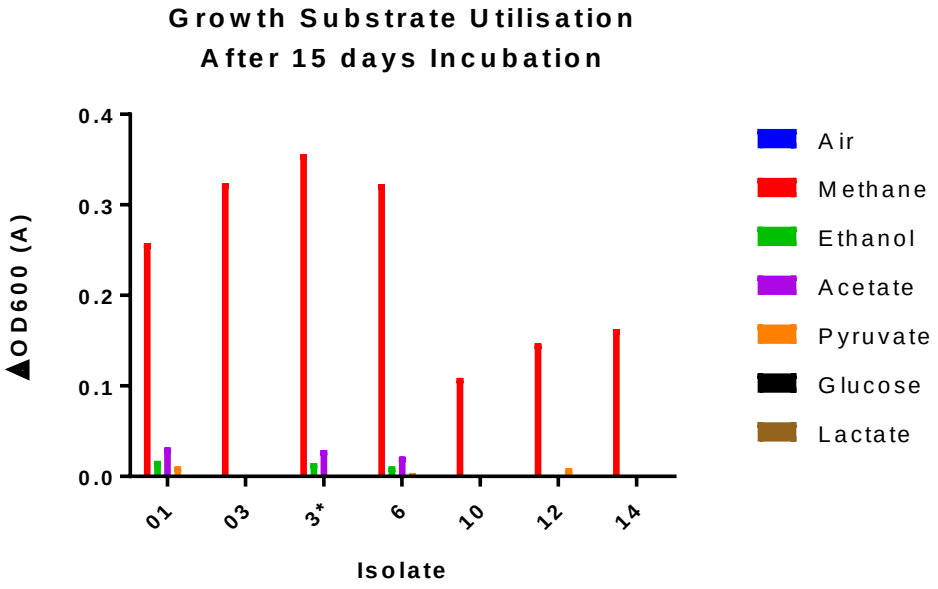


Figure 25 – Growth after 15 days of isolates in serum bottles with a variety of carbon sources (including: methane, ethanol, acetate, pyruvate, glucose and lactate) indicated by ΔOD600. Samples in singlicate.

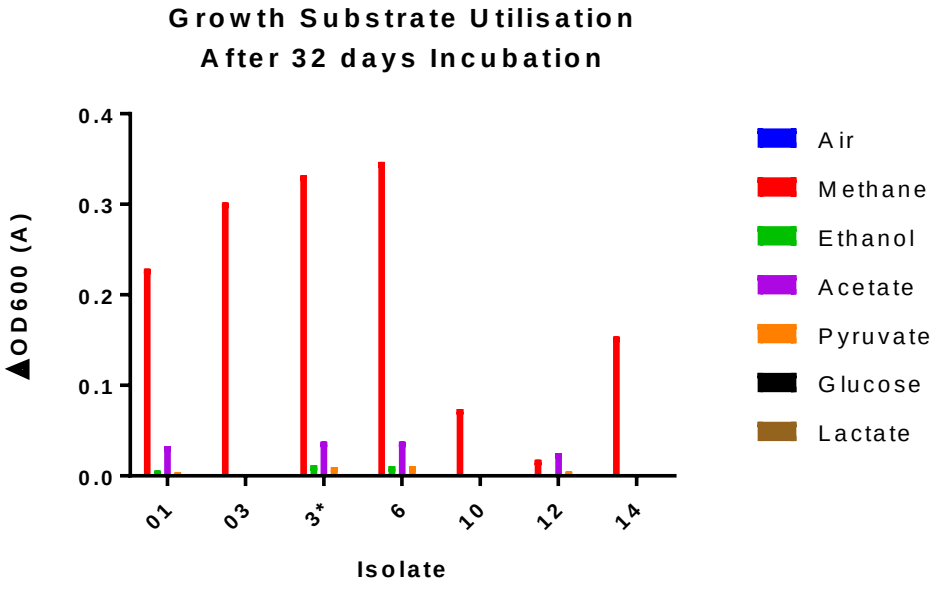


Figure 26 - Growth after 32 days of isolates in serum bottles with a variety of carbon sources (including: methane, ethanol, acetate, pyruvate, glucose and lactate) indicated by ΔOD600.

4.5.1.8 Methanol Utilisation

Many methanotrophs can grow on methanol as a sole source of carbon. As seen in Figure 27 isolate 01 and 6 grow on methanol to different extents. Isolate 01 grows well on methanol up to 0.2 at 0.01% which was the best growth observed. Other isolates including isolate 03 and 10 exhibited limited growth.

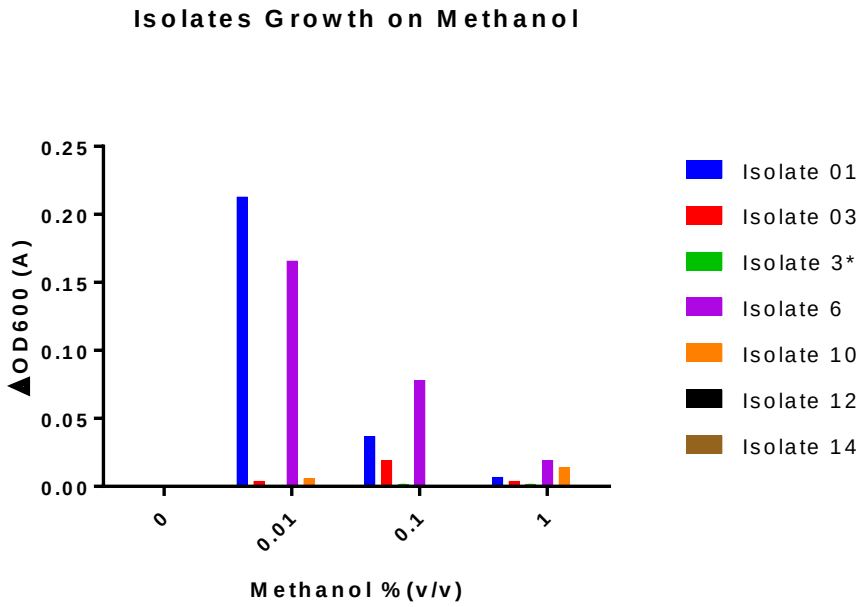


Figure 27 - Isolates grown on varying concentrations of methanol.

4.5.1.9 Tolerance to salt

A number of isolates ability to grow in increased sodium chloride conditions were tested. As seen in Figure 28 isolate 3* and 6 could not grow at 0.25% whereas isolate 12 could grow at 0.25% but not at 0.5%. Isolate 10 showed growth up to 1% though not at 5%, also the 0% sample did not growth and so the isolate 10 experiment was repeated.

The standard deviation in the plate was much greater as often the cell biomass can settle and adhere to the bottom of the plate giving unreliable readings, taking multiple reads per well as seen in subsequent experiments (See Figure 30) can greatly reduce this deviation.

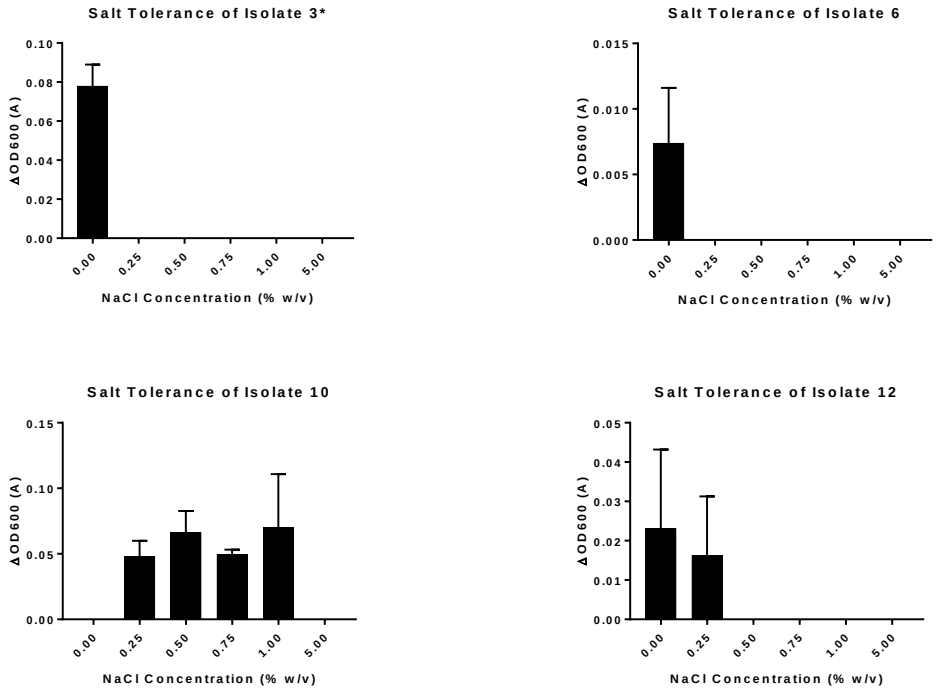


Figure 28 – Growth of isolates 3*, 6, 10 and 12 in increasing concentrations of NaCl.

The repeat experiment, seen in Figure 29 shows that isolate 10 can tolerate sodium chloride concentrations up to 2% (w/v).

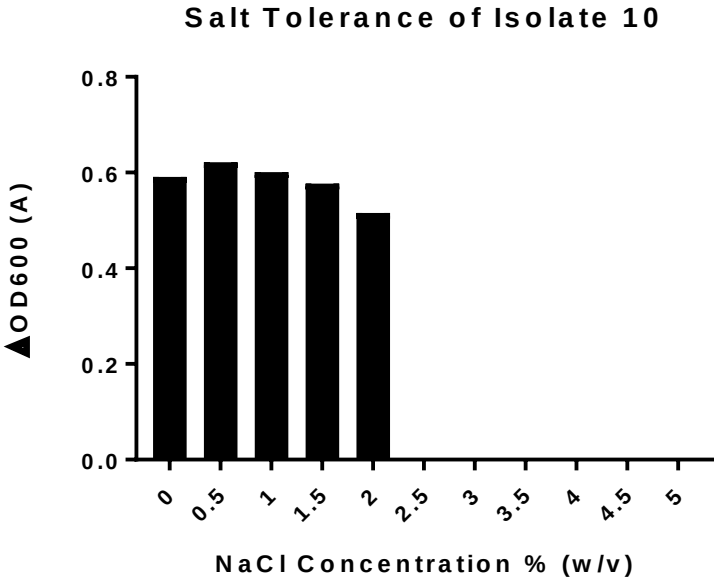


Figure 29 – Growth of Isolate 10 in increasing concentrations of NaCl.

4.5.1.10 Nitrogen source utilisation

A typical nitrogen source of methanotrophs includes potassium nitrate (NMS media) or ammonium chloride (AMS media). As seen in Figure 30 a variety of nitrogen sources were utilised by some of the isolates for growth. The isolates able to grow on NMS include 3*, 6, 10 and 12; on potassium nitrate 3*, 6, 10 and 12; on ammonium chloride 3*, 6, 10 and 12; on asparagine only 3*; on glutamine 3*, 6, 10 and 12; on ornithine 3*, 6, 10, 12 and no growth was seen on aspartate lysine or putrescine.

Standard deviation was greatly reduced by re-suspending cell mass that had adhered to the plate and also taking 9 reads at different locations in each individual well and taking an average of the well.

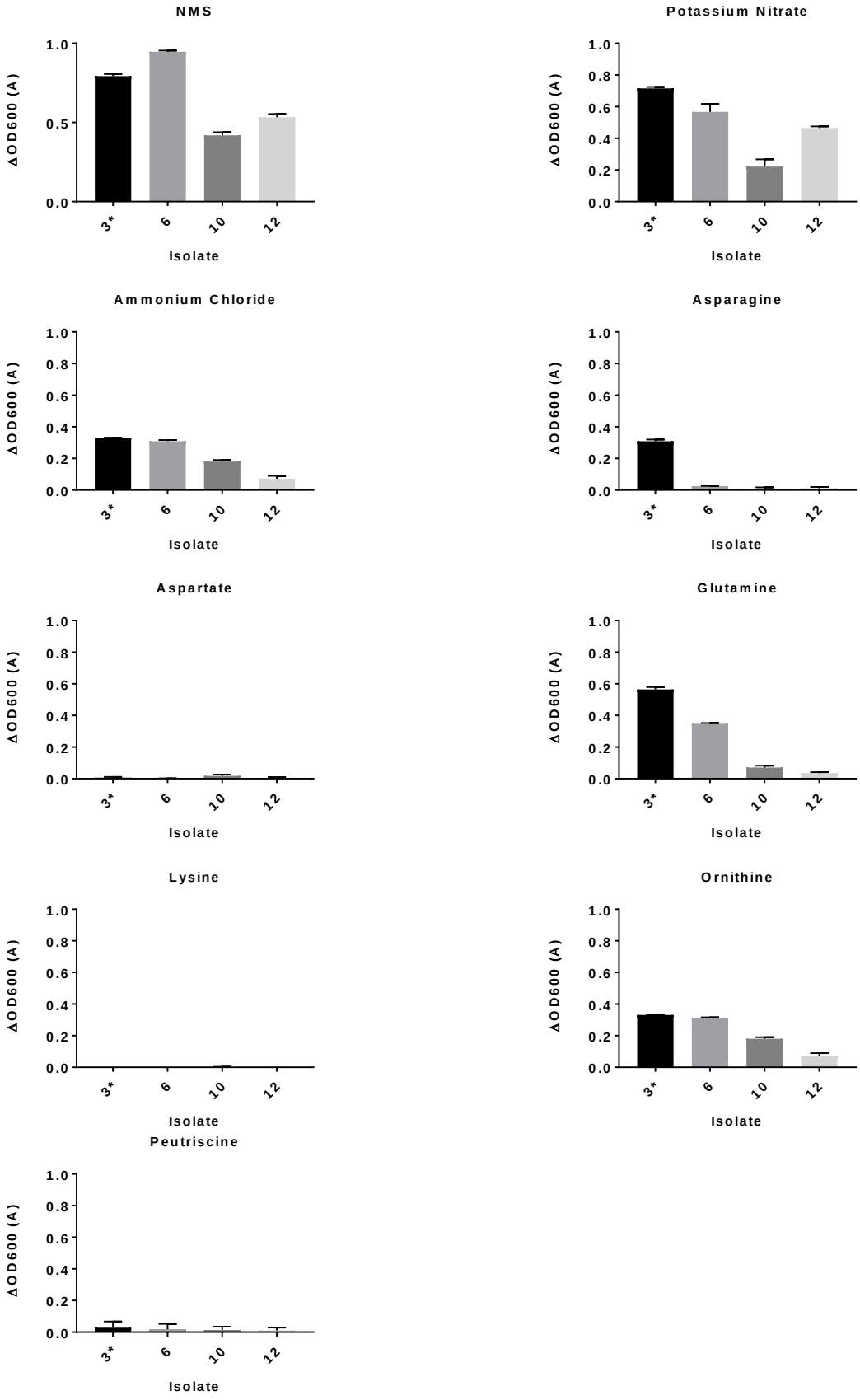


Figure 30 - Growth of isolate 3*, 6, 10 and 12 on a variety of nitrogen sources.

4.5.1.11 Motility and morphology of isolate 10

The motility and morphology of isolate 10 was assessed for morphology and motility to distinguish the isolate from other species within the genus. To monitor growth of the culture absorbance at OD600 was recorded at the following: 0 h, 0.051; 24 h, 0.122; 48 h, 0.173; 72 h, 0.271; 96 h, 0.350; 120 h, 0.434. Motility was assessed by looking for evidence of movement in the media other than flow of the liquid, which can be seen as many cells moving in one direction or Brownian motion in which cells typically move around a fixed position. No cells showed motion indicative of flagellar propulsion at any of the time-points. Furthermore as seen in Figure 31 no only cocci morphology was observed along with diplococci formations from dividing cells.

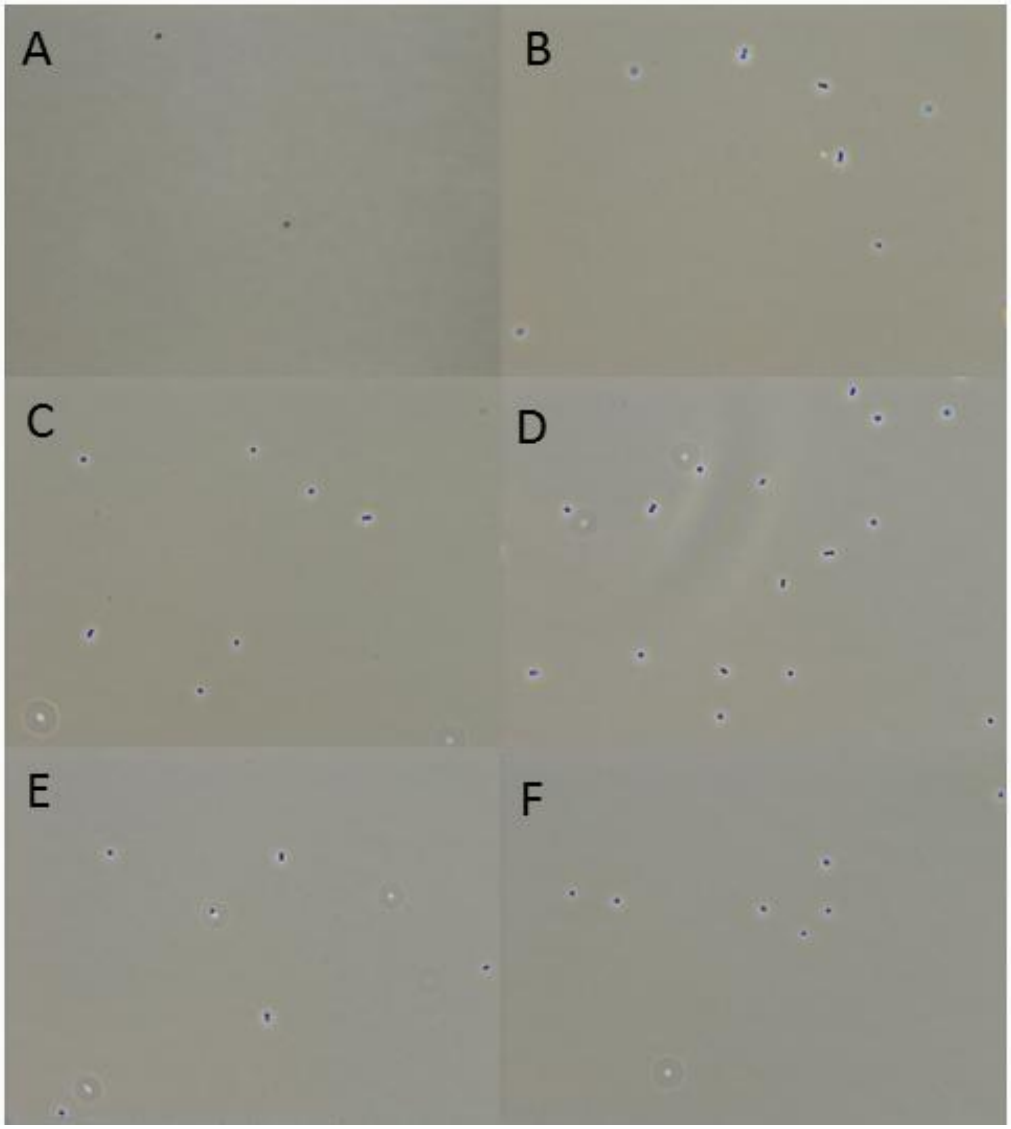


Figure 31 – Phase contrast microscopy of isolate 10 with x100 magnification at different at different growth phases. (A) 0 hours, (B) 24 hours, (C) 48 hours, (D)72 hours, (E) 96 hours, (F) 120 hours.

4.5.1.12 Isobutanol tolerance

Before engineering the isobutanol synthetic pathway into the host methanotrophic host it is essential to confirm that the isobutanol end product is not lethal. As seen in Figure 32 isobutanol, although inhibitory, is not lethal to both Isolate 6 and isolate 14. Isobutanol can be tolerated by isolate 6 at 0.025% which exerts and inhibitory effect on growth, to 0.050% absorbance at decreased from an average OD600 of 0.841 to 0.304. Isolate 14 has a superior tolerance to isobutanol which was almost unchanged

from 0% to 0.05%. at 0.1% tolerance decreases as shown by and a drop in OD600 down to 0.191.

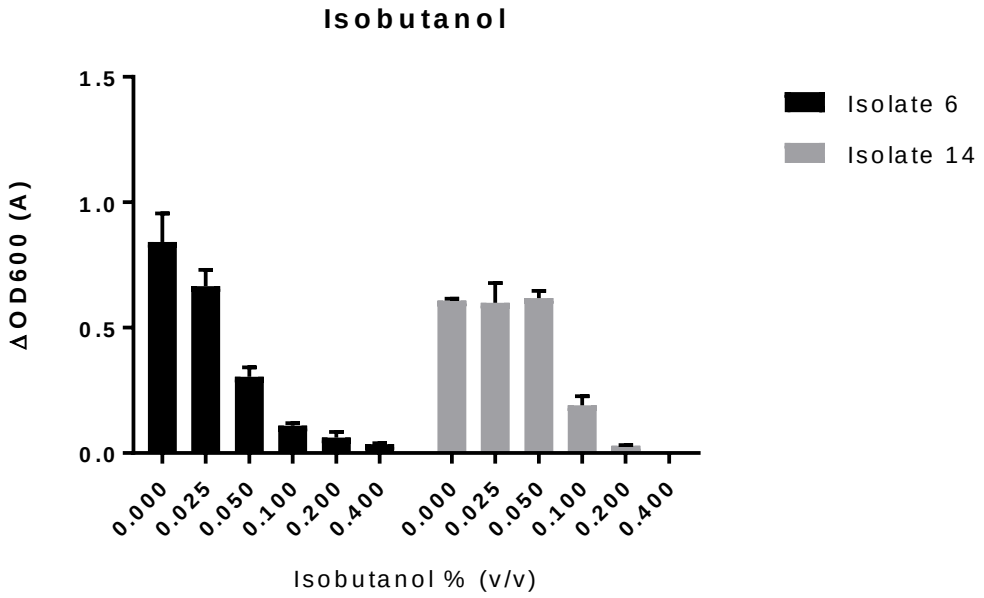


Figure 32 - Tolerance of isolate 6 and 14 to the potential end product of isobutanol.

4.5.1.13 Total phenotypes of isolates

Table 12 shows the total of the data collected on the phenotypic characterisation of all the methanotrophic isolates.

Table 12 - Total phenotype of all methanotrophic isolates. +, positive for the trait .-, negative for the trait. ND, no data.

	Isolate 01	**Isolate 03**	**Isolate 3***	**Isolate 6**	**Isolate 10**	**Isolate 12**	**Isolate 14**
NCBI BLAST Hit	*Methylocystis*	*Methylocystis*	*Methylocystis*	*Methylocystis*	*Methylocaldum*	*Methylocystis*	*Methylococcus*
Closest Phylogenetic relative (divergent or identical relationship)	*Methylocystis/ Methylosinus* (divergent)	*Methylocystis/ Methylosinus* (divergent)	*Methylocystis rosea* (identical)	*Methylocystis rosea* (identical)	*Methylocaldum gracile* (divergent)	*Methylocystis parvus* (identical)	*Methylococcus capsulatus* Bath (identical)
Pairwise alignment to closest related species	*M.echinoides* 99.14%	*M.echinoides* 99.14%	*M. rosea* 100.00%	*M. rosea* 100.00%	*M.gracile* 98.68%	*M. parvus* 100.00%	*M. capsulatus* 100.00%
Isolation location	Wollaton park lake	UoN lake	Moseley bog	UoN lake	S.B Cow manure	Moseley bog	Roman bath
Isolation method	Extinction dilution	Extinction dilution	Miniaturised E.D	Extinction dilution	Extinction dilution	Miniaturised E.D	Extinction dilution
Isolation temp (°C)	30	30	30	30	37	30	45
Colony piment	Cream	Cream	Pink	Pink	Light brown	Cream	Cream
pMMO	+	+	+	+	+	+	+
sMMO (PCR)	-	-	-	-	-	-	+
sMMO (Naphthalene assay)	-	+	+	+	+	-	+
Cell morphology	ND	ND	Polymorphic/ dumbbell	Polymorphic/ dumbbell	Cocci	Polymorphic (coccobacilli)	Cocci
ICM	ND	ND	Type II	Type II	Type I	Type II	Type I
Fimbriae	ND	ND	+	-	-	-	-
Capsule formation	ND	ND	-	-	+	-	+
Carbon storage granules	+	+	+	+	+	+	+
Poly-3-hydroxybutyrate	+	+	+	+	-	+	-
Motility	ND	ND	ND	ND	-	ND	ND

	Isolate 01	Isolate 03	Isolate 3*	Isolate 6	Isolate 10	Isolate 12	Isolate 14
Salt tolerance	ND	ND	<0.25%	<0.25%	<2.5%	0.5%	ND
Utilisation of nitrogen source:							
potassium nitrate	ND	ND	+	+	+	+	ND
ammonium chloride	ND	ND	+	+	+	+	ND
asparagine	ND	ND	+	-	-	-	ND
glutamine	ND	ND	+	+	+	+	ND
ornathine	ND	ND	+	+	+	+	ND
aspartate	ND	ND	-	-	-	-	ND
lysine	ND	ND	-	-	-	-	ND
putrescine	ND	ND	-	-	-	-	ND
Utilisation of carbon source (and/or media):							
Methane (NMS)	+	+	+	+	+	+	+
Methanol (NMS)	0.01-1%	0.10%	-	0.01-1%	0.01-1%	-	-
Ethanol (NMS)	+	-	+	+	-	-	-
Acetate (NMS)	+	-	+	+	-	-	-
Pyruvate (NMS)	+	-	+	+	-	+	-
Glucose (NMS)	-	-	-	-	-	-	-
Lactate (NMS)	-	-	-	-	-	-	-
(Lysogeny Broth media)	-	-	-	-	-	-	-
(Lysogeny Broth media 10%)	-	-	-	-	-	-	-
(Tryptic soy agar)	-	-	-	-	-	-	-
(Tryptic soy agar 10%)	-	-	-	-	-	-	-

4.5.2 *Genotypic Characterisation*

4.5.2.1 Genome Sequencing

Genomic DNA from Isolate 14 and 6 were sequenced using the long read *de novo* sequencing method PacBio [103] by Genome Quebec, Canada. The same DNA was sequenced using Illumina MiSeq benchtop sequencer (Deepseq, University of Nottingham). The sequence reads were mapped onto the PacBio sequence using CLC Genomics workbench 9 and the resulting genome was annotated using a PROKKA pipeline.

Isolate 6 possesses a 3,386,331bp genome with a GC content of 63.0%. This genome contains 3268 CDS and also 52 tRNAs. Isolate 6 contains the particulate methane monooxygenase gene cluster *pmoCAB* in addition to a separate gene cluster containing only *pmoAB*. A copy of *mmoC* is annotated within the genome though this may be a misannotation due to the fact this belongs to the sMMO and no other components are present. Upon BLAST search the sequence is 89% homologous to an Oxidoreductase enzyme from *Methylocystis* SC2 and so was re-annotated.

Other genes involved in the H_4MPT linked C1 transfer pathway (Figure 33) including formaldehyde activating enzyme (*fae*), NAD(P)-dependent methylenetetrahydromethanopterin dehydrogenase (*mtdB*), Methenyl-HMPT cyclohydrolase (*mch*), formyltransferase/hydrolase complex (*fhc*) and formate dehydrogenase (*hyc*) were all present.

Key enzymes involved in serine cycle (see Figure 33) including: Serine hydroxymethyl transferase (*gly*), hydroxypyruvate reductase (*ttuD*), malate dehydrogenase (*mdh*), malate thiokinase and malyl coenzyme A lyase (*mcl*) were all present suggesting the serine cycle is used for carbon assimilation.

Genes involved in RuMP cycle such as hexulose-6-phosphate isomerase (*rmpB*), glucose phosphate isomerase (*pgi*), glucose-6-phosphate dehydrogenase (*zfw*), 6-phosphogluconate dehydrogenase, hexulose-6-phosphate synthetase (*rmpA*) were not present in the genome.

Interestingly genes involved in growth on ethanol, seen in Figure 33 such as alcohol dehydrogenase (*adhA*), acetaldehyde dehydrogenase (*aldA*) and acetyl-CoA synthetase (*acsA*) which catalyse the reaction from ethanol to acetyl-CoA are present. This was also seen in Illumina data of isolate 3*

Genes catalysing carbon storage were looked for within the genome. Genes encoding PHB production including acetyl CoA acetyltransferase and acetoacetyl CoA reductase (*phaAB*) with also two independent copies of poly-3-hydroxyalkonate polymerase (*phaC*) were seen. *glgXACB* operon involved in glycogen synthesis were not present.

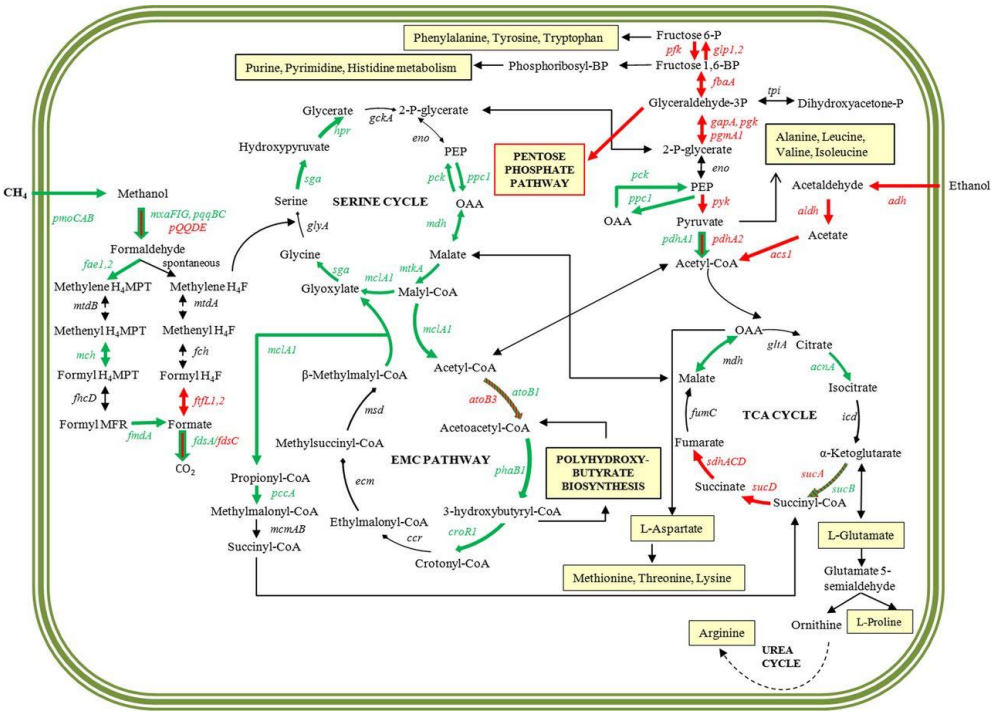


Figure 33 – Central metabolism of Methylocystis sp. strain SB2 for growth on methane or ethanol. As described by Vorbev *et al*, genes highlighted in green or red (and corresponding steps were significantly upregulated on growth on methane and ethanol, respectively. Some steps could be performed via products of multiple genes that were differentially expressed in methane versus ethanol grown cultures. These steps are detonated by a single red and green arrow [104].

Isolate 14 possess a genome that is 3,357,575bp with a GC content of 63.5% and 3093 CDS. Isolate 14 contains two *pmoCAB* encoding pMMO and one *mmoXYBZDC* operon encoding sMMO. Enzymes involved in the serine cycle and also the RuMP cycle as described above are present. In addition to this the ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) use in the Calvin Benson Bassham cycle for carbon dioxide fixation. None of the *pha* operon are present within the genome though *glgX*, two copies of *glgC glgBA* and *glgAE* are present within the genome.

4.5.2.2 Restriction systems

Using the auto-annotation pipeline service provided by The Restriction Enzyme Database (REBASE) The genome sequence for isolate 14 was submitted for analysis of Restriction Modification Systems (RMS).

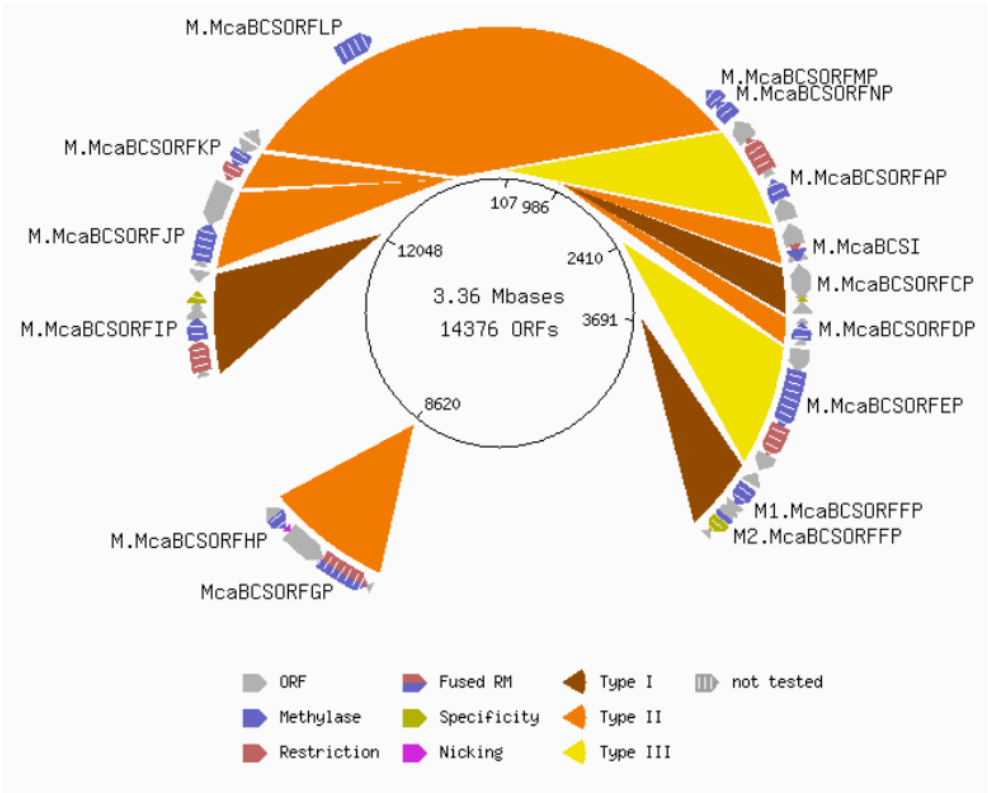


Figure 34 – Annotation of potential restriction systems within isolate 14 annotated by The Restriction Enzyme Database (http://rebase.neb.com/rebase/rebase.html).

As outlined by Figure 34 Isolate 14 contains multiple regions homologous to known RMS.

Three regions have been highlighted to contain possible type I RMS genes though only one of these contains the restriction, methylase and specificity genes necessary for a type I RMS though for this RMS. The RMS identified has a recognition site ACCNNNNNGCTC (the blue letters indicate where methylation occurs) though the sequence cannot be unambiguously matched to the specificity gene.

Similarly a type II restriction site was found (GAGAGC) though again it could not be unambiguously matched. A more likely match for a type II restriction enzyme was seen in M.McaBCSI where the restriction and modification genes were fused and the recognition sequence was RGATCY. This is identical to the commercially available *BstYI*. Two regions that potential resemble type III RMS are present though no recognition sequence could be attained.

4.5.2.3 Mauve comparison

As the 16S rRNA of isolate 14 and isolate 6 were highly homologous to known species mauve comparison tools was used to visualise the similarities between the genomes on a genome wide scale.

Using the Mauve alignment tool with a progressive mauve alignment algorithm the comparisons of isolate 14 to *M. capsulatus* (Bath) and *Methylocystis rosea* and isolate 6 were compared as seen in Figure 35 and Figure 36 respectively.

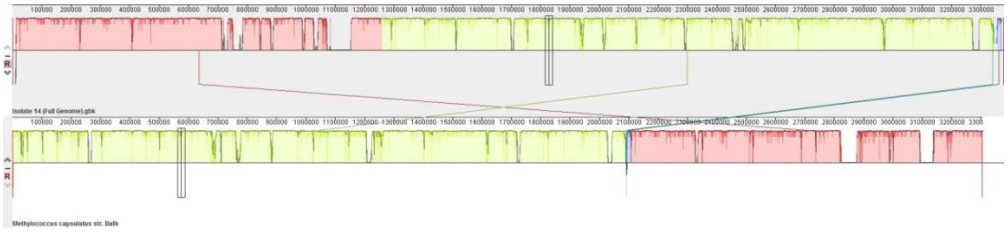


Figure 35 – Whole genome comparison of *M. capsulatus* (Bath) and Isolate 14 using Mauve comparison. Gaps, represent insertion/deletions present or not present on the genome as compared to the other. Red and yellow coloured blocks indicate homologous regions of genome sequence.

Figure 35 shows that Mauve has taken two regions that are homologous. As Mauve treats the input DNA as linear and not as a circular bacterial chromosome it can be considered that the genomes are highly similar with indels seen throughout the

genome showing the genomes are genetically similar but not identical.

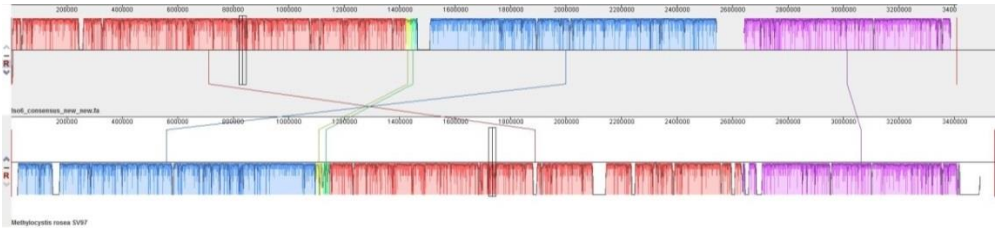


Figure 36 - Whole genome comparison of *M. rosea* and Isolate 6 using Mauve comparison. Gaps, represent insertion/deletions present or not present on the genome as compared to the other. Red, yellow, green blue and purple coloured blocks represent homologous regions of genome sequence.

Similarly Figure 36 shows that the sections of the genome identified by Mauve are similar when considering a circular genome. Again there are many indels throughout the genome suggesting many differences in the genome.

4.5.2.4 Illumina variant analysis to closest type strain

To quantify the differences in the genomes of isolate 14 and 6 to their most closely related type strain short read Illumina sequencing data of the isolate was mapped to the genome of the type strains using CLC Genomics Workbench 9.

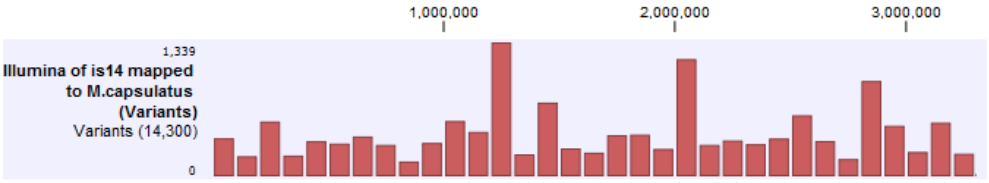


Figure 37 – Visualisation of the single and multiple nucleotide variations between the isolate 14 illumina data and the genome of *M. capsulatus* (Bath).

Whole genome comparison of isolate 14 illumina sequencing to *M. capsulatus* (Bath) genome, seen in Figure 37, sequence revealed 14,300 nucleotide variances throughout the genome.

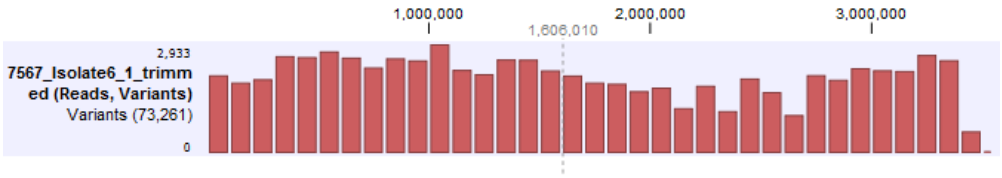


Figure 38 - Visualisation of the single and multiple nucleotide variations between the isolate 6 Illumina data and the genome of *M. rosea*.

Whole genome comparison of Isolate 6 illlumina sequencing to *M. rosea*, seen in, showed 73, 261 nucleotide variances distributed throughout the genome.

4.6 Discussion

4.6.1 *Isolate comparison tables*

To identify the environmentally isolated methanotrophs the phenotypes were accumulated and compared to representatives of the respective existing Genus.

4.6.1.1 Methylocystis isolates

A comparison of the *Methylocystis* isolates to known representatives of the *Methylocystis* genus can be seen in Table 13.

Table 13 - Isolate comparison table comparing type II isolates to closely related Methylocystis species. +, possesses the trait. -, does not possess the trait. ND, no data available. [105-110]

	Isolate 01	Isolate 03	Isolate 3*	Isolate 6	Isolate 12	*hirsuta*	*echinoides*	*bryophila*	*rosea*	*parvus*	*heyeri*	*SB2*
NCBI BLAST Hit	*Methylocystis*	*Methylocystis*	*Methylocystis*	*Methylocystis*	*Methylocystis*	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Closest Phylogenetic relative (divergent or identical relationship)	*Methylocystis/ Methylosinus* (divergent)	*Methylocystis/ Methylosinus* (divergent)	*Methylocystis rosea* (identical)	*Methylocystis rosea* (identical)	*Methylocystis parvus* (identical)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Pairwise alignment to closest related species	*M.echinoides* 99.14%	*M.echinoides* 99.14%	*M. rosea* 100.00%	*M. rosea* 100.00%	*M. parvus* 100.00%	N/A N/A	N/A N/A	N/A N/A	N/A N/A	N/A N/A	N/A N/A	N/A

	Isolate 01	Isolate 03	Isolate 3*	Isolate 6	Isolate 12	*hirsuta*	*echinoides*	*bryophila*	*rosea*	*parvus*	*heyeri*	*SB2*
Isolation location	Wollaton park lake	UoN lake	Moseley bog	UoN lake	Moseley bog	Groundwater aquifer	ND	Acidic Sphagum peat	Wetland soil	Coal mine drainage	Acidic peat bog/acidic forest soil	Acidic Sphagum Peat
Isolation method	Extinction dilution	Extinction dilution	Miniaturised E.D	Extinction dilution	Miniaturised E.D	ND	ND	Plate restreaking	Enrichment and restreaking	Extinction dilution	Enrichment and restreaking	Plate restreaking
Isolation temp (°C)	30	30	30	30	30	ND	ND	30	20		ND	
Colony pigment	Cream	Cream	Pink	Pink	Cream	Cream	White/pale pink	Light Cream	Pink/red	Diffusible brown pale pink	White	White, pink, red and brown
pMMO	+	+	+	+	+	+	+	+	+	+	+	+
sMMO	-	-	-	-	-	+	-	+	-	-	+	-
NifH	+	+	+	+	+	+	+	+	+	+	+	+
Cell morphology	ND	ND	Polymorphic/ dumbbell	Polymorphic/ dumbbell	Polymorphic (coccobacilli)	Polymorphic/dumbell	coccobacilli/rods	Curved cocci/short rods	Polymorphic, straight/curved fat rods	coccobacillus	Polymorphic/ straight curved rod	Curved rod
ICM	ND	ND	Type II	Type II	Type II	Type II	Type II	Type II	Type II	Type II	Type II	Type II
S-layer feature	ND	ND	Fimbrae	-	-	Sharp spines	Tubular spines	-	-	-	-	-
Capsule formation	ND	ND	-	-	-	ND	ND		-		+	+
Carbon storage granules	+	+	+	+	+	+	-	+	+	+	+	+
Poly-3-hydroxybutyrate	+	+	+	+	+	+	-	+	+	+	+	+
Motility	ND	ND	ND	ND	ND	-	-	-	-	-	-	-
Salt tolerance	ND	ND	<0.25%	<0.25%	0.50%	ND	ND	<0.4%	1.00%	ND	<0.8%	ND
<u>Utilisation of nitrogen source:</u>												

	Isolate 01	Isolate 03	Isolate 3*	Isolate 6	Isolate 12	*hirsuta*	*echinoides*	*bryophila*	*rosea*	*parvus*	*heyeri*	*SB2*
potassium nitrate	ND	ND	+	+	+	+	ND	+	+	+	+	+
ammonium chloride	ND	ND	+	+	+	ND	ND		+	+	+	+
asparagine	ND	ND	+	-	-	+	+	+	ND	+	ND	-
glutamine	ND	ND	+	+	+	+	+	+	ND	+	+	+
ornathine	ND	ND	+	+	+	+	-	ND	ND	+	ND	ND
aspartate	ND	ND	-	-	-	+	+	ND	ND	+	ND	ND
lysine	ND	ND	-	-	-	-	-	ND	ND	+	ND	-
putrescine	ND	ND	-	-	-	+	-	ND	ND	+	ND	ND
Utilisation of carbon source (and/or media):							ND					
Methane (NMS)	+	+	+	+	+	+	+	+	+	+	+	+
Methanol (NMS)	0.01-1%	0.10%	-	0.01-1%	-	+	0.2	<0.1%	-	+	+	-
Ethanol (NMS)	+	-	+	+	-	ND	ND	+	-	+	-	+
Acetate (NMS)	+	-	+	+	-	-	w	+	-	-	-	+
Pyruvate (NMS)	+	-	+	+	+	ND	ND	+	ND	ND	ND	-
Glucose (NMS)	-	-	-	-	-	-	ND	-	ND	ND	ND	-
Lactate (NMS)	-	-	-	-	-	ND	ND	-	ND	ND	ND	ND

4.6.1.2 Methylocaldum isolate

The *Methylocaldum* isolate was compared to known representatives of the *Methylocaldum* genus.

Table 14 - Isolate comparison table comparing *Methylocaldum* isolates to closely related Methylocystis species. +, possesses the trait. -, does not possess the trait. ND, no data available. [90, 111-113]

	Isolate 10	***tepedium***	***szegediense***	***gracile***	**O12**	**H11**	***marinum***
NCBI BLAST Hit	*Methylocaldum*	N/A	N/A	N/A	N/A	N/A	N/A
Closest Phylogenetic relative (divergent or identical relationship)	*Methylocaldum* gracile (divergent)	N/A	N/A	N/A	N/A	N/A	N/A
Pairwise alignment to closest related species	*M.gracile 98.68%*	N/A	N/A	N/A	N/A	N/A	N/A
Isolation location	S.B Cow manure	Soil	Hot spring	Activated sludge	Silage	Manure	Marine sediment
Isolation method	Extinction dilution	Extinction dilution	Extinction dilution	Extinction dilution	Extinction dilution	Extinction dilution	Extinction dilution
Isolation temp (°C)	37	37	45	ND	55	55	ND
Colony pigment	Light/dark brown	Light brown	Brown to dark brown	Brown to dark brown	cream	light cream	brown to dark brown
pMMO	+	+	+	+	+	+	+
sMMO (PCR)	-						
sMMO (Naphthalene assay)	+	-	-	-	-	-	+
Cell morphology	Cocci	Rod/pleomorphic	Rod/pleomorphic	Thin rod/coccus	Rod/coccus	Rod/coccus	Rod/coccus
ICM	Type I	Type I	Type I	Type I	Type I	Type I	Type I
EPS	+	-	+	ND	+	-	ND
Carbon storage granules	+	ND	ND	ND	-	-	ND
Motility	-	+	+	+	+	+	-
Salt tolerance	<2.5%	ND	ND	ND	<0.5%	<0.5%	5%

	Isolate 10	*tepedium*	*szegediense*	*gracile*	O12	H11	*marinum*
Chain formation	-	+	+	-	-	-	+
Utilisation of nitrogen source:							
potassium nitrate	+	ND	ND	ND	+	+	ND
ammonium chloride	+	ND	ND	ND	+	+	ND
asparagine	-	ND	ND	ND	-	-	ND
glutamine	+	ND	ND	ND	-	-	ND
ornathine	+	ND	ND	ND	-	-	ND
aspartate	-	ND	ND	ND	-	-	ND
lysine	-	ND	ND	ND	-	-	ND
putrescine	-	ND	ND	ND	-	-	ND
Utilisation of carbon source (and/or media):							
Methane (NMS)	+	+	+	+	+	+	+
Methanol (NMS)	0.01-1%	-	-	-	-	-	+
Acetate (NMS)	-	ND	ND	ND	-	-	ND
Pyruvate (NMS)	-	ND	ND	ND	-	-	ND
Glucose (NMS)	-	ND	ND	ND	-	-	ND
Lactate (NMS)	-	ND	ND	ND			ND

4.6.2 *Taxonomic identification of methanotrophic isolates*

Characterisation of each isolate was performed to gain a basic understanding of the microbial chassis from an engineering perspective and also to assess novelty of the strains.

Overall findings confirmed methanotrophy and associated characteristic within all isolates, such as the presence of ICMs and the presence of MMOs. Pre-existing phylogenetic framework suggesting type I (*Gammaproteabacteria*) methanotrophs have stacked anti-parallel membranes and do not produce PHB, this was observed in both type I methanotrophs (isolate 10 and 14). Further associated traits of type I methanotrophs such as RuMP pathway genes were also seen in the type I strain isolate 14. Type II (*Alphaproteobacteria*) strains also followed the existing phylogenetic framework as all strains produced PHB and had type II ICMs. Genomic analyses again revealed the presence of the predicted serine pathway for carbon assimilation and genes catalysing PHB production.

Specifically Isolate 01 and 03's phylogenetic relationship was not convincingly resolved using the phylogenetic tree due to low bootstrap confidence. Pairwise alignment without phylogenetic tree generation and some phenotypic characteristics (compared in Table 13) such as colony pigmentation and growth on methanol suggests it's closely related to *M.echinoides*. Taking this into account isolate 01 showed characteristics that differed from the published *M.echinoides* strain such as PHB accumulation. As a result, its identity cannot be putatively resolved without further characterisation.

Isolate 03 which produces a similar 16S rRNA homology score which also accumulates PHB though does not show any evidence of facultative methanotrophy.

The lack of evidence of growth on acetate is not a significant characteristic as the growth could be undetectably slow as facultative growth on acetate typically is. As a result, and with a less complete characterisation, this strain can be putatively identified as a PHB producing *M.echinoides* strain and further investigation would be needed to confirm the obligate methanotrophic metabolism.

Isolate 3* and 6 have very similar 16S rRNA sequences and phenotype (compared in Table 13) to *M. rosea* due to similarities in: 16S rRNA sequence, distinct pink pigmentation, polymorphic cell morphology and PHB accumulation. Interestingly though *M. rosea* is an obligate methanotroph whereas isolate 3* and 6 were able to grow on ethanol, acetate and pyruvate (albeit trace growth). One example of a strain with similar characteristics, though is not fully characterised is the Methylocystis SB2 strain that possesses pink colony morphology, and is closely related to *M. rosea* though can grow on acetate and ethanol [110]. This is a possible identification for isolate 3* though the presence of fimbriae prevent the identification from being putative. Growth of certain *Methylocystis* is also seen in fully characterised species, for example strain H2S (later named M.bryophila) was seen to grow on pyruvate and ethanol reaching 0.05-0.06 in 1.5 months and growth was seen on acetate at $0.006h^{-1}$ (doubling time 115 h) which reflects the slow growth seen in the obtained isolates [114]. The genomics data of isolate 6 further confirming the phenotype suggests isolate 6 is a facultative methanotroph. Isolate 3* contains fimbriae which distinguish the two strains. Fimbriae, or attachment pilli, are used for attachment to surfaces. This characteristic is significant as fimbriae, to my knowledge, have not been seen in methanotrophs to date. Genomics data of a *Methylomicrobium* sp. inferred to presence of pili in methanotrophs [115] though the phenotype was not confirmed. The genes for pili biogenesis in the isolated *Methylomicrobium* sp. included *PilT*, *PilW*, *PilQ*, *PilP*,

PilO, *PilN*, *PilM* and *PilZ* were seen within the genome. Interestingly these genes were not found in isolate 3* implying that distantly related genes may be encoding the pillin sub-units as they are not annotated in the genome currently.

Further dissimilarities of isolate 3* and 6 are the polymorphic morphologies look more similar to *M. hirsuta* than *M. rosea* as seen in Figure 39 and Figure 40 respectively. In contrast to this only one image of an *M. rosea* cell is available so this difference cannot be viewed as significant.

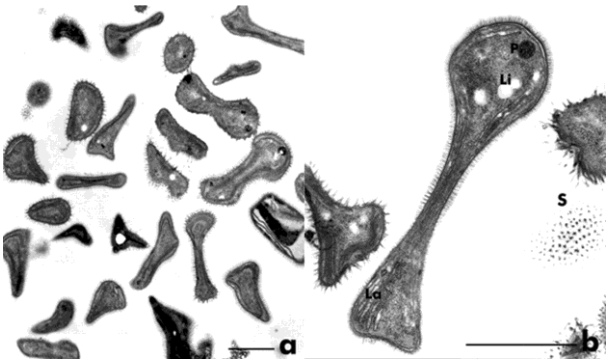


Figure 39 – Transmission electron microscopy of *Methylocystis hirsuta*. Bar, 1 µm [107].

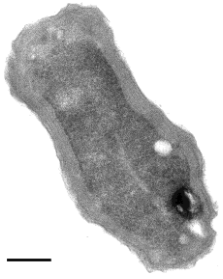


Figure 40 - Transmission electron microscopy *Methylocystis rosea*. Bar, 200 nm [106].

Isolates 3* and 6 are difficult to taxonomically identify as phenotypically they are unique within the *Methylocystis* genus, though using 16S rRNA they are highly

similar to *M. rosea*. Isolate 3* could possibly be considered novel due to morphology and fimbriae, whereas isolate 6 more closely represents *Methylocystis* SB2.

Isolate 12 was given a putative identification of *M. parvus* due to high homology of 16S rRNA and most of the phenotype being identical apart from the fact that *M. parvus* type strain grows on ethanol which is known to be trace growth so may not have been detected.

Isolate 10 differs most significantly from known species of methanotrophs. As seen in Table 14 it differs from the most closely related *M.gracile* because of: motility, morphology, methanol consumption and divergent 16S rRNA sequence.

It also possesses novel traits to the *Methylocaldum* genus which include can grow in salt concentrations up to 2.5% though does not require salt to grow like the *M.marinum* strain (that has a tolerance up to 5% and requires salt for growth). Other novel characteristics not seen in the *Methylocaldum* genus include: granule formation (presumably glycogen in a type I methanotroph) and use of amino acid as a nitrogen source.

Taking into account the unique 16S rRNA sequence and phenotype there is reasonable ground to suggest isolate 10 is a novel species of the *Methylocaldum* genus.

Isolate 14 was a re-isolated strain of *M. capsulatus* (Bath) from the same body of water the type species was obtained from. Using a similar isolation technique the species was re-isolated and can be putatively identified as *M. capsulatus* . Isolate 14's identification was made primarily on the basis of the organisms identical 16S rRNA sequence, type I ICM and growth at 45°C. Genomic analysis further identified shared traits common to the type X organism such as the presence of RuMP and Serine cycle

enzymes and the presence of pMMO and sMMO. In contrast to key metabolic features divergence was seen on wider genomic scale, for example isolate 14 (compared to *M. capsulatus* (Bath)) genome was: 3,357,575 bp (3,304,697 bp), 63.5 % (63.6 %) GC content and contained 3093 (3120) coding sequences. Further to this indels are seen throughout the genome showing sequence divergence. Comparing specific genetic changes in the context of adaption over time from the original *M. capsulatus* (Bath) strain is beyond the scope of this study. This comparison and justification through environmental selective pressure would require data on specific microenvironments and also a direct comparison would imply isolate 14 is direct descendant of the *M. capsulatus* (Bath) strain whereas the species diversity of *M. capsulatus* species within that environment may vary greatly.

4.6.2.1 Genus ammendments

Due to the discovery of novel traits from phenotypic analysis of the isolates the Respective genera are expanded as seen below. The added ammendments are underlined.

Methylocystis genus description

Gram-negative cells that are reniform, coccobacillary or rod-shaped, 0.3–1.2 μm wide by 0.5–4.0 μm long. Reproduces by normal cell division. Does not form either rosettes or exospores. May form a lipid cyst. Non-motile. Encapsulated. May accumulate poly-β-hydroxybutyrate and polyphosphate. May form spinae **or fimbrae** on cell surfaces. Contains type-II ICM that are aligned parallel to the cell wall. Possess pMMO; some strains may possess sMMO. Grows at 5–40 °C and at pH 4.5–9.0. Organic growth factors and NaCl are not required for growth. Aerobic chemolithotroph. Utilizes C1 compounds via the serine pathway. May utilize acetate or ethanol. No growth occurs on complex organic media. Does not contain the Benson–Calvin cycle for

CO_2 fixation but contains a complete tricarboxylic acid cycle. Capable of dinitrogen fixation. Produces oxidase and catalase. All representatives possess C 18:1ω8 c as the predominant PLFA; some species may also possess C 16:1ω8 c as a major PLFA. In methanotrophic bacteria, the PLFAs C 18:2ω7,12 c and/or C 18:2ω6,12 c have only been demonstrated to be present in the genus *Methylocystis* , and can therefore be regarded as diagnostic lipids for this genus. The major quinone is ubiquinone Q-8. The DNA base composition ranges from 61.5 to 67.0 mol% G+C. Belongs phylogenetically to the *Alphaproteobacteria* and is closely related to the genus *Methylosinus* . *Methylocystis parvus* is the type species.

Methlocaldum genus description

Cells are aerobic, Gram-staining-negative and may vary in shape from coccoid to long rods and **cells may be encapsulated**. Moderate thermophiles that grow at temperatures exceeding 40 °C. DNA G+C content varies from 57 to 60 mol%. The major phospholipid fatty acid is C 16:0. The major quinone type is 18-methylene ubiquinone 8. Do not grow on compounds containing carbon–carbon bonds. They possess key enzymes for both the RuMP and serine pathways of formaldehyde assimilation and can **utilise amino acids as nitrogen sources and may posses carbon storage granules**. They possess particulate methane monooxygenase and produce cysts. Known natural habitats are soil, hot springs and marine sediments. The type species is *Methylocaldum szegediense*.

4.6.3 *Notable industrial traits*

As outlined in the introduction methanotrophs naturally produce a range of value added compounds from their native metabolism. The isolates obtained produce a

variety of industrially relevant compounds and have traits that make them industrially relevant.

The most industrially relevant compound, poly-3-hydroxybutyrate, is produced by all the *Methylocystis* isolates. Isolate 12 was seen to accumulate significantly more than the other isolates making the strain industrially relevant [116]. Within the *Methylocystis* isolates Isolate 3* and 6's pink pigmentation is indicative of carotenoid biosynthesis which also is an industrially relevant compound [117].

Isolate 10, as seen by transmission electron microscopy is seen to produce exopolysaccharide which it produces at higher temperatures and does not produce at 30°C (data not shown). The origin of the thermotolerant phenotypes could provide resistance against the deleterious effects of temperature found within the ruminant's digestive tract which would come from the animals' core body temperature. EPS production is seen in other members of the *Methylocaldum* genus for example species *M. szegediense* [112]. Heat (and also salt) tolerance by mans of EPS has been seen by the secretion of xanthan produced by *Xanthomonas* spp. [118]. This EPS is in industrially relevant compound which is secreted to the media for easy extraction. Many halotolerant methanotrophs produce the high value compound ectoine to balance osmotic pressure in saline environments [119]. It was initially suspected the halotolerant phenotype in isolate 10 was as a result of ectoine biosynthesis though genes that typically catalyse this reaction including *EctA*: diaminobutyric acid, *EctB*; diaminobutyric acid transaminase and *EctC*; ectoine synthase were not present in the annotated Illumina sequencing data.

Isolate 14 (*M. capsulatus* (Bath)), as previously described, is an industrial strain commercially used to create single cell protein from methane and so also has industrial relevance.

Isolate 14 and 6 were chosen as chassis for metabolic engineering due to their robust growth characteristics and distinct metabolism from each other. Isolate 14 and 6 were shown to be able to tolerate low concentrations of isobutanol and so were adequate candidates for further metabolic engineering.

4.6.4 *Conclusion*

Seven methanotrophic isolates were obtained of which five belonged to the *Methylocystis* and identified as followed: Isolate 01; *Methylocystis* sp., isolate 03; *Methylocystis* sp., isolate 3*; *Methylocystis* nov sp. and isolate 6; *Methylocystis* SB2. Novel characteristics of methanotrophs such as fimbriae were viewed in isolate 3*. Two type I methanotrophs were isolated with isolate 14 *M. capsulatus* and isolate 10 *Methylocaldum* nov sp. which exhibited novel characteristics for the genus. Isolate 14 and 6 were chosen to continue to establish genetic tools with the intention of isobutanol production.

5 *Characterisation of Molecular Tools in Methanotrophic Isolates*

5.1 Chapter Introduction

For metabolic engineering of prokaryotes a variety of tools are required for manipulation and expansion of native metabolic networks. Metabolic engineering seeks to implement or improve the production of desired compounds. For the bio-conversion of methane to isobutanol overexpression of multiple genes is required. For a traditional plasmid based approach a replicative plasmid vector that contains an antibiotic selection marker and the operon harbouring the heterologous enzymes that catalyse the steps to isobutanol is required. Further to this the isobutanol operon must be expressed by a suitable promoter so that sufficient amounts of the enzymes are present to yield detectable amounts of the desired product. It is desirable to knockout competing pathways to increase metabolic flux to the desired compound. Key examples of these approaches is the expansion of genetic tools for the industrially promising *M. buryatense* where in which broad host range replicable plasmids containing the pBBR1 replicon was proved functional, the fluorescent reporter dTomato and GFP were used to characterise constitutive and inducible promoters, and genetic deletions were produced using *sacB* based allelic exchange [58, 120].

Within this study the most successful constitutive promoter in the *M. buryatense* study was the endogenous methanol dehydrogenase (*mxaF*) promoter, Pmxaf, an approach which was also successfully used for metabolic engineering of *M. capsulatus* (Bath) [65]. Limited inducible promoters are available in methanotrophs, for *M. buryatense* anhydrotetracycline was shown to be functional and in *M. capsulatus* IPTG inducible systems were shown to be functional. In regards to genetic manipulation via allelic

exchange, *M. capsulatus* mutants have shown to be generated using the pPVW87 plasmid derived from pJQ200SK plasmid containing p15a suicide replicon with *sacB* counter selection, gentamycin resistance and 1kb homology arms [121]. *Methylocystis* mutants are typically generated using allelic exchange with knockout plasmids containing kanamycin and ampicillin markers where in which mutants are selected for by resistance to kanamycin but sensitivity to ampicillin [122].

Knockout of glycogen biosynthesis has been achieved in *M. buryatense* by deletion of *glgA1* and *glgA2* glycogen synthase homologues [57], the resulting strain had an improved specific methane uptake of 3.5 from 2.4 mmol/gDCW/h [123]. PHB biosynthesis has been knocked out in *C.necator* by removing *phaC* polyhydroxyalkonate polymerase [124]. This suggests these pathways are not essential and can most likely be knocked out in isolate 14 and 6. A similar approach to [121] will be taken to create carbon storage mutants where a suicide plasmid will be introduced and via allelic exchange single crossover integration will be selected for using a Km marker, further homologous recombination for double crossovers removing the gene will be selected for via counters selection of sucrose sensitivity conveyed by *sacB* (levansucrase). To create a modular suicide vector the p15a replicon which is only able to replicate in *Enterobacteria* [125] and so can replicate *E. coli* but not in *M. capsulatus* [121] (isolate 14) or *M. rosea* (isolate 6) will be used.

A robust plasmid format for basing this work in will be the pMTL plasmid series. The pMTL series are modular shuttle vectors originally designed for *Clostridia* [126]. All pMTL vectors follow the same format as seen in Figure 41 though different branches of the series exist. The published pMTL80000 series parts are designed for *Clostridia* species though pMTL7 series exists for *Cupriavidus* species and, as discussed later on,

the pMTL9 series for methanotrophs (including but not limited to *Methylococcus*, *Methylocystis* and *Methylocaldum* spp.). Four main modules are found on the plasmid which include: Gram positive replicon, selection marker, Gram negative replicon and application specific module. Each module is given a numbers which together given a pMTL8 prefix designate the name of the plasmid. For example pMTL80651 is composed of 0 = spacer, 6 = kanamycin marker selection marker, 5 = ColE1 + tra replicon, 1 = multiple cloning site. Each module is separated by uncommon restriction sites (*SbfI*, *AscI*, *FseI* and *PmeI*) to allow quick changes of the modules to produce new constructs with new function without complex cloning procedures. Applications not in use such as Gram positive replicons can be replaced with short spacer sequences.

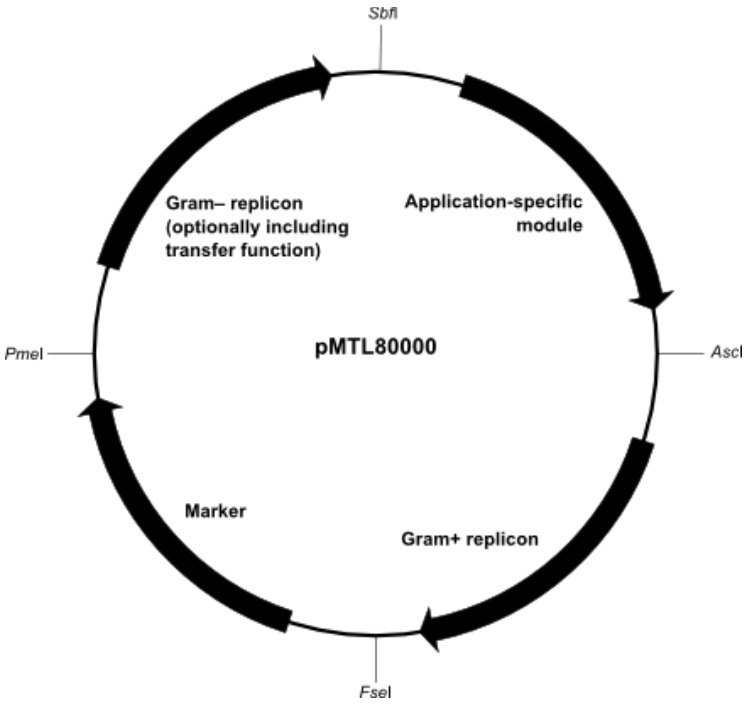


Figure 41 – Simplified schematic of the pMTL80000 modular vector that contains the 5 main modules: Gram negative replicon, application specific module, gram positive module and antibiotic selection marker. [127]

5.2 Strains and Plasmids

The *E. coli* strains and methanotrophic organisms used in the following chapter are detailed below.

Table 15 - Strains used in this chapter

Strain	Description	Reference
Escherichia coli		
DH5α	*F' endA1 hsdR17*(r_k^-,m_k^-) *supE44 thi-1 recA1 gyrA* (Nal^r) relA1Δ(*lacIZYA-argF*) U169 *deo*R (φ *80*d*Lac* Δ(*lacZ*)*M15*)	[128]
S17-1	*Thi, pro hsdR*$^-$*, hsdμ*$^+$*, recA; integrated plasmid RP4-Tc::Mu-Kn::Tn7*	[129]
XL1-BLUE	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIq ZΔM15 Tn10 (Tetr)	(NEB)
TOP10	mcrA, Δ(mrr-hsdRMS-mcrBC), Phi80lacZ(del)M15, ΔlacX74, deoR, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL(SmR), endA1, nup G	(ThermoFisher)
Methanotrophs		
Methylococcus capsulatus (Bath)	*Methylococcus capsulatus* (Bath) kindly donated by Dr Andrew Crombie	[28]
Isolate 03	Methanotroph isolated from University of Nottingham lake	This study
Isolate 6	methanotroph isolated from University of Nottingham lake	This study
Isolate 14	Methanotroph isolated from the Roman Bath in Bath	This study

The plasmid constructions used in the following chapter are detailed below.

Table 16: Plasmids used in this study. SBRC CC, Synthetic Biology Research Centre Culture Collection. KmR, Kanamycin resistant selection marker. AmpR, ampicillin resistant selection marker. CmR, Chloramphenicol resistant marker. EryR,Erythromycin resistant selection marker. TetR, tetracycline resistant selection marker. EYFP, enhanced yellow fluorescent protein.

Plasmid	Description	Reference
pMHA199	Promoter probe plasmid containing the reporter gene XylE with no fused promoter, Col E1 replicon and KmR	[61]
pBBR1-2USER_NPR3-	Promoter probe plasmid containing	This study (Dr

EYFP	pBBR1 replicon, EYFP reporter and KmR	Naglis Malys)
pBBR1MCS_2_PphaC	Promoter probe plasmid containing pBBR1 replicon, *phaC* promoter and EYFP reporter	This study (Dr Naglis Malys)
pMTL80451	Col E1 replicon, TetR and multiple cloning site	This study (SBRC CC)
pMTL71101	pBBR1 replicon and multiple cloning site	This study (SBRC CC)
pMTL80651	pMTL80541 with selection marker replaced with KmR from pBBR1MCS_2_Pphac	This study (SBRC CC)
pMTL71401	pMTL71101 with selection marker replaced with KmR from pBBR1MCS_2_Pphac	This study (SBRC CC)
pMTL90882	Modular methanotroph plasmid containing Col E1,KmR and MCS	This study (Emily Harding/ Martina Pasini)
pBluelux	Template for AmpR	
pMTL8215_USER	Template for CmR	This study (SBRC CC)
pMTL8225-PtcdR-alsS-yqhD	Template for EryR	This study (SBRC CC)
pMTL90882 (amp)	pMTL90882 with AmpR	This study
pMTL90882 (cml)	pMTL90882 with cmlR	This study
pMTL90882 (ery)	pMTL90882 with eryR	This study
pMTL71401-EYFP	pMTL71401 (pBBR1, KmR, MCS)with EYFP introduced into the MCS	This study
pMTL71401-Ppyk-EYFP	Pyruvate kinase constitutive promoter probe vector	This study
pMTL71401-PmxaF-EYFP	Methanol dehydrogenase constitutive promoter probe vector	This study
pMTL71401-Pfae-EYFP	Formaldehyde activating enzyme constitutive promoter probe vector	This study
pMTL71401-Ptac-EYFP	Tac constitutive promoter probe vector	This study
pMTL71401-PphaC-EYFP	Polyhyroxyalkonate polymerase constitutive promoter probe vector	This study
pMTL71401-Acetate-EYFP	Acetate inducible promoter probe vector	This study
pMTL71401-Acetoin-EYFP	Acetoin inducible promoter probe vector	This study
pMTL71401-Arabinose-EYFP	Arabinose inducible promoter probe vector	This study
pMTL71401-Benzoate-EYFP	Benzoate inducible promoter probe vector	This study
pMTL71401-DAPG-EYFP	2,4-Diacetylphloroglucinol inducible promoter probe vector	This study
pMTL71401-IPTG-	Isopropyl β-D-1-thiogalactopyranoside	This study

EYFP	inducible promoter probe vector	
pMTL71401-Rhamnose-EYFP	Rhamnose inducible promoter probe vector	This study
pMTL71401-Xylose-EYFP	Xylose inducible promoter probe vector	This study
pMTL8S141	Modular suicide plasmid containing sacB (S),	This study (SBRC CC)
pMTL8S141 (Km)	pMTL8S141 with selection marker swapped for KmR	This study
pMTL84422	Template for pBBR1 for pMTL8S141 (Km) – pBBR1	This study (SBRC CC)
pMTL8S141 (Km) – pBBR1	Replicative plasmid in isolates containing *sacB*	This study
pMTL8S141 (Km) – Col E1	Replicative plasmid in isolates containing *sacB*	This study
pMTL8S141 (Km) – glgA1	Suicide plasmid containing homology arms for allelic exchange of *glgA* homologue 1	This study
pMTL8S141 (Km) – glgA2	Suicide plasmid containing homology arms for allelic exchange of *glgA* homologue 2	This study
pMTL8S141 (Km) - phaC	Suicide plasmid containing homology arms for allelic exchange of *phaC*	This study

5.3 Primers

The primers used in the following chapter are detailed in Table 17.

Table 17 - Cloning primers used in this study. Underlined nucleotides represent restriction sites.

Name	Sequence (5' to 3')	Gene/Description
Km_FseI_F/ Km_PmeI_R	F-TATACGGCCGGCCATGTCAGCTACTGGGCTATC R:TGCTGAGGGTTTAAACTCATTTCGAACCCCAGAGTCCC	Modularisation of Km marker from pBBR1MCS_2_PphaC
Amp_F_(PmeI)/ Amp_R (FseI)	F – GTTTAAACATGAGTATTCAACATTTCCG R - GGCCGGCCTTACCAATGCTTAATCAGTGAGGCAC	Modularisation of Amp marker from pBluelux
Pyk_F(NotI)/ pyk_R(NdeI)	F – ATTCGGTGCGGCCGCCGATGGAAGGTTTCCTGCAT R - ATTCGGTCATATGCCTGCTCCACTACTAGCGCT	Cloning Ppyk into promoter probe vector
Fae_F(NotI)/ Fae_R(NdeI)	F-ATTCGGTGCGGCCGCACCTTCGGACACGTGGGGCC R - ATTCGGTCATATGAATTTCCTCTTACTACTAGC	Cloning Pfae into promoter probe vector
mxaF_F(NotI)/ mxaF_R (NdeI)	F – ATTCGGTGCGGCCGCGAGGTTCAGGCGAAACCGCA R - ATTCGGTCATATGTCTCCTCCACTACTAGCGCT	Cloning PmxaF into promoter probe vector
phaC_F (NotI)/ phaC_R	F – ATTCGGTGCGGCCGCAAATACCTGTGACGGAAGAT R - ATTCGGTCATATGTATATCTCCTTCTT	Cloning PphaC into promoter probe vector
Tac_F (NotI)/ Tac_R	F - ATTCGGTGCGGCCGCAAATACCTGTGACGGAAGAT	Cloning Ptac into promoter probe vector
araB_F (NotI)/ araB_R	F - ATTCGGTGCGGCCGCTTATGACAACTTGACGGCTA R - ATTCGGTCATATGTATATCTCCTTCTT	Cloning inducible arabinose promoter into promoter probe vector
Hifi-Acetate-F/ Hifi-Acetate-R	F – ATCAGGAAACAGCTATGACCGCGGCCGCATAAAACGAAAGGCTCAGTC R - CCTCGCCCTTGCTCACCATATGATGTATATCTCCTTCTTAAAAGATCTTT	Cloning inducible acetate promoter into promoter probe vector
T1_F(NotI)/ Acetoin_R	F – TAAGCAGCGGCCGCATAAAACGAAAGGCTCAGTCGAAAG	Cloning inducible acetoin promoter into promoter probe vector

	R - actcgccatatgctgtctcc	
T1_F(NotI)/ RBAX_R	F – TAAGCAGCGGCCGCATAAAACGAAAGG CTCAGTCGAAAG R - actcgccatatgtatatctccttctt	Cloning inducible benzoate promoter into promoter probe vector
T2_F(NotI)/ DAPG_R	F – TAAGCAGCGGCCGCAAAAGGCCATCCGT CAGGA R - actcgccatatgcctcttga	Cloning inducible DAPG promoter into promoter probe vector
Lac_F(NotI)/ Lac_R(NdeI)	F – TAAGCAGCGGCCGCGACACCATCGAATG GTGCAA R - TAAGCACATATGAGCTGTTTCCTGTGTG AAATTGT	Cloning inducible IPTG promoter into promoter probe vector
T1_F(NotI)/ RBAX_R	F – TAAGCAGCGGCCGCATAAAACGAAAGG CTCAGTCGAAAG R - actcgccatatgtatatctccttctt	Cloning inducible rhamnose promoter into promoter probe vector
Hifi-Xyl-F/ Hifi-Xyl-R	F – atcaggaaacagctatgaccgcggccgcaaaaggccatccgtca ggat R - agctcctcgcccttgctcaccatatgtatatctccttcttaaattaagt gaacaa	Cloning inducible xylose promoter into promoter probe vector
glgA1_F/glgA1 _R_A	F – ccgcggccgctgtatccataGCGTCGATGCCGTCGC CTCG R – CCTGACCGCCTTTGGCGGCTTCGTCGCC CGGCGTTCCGCA	PCR amplification for homology arms for *glgA1* for Hifi assembly
glgA1_F/glgA1 _R_B	F – TGCGGAACGCCGGGCGACGAAGCCGCC AAAGGCGGTCAGG R – gcttcttatttttatgctagGGGTAATGACGGCGGGC GAT	PCR amplification for homology arms for *glgA1* for Hifi assembly
glgA2_F/glgA2 _R_A	F- ccgcggccgctgtatccataTGTCCTCCTGGAACAC CGGA R- CGCCTCCGGTTGCCGCGGAGCCGGCCAT GAGGATTTACAA	PCR amplification for homology arms for *glgA2* for Hifi assembly
glgA2_F/glgA2 _R_B	F- TTGTAAATCCTCATGGCCGGCTCCGCGG CAACCGGAGGCG R- gcttcttatttttatgctagGCACGCAGCCGCCGAAC ACA	PCR amplification for homology arms for *glgA2* for Hifi assembly
PhaC1_F/ PhaC1_R	F- ccgcggccgctgtatccataTCGTCTTCGCGGTGAT GCCG R- GAAAACAGCAGGTTTAGGGCCTTCTTTT GAACGAATGCATTTTTTGTAGGG	PCR amplification for homology arms for *phaC1* for Hifi assembly
PhaC1_F/ PhaC1_R	F – ATGCATTCGTTCAAAAGAAGGCCCTAAA CCTGCTGTTTTCGAATGA	PCR amplification for homology arms for

	R – gcttcttatttttatgctagGATTGCCGTTGCTCGCGCCG	*phaC1* for Hifi assembly
PhaC2_F/ PhaC2_R	F – gcggccgctgtatccatatgTCGCTTCATTTTATCGGACGTTCGGC R – GCGCAGAGCAGACATCATCCCGCGCGGGGATGCGCTACTC	PCR amplification for homology arms for *phaC2* for Hifi assembly
PhaC2_F/ PhaC2_R	F – GAGTAGCGCATCCCCGCGCGGGATGATGTCTGCTCTGCGCGC R – cttcttatttttatgctagcCCACACCCGCACCTCGCCGC	PCR amplification for homology arms for *phaC2* for Hifi assembly
pMTL8S141 (Km) - p15a + OriT	F – GCGGCGAGGTGCGGGTGTGGgctagcataaaaataagaagcctgc R – CGTCCGATAAAATGAAGCGAcatatggatacaggccgc	PCR amplification for homology arms for *phaC* backbone for Hifi assembly

5.4 Methods used in Chapter

5.4.1 *Transformation of methanotrophs*

5.4.1.1 Plasmid construction

Plasmids used for initial transformations include pMHA199, pMTL80651, pMTL71401 and pBBR1-2USER_NPR3-EYFP. Plasmid pMHA199 was synthesised from [61] and is a partial construct (contains no promoter for the reporter) of a promoter probe vector that contain Col E1 replicon, KmR and *XylE* (catechol 2,3-dioxygenase) as a reporter gene. The study reports conjugation into *M. capsulatus* (Bath) and so acts as a positive control for conjugation. pBBR1-2USER_NPR3-EYFP is a promoter probe vector containing the Enhanced Yellow Fluorescent Protein (EYFP) driven by a Tac promoter and contains the pBBR1 replicon, this was kindly donated by Dr Naglis Malys.

Plasmids pMTL80651 and pMTL71401 were constructed with Col E1 and pBBR1 replicons respectively and both contained Km selection markers. To construct these a Km selection marker from pBBR1MCS_2_Pphac was PCR amplified, using Q5 High-Fidelity 2x Master Mix adding restriction sites *PmeI* and *FseI* using Km_FseI_F/Km_PmeI_R. The PCR product was cleaned using QAIkit PCR Purification Kit. PCR products along with pMTL80451 and pMTL71101 were digested using *PmeI* and *FseI* (NEB cutsmart). The modularised PCR products were then ligated, using NEB T4 Ligase, into the backbones to produce new constructs with the selection markers replaced for Km (now called pMTL80651 and pMTL71401). These were transformed into *E. coli* DH5α and *E. coli* S17-1 via electroporation. Transformants were selected for on LB agar containing 50 µg/ml kanamycin and plasmids were confirmed by diagnostic digest using PmeI and FseI (as previously described) of mini-prep from a 5 ml overnight culture.

Another commonly used plasmid was pMTL90882 which was from the pMTL9 series designed for methanotrophs. The modules represent 0 = spacer, 8 = KmR, 8 = Col E1 (*OriT*, *OriV*, *trfa*), 2 = Pthl + MCS. This construct was made by Emily Harding and Dr Martina Pasini.

5.4.1.2 Conjugation

Conjugation was performed as described in 2.5 with plasmids pMTL80651, pMTL71401, pBBR1-2USER-NPR03-EYFP and pMHA199.

5.4.1.3 Electroporation

To improve the speed of transformation isolates 14 and 6, electroporation was attempted. In addition to isolate 14 and 6 *Methylococcus capsulatus* (Bath) and *Methylocystis parvus* were transformed with the same protocol. Plasmids pMTL90882

(Col E1 replicon) and pMTL71401 (pBBR1 replicon) were used, both contain Km selection markers. The electroporation technique was adapted from [130] which outlines electroporation of *M. buryatense* a Type I organism.

One loop full of isolate 14, isolate 6, *M. capsulatus* (Bath) and *M. parvus* cells spread across an NMS plate and incubated for 3 days and 50 ml liquid culture cultivated to stationary phase. Cells grown on solid agar harvested by scraping plate liquid sample centrifuged at 5,000 rpm at 4°C for 10 minutes. Cell suspension resuspended in 50 ml room temperature sterile water and harvested by centrifugation (see above). The wash step was repeated and the resulting pellet re-suspended in 100 µl water and 50 µl transferred into a separate Eppendorf tube then placed on ice. Of the chilled sample 50 µl mixed with 1 µl of pMTL90882 and pMTL71401 then transferred to ice-cold 2 mm electrocuvette.

The cuvette was then pulsed at 12.5 kV/cm 25 µF at 200Ω and then immediately 1 ml of NMS added. The cell suspension was then transferred to 60 ml serum bottles containing 10 ml of NMS medium and incubated with 30 ml of methane added to the headspace and incubated overnight. Cells have harvested at 5k rpm at room temperature for 10 minutes and re-suspended in 1 ml of NMS. The samples were then diluted 1 in 10 and 100 and 500 with NMS media then 50 µl spread over selective NMS plates (Iso 6 + *M.paruvs* Km50 and Iso 14 + *M.caps* Km15). Methane air headspace was refreshed every other day.

5.4.2 *Alternative markers*

To establish alternative markers for potential use of two plasmid expression or knockout plasmids a minimum of two selection markers were selected for testing. In the chosen methanotroph isolates ampicillin (Amp), chloramphenicol (Cm) and

erythromycin (Ery) were chosen as isolate 6 is sensitive to Amp, Ery and Cm and isolate 14 is sensitive to Amp and Ery. AmpR was PCR amplified to include modular restriction sites for the pMTL series from the template pBluelux using Amp_F_(*PmeI*)/Amp_R (*FseI*). This PCR product along with pMTL8215_USER containing Cm, pMTL8225-PtcdR-alsS-yqhD containing Ery and pMTL90882 containing the vector backbone were subject to restriction digest using *PmeI* and *FseI* (NEB). Samples were visualised by gel electrophoresis and extracted using Qaigen gel extraction kit. Respective fragments were ligated with T4 DNA ligase at a 3:1 insert:vector ratio (NEB) and transformed into chemically competent *E. coli* DH5α and *E. coli* S17-1 via heat-shock and selected for on LB agar with 50 µg/ml kanamycin. Resulting colonies were confirmed with diagnostic digests of plasmids extracted by Monarch® Plasmid Miniprep Kit. The resulting plasmid were: pMTL90882 (Amp), pMTL90882 (Cm) and pMTL90882 (Ery). Isolate 14 and 6 were transformed with the above described selective marker plasmids via conjugation using *E. coli* S17-1. Subsequent transformants were plated on respective antibiotics at 5, 10 and 50 µg/ml. transformants confirmed using colony PCR and restreaked to prove true growth.

5.4.3 *Promoter validation*

5.4.3.1 Anhydrotetracycline sensitivity

To assess if anhydrotetracycline (aTc) can be used as an inducer molecule for inducible gene expression aTc tolerance was assessed. aTc inhibits growth of *M. buryatense* after 1 µg/ml after 72h of growth and aTc inducible promoter can be used [58]. To assess isolate 6 for aTc tolerance a pre-culture of Isolate 6 grown and sub-cultured into 5 ml of dNMS to 0.1 OD600 in triplicate with aTc concentrations: 0,

0.25, 0.5, 1, 2, 4, 8 µg/ml in 60 ml serums bottles. Cultures were then over-pressured with 30 ml of methane and incubated at 30°C with agitation at 200 rpm for 72h.

5.4.3.2 Construction of constitutive promoter probe plasmids

An initial promoterless enhanced yellow fluorescent protein (EYFP) plasmid was constructed by digesting pMTL71401 (containing pBBR1 replicon, Km selection marker and multiple cloning site (MCS)) and pBBR1-2USER_NPR3-EYFP (containing EYFP) with NdeI and NheI (NEB). Following gel extraction of the desired fragments using Monarch DNA Gel Extraction Kit® EYFP was ligated into the pMTL71401 MCS using NEB T4 ligase reaction. Ligation reaction was transformed using heat-shock transformation into *E. coli* One Shot™ TOP10 chemically competent *E. coli* and selection on LB agar containing 50 µg ml^{-1} Kanamycin. Resulting transformants were assessed for the recombinant construct using a diagnostic digest. The resulting construct was pMTL71401-EYFP.

Following the construction of the promoterless promoter probe vector a variety of promoter candidates were selected. Presumed strong native promoters controlling expression of central carbon metabolism were targeted using KEGG's metabolic pathway viewer. Genes selected from the *M. capsulatus* genome included: pyruvate kinase (*pyk*, MCA2597), formaldehyde activating enzyme (*fae*, MCA2866) and methanol dehydrogenase (*mxaF*, MCA0779). The 200bp upstream region of the start codon was taken and the promoter region was identified using the promoter predictor tool found at (www.fruitfly.org/seq_tools/promoter). The σ factor -10 and -35 regions were identified using the Softberry promoter sequence identifier tool (http://www.softberry.com/berry.phtml?topic=promoter). Non-native promoters including the tac promoter derived from the *trp* and *lac* UV5 promoters [131] though there was no operator sequence present so expression was constitutive and not

inducible. The other non-native promoter used was a *phaC* promoter controlling the expression of poly-3-hydroxybutyrate polymerase in *Cupriavidus necator*.

For construction of constitutive promoter probe vectors (seen in Figure 42 and Figure 43) Native promoters were synthesised by ThermoFischer Scientific Gene Art String with *AfeI* restriction site separating the Ribosome binding site (RBS) to the rest of the promoter for future RBS comparison work. Native promoters from Gene Strings, *tac* promoter from pMTL71101-USER_NPR3-EYFP_Ptac plasmid and *phaC* promoter from pBBR1-2USER_NPR01-EYFP plasmid were amplified using primers: Pyk_F/Pyk_R, Fae_F/Fae_R, mxaF_F/mxaF_R, Tac_F/Tac_R and PhaC_F/PhaC_R. This PCR amplification added *NotI* and *NdeI* of the upstream and downstream promoter if not already present. PCR amplification was performed using Q5 High-Fidelity 2X Master Mix (NEB). Fragments were visualised via gel electrophoresis, excised and purified using Monarch DNA Gel Extraction Kit® and the resulting purified DNA along with pMTL71401-EYFP was digested with the restriction enzymes *NotI* and *NdeI* (ThermoFischer Fast Digest). The pMTL71401-EYFP backbone was extracted by gel extraction as previously described and the digested PCR products were purified using Monarch PCR Clean-up Kit. Each promoter was ligated into the backbone using T4 Ligase (NEB) and transformed into chemically competent *E. coli* DH5α using heat-shock transformation and plated onto LB agar containing 50 μg ml^{-1} kanamycin. The resulting plasmids were: pMTL71401-Ppyk-EYFP, pMTL71401-PmxaF-EYFP, pMTL71401-Pfae-EYFP, pMTL71401-Ptac-EYFP amd pMTL71401-PphaC-EYFP. These were confirmed using Source Bioscience Sanger Sequencing Service.

Constitutive promoter probe plasmids were subsequently transformed into conjugative donor strain *E. coli* S17-1 via heat-shock for conjugation into methanotrophic strains and confirmed by PCR.

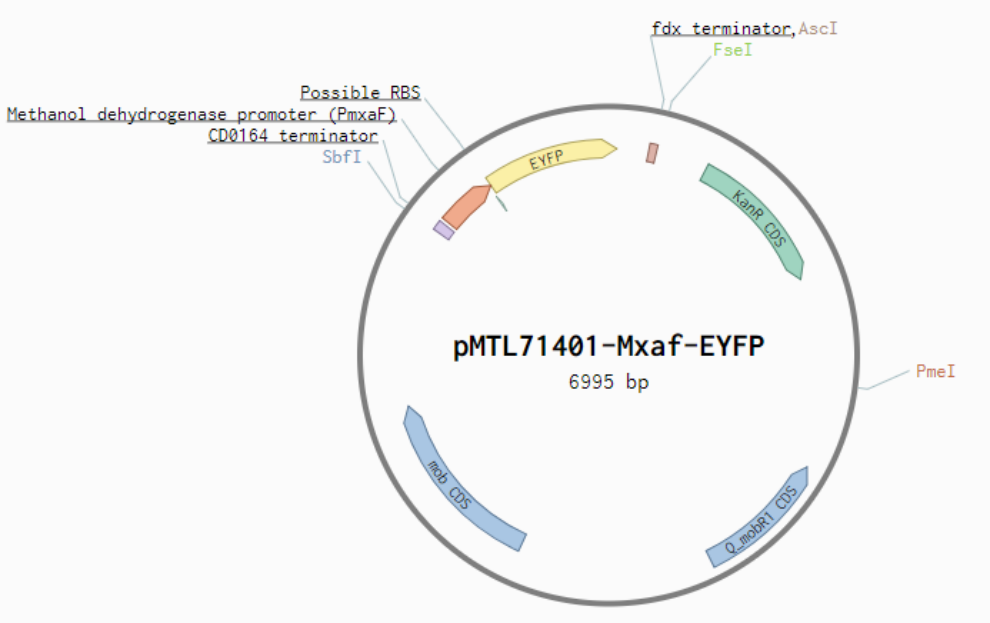


Figure 42 – Schematic layout of genetic elements in the modular pMTL71401 – mxaF – EYFP plasmid. PmxaF, methanol dehydrogenase promoter. EYFP, enhanced yellow fluorescent protein, KanR, kanamycin antibiotic selection marker. Mob, *mob* gene for mobilisation via conjugation. MobR1, pBBR1 replicon.

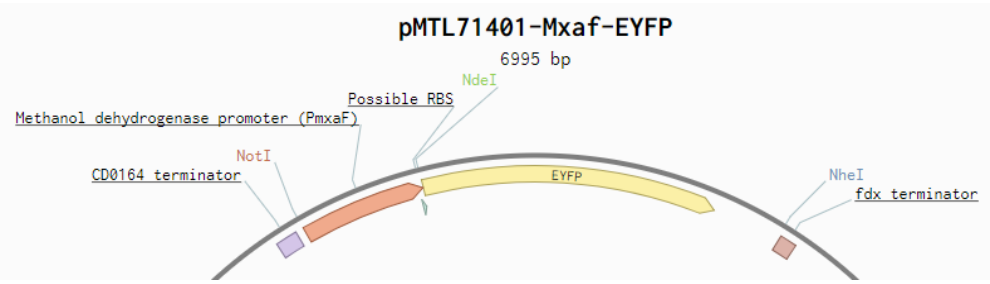


Figure 43 – Promoter probe module showing fusion of the methanol dehydrogenase promoter and EYFP (enhanced yellow fluorescent protein).

5.4.3.3 Construction of inducible promoters

Due to the limited range of inducible promoters available a variety of promoters were selected for activity in the isolates. IPTG inducible promoter selected from pGex-6P-1

and the arabinose inducible promoter was selected form the pKTrfp plasmid. A number of other inducible promoters were taken from plasmids kindly donated by Erik Hanko. The plasmid and respective inducer molecule include: pEH016_alsSDTrfp (Acetate) [132], pEH052_CnacoPrfp (Acetoin) [133], pEH041_benABCTrfp (Benzoate) [134], pEH043_phlArfp (2,4-diacetylphloroglucinol) [12], pEH002_rhaBADTrfp (Rhamnose) [135, 136] and pEH038_xylABrfp (Xylose) [137]. All these promoters are σ70 RNA polymerase-dependant promoters [65] that originate from the respective organisms: *Bacillus subtilis* 168, *Cupriavidus necator* H16, *Pseudomonas putida* KT2440, *Pseudomonas protegens* CHA0, *Esherichia coli* K12 and *Bacillus megaterium* DSM319.

Promoter probe vectors were created using the same format as the constitutive promoter probe vectors (see Figure 44 and Figure 45). Promoters were PCR amplified using Q5® High Fidelity 2X Master Mix from the above described templates using the primers described in Table 17. Restriction sites *NotI* and *NdeI* were incorporated if not already present. All subsequent PCR products aside from xylose and acetate were purified using Monarch ® PCR & DNA Cleanup Kit, digested with NdeI and NotI (NEB) along with the backbone pMTL71401-EYFP then respective fragments ligated into the backbone via NEB T4 DNA Ligase. Acetoin and xylose promoter PCR products were assembled using NEBuilder ® Hifi DNA Assembly Master Mix with the digested pMTL71401-EYFP backbone described in the previous step. All assembled DNA solutions were transformed into chemically competent *E. coli* S17-1 and DH5α via heat-shock transformation and selected or on LB agar containing 50 µg/ml kanamycin. The final constructs were named: pMTL71401-Acetoin-EYFP, pMTL71401-Acetate-EYFP, pMTL71401-Arabinose-EYFP, pMTL71401-Benzoate-EYFP, pMTL71401-IPTG-EYFP, pMTL71401-Rhamnose-EYFP, pMTL71401-

Xylose-EYFP. Constructs were confirmed using SourceBioscience Sanger sequencing service.

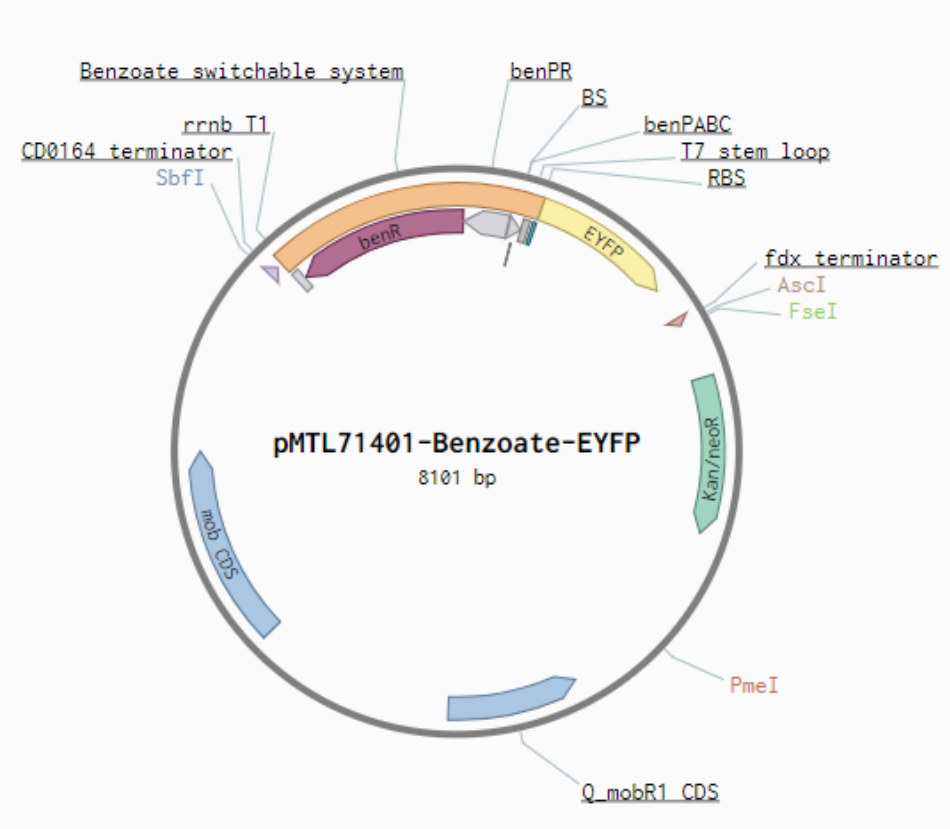


Figure 44 – Schematic layout of genetic elements within the modular pMTL71401- Benzoate – EYFP plasmid. EYFP, enhanced yellow fluorescent protein, KanR, kanamycin antibiotic selection marker. Mob, *mob* gene for mobilisation via conjugation. MobR1, pBBR1 replicon.

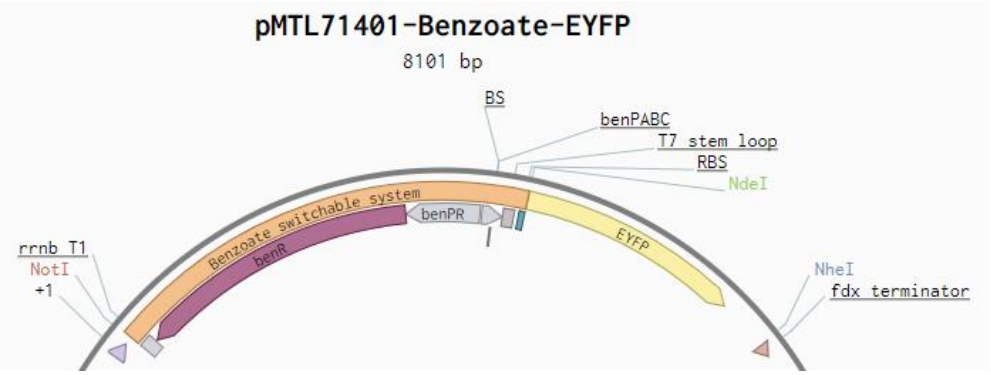


Figure 45 – Schematic layout of promoter probe module containing the fused benzoate inducible promoter and enhanced yellow fluorescent protein. benPR,. benR,. BS,. benABC,. RBS,. Rrnb ,.

5.4.3.4 Quantifying strength of promoters

E. coli or Isolate 14 and 6 pre-cultures harbouring the promoter probe vectors were diluted in respective media (LB or NMS) containing respective kanamycin concentration to OD600 0.1 into a Greiner Black 96 well plate (*E. coli* samples were diluted into plates 1 in 2 diluted with sterile water). The total volume in each well was 100 µl unless stated otherwise. Each row of the plate contained triplicate wells containing the specific host with a promoterless promoter probe vector (pMTL71401-EYFP) and also a media triplicate. Samples were put in adjacent cells. Readings of OD600 and fluorescence were recorded using a Tecan Infinite® M1000 PRO. Fluorescent readings were taken with excitation at 495 nm, emission at 530 nm, mode "read from bottom", gain 70 and kinetic cycles 5-10.

Inducible promoters were tested in a similar way though inducers were added at respective concentrations. To allow for inductive expression of EYFP *E. coli* S17-1 was incubated in a 96 plate at 37°C at 200 rpm. Incubation of isolate 14 and 6 for induction was performed either within the plate and methane atmosphere was created using the gas tight box (as described in Materials and Methods) or within serum bottles with incubation at 45/30°C and 200 rpm agitation.

Normalised fluorescence values were calculated by subtracting the value of fluorescence of both media and promoterless vector control and then normalising by OD600 (OD600 corrected by subtracting OD of medium first). Negative values suggesting less fluorescence than the promoterless EYFP are given a 0 value.

5.4.4 *Allelic Exchange using SacB as a negative selection marker*

5.4.4.1 Plasmid construction

pMTL8S141 from the SBRC culture collection containing p15a + TraJ replicon had its selection marker changed to one compatible with methanotrophs, kanamycin, by restriction digestion of pMTL8S141 and pMTL90882 using *PmeI* and *FseI*. Correct fragments were obtained using agarose gel electrophoresis followed by gel extraction (Monarch® DNA Extraction Kit). Ligation of the kanamycin selection marker into the backbone was performed using T4 ligase (NEB) and resulting ligation mixture was transformed using chemically competent *E. coli* XL1-BLUE, *E. coli* DH5α and *E. coli* S17-1 via heatshock transformation. Transformants were confirmed using Eurofins Genomics TubeSeq service and the resulting plasmid was named pMTL8S141 (Km).

Firstly to confirm *SacB* was functional in the isolates construct were made containing *SacB* and functional replicons for both isolates. Construction of these vectors was performed by double digestions of the backbone pMTL8S141 (Km) along with pMTL84422, pMTL71401-EYFP and pMTL90882 containing p15a, pBBR1 and ColE1 replicons respectively. Digestion was performed using *PmeI* and *SbfI* (ThermoFischer, Fast Digest), the correct size bands were excised following gel electrophoresis and gel extraction (Monarch® DNA Extraction Kit). Ligations of the replicons into the backbone was performed using T4 ligase (NEB) and resulting ligation mixture was transformed using chemically competent *E. coli* XL1-BLUE and *E. coli* DH5α and S17-1 via heatshock transformation. Transformants were confirmed using Eurofins Genomics TubeSeq service. The plasmids were named pMTL8S141 (Km) – pBBR1 and pMTL8S141 (Km) – ColE1.

Suicide plasmids containing homology arms were constructed by first locating *glgA* and *phaC* genes in isolate 14 and 6 respecitvely. Two homologues of each were discovered within the genomes. Homology arms were designed by taking a 1kb of

each terminal end of each gene. Primers were designed using Benchling DNA assembly tool for NEB Hifi DNA assembly that contained 20 bp overlap for multiple fragment assembly. Fragments were PCR amplified using 2 µl of isolate boilate using Q5® 2X High-Fidelity Master Mix. Primers for this reaction are detailed in Table 17 pMTL8S141 – Km was digested using *NdeI* and *NheI* (NEB) to provide a backbone for the reaction, this fragment was purified using gel electrophoresis and gel extraction as previously described. The DNA assembly was done using NEBuilder® Hifi DNA Assembly Master Mix (NEB). Resulting assembly mix was them transformed into *E. coli* XL1-Blue and *E. coli* S17-1. Constructs were named pMTL8S141 (Km) – glgA1, pMTL8S141 (Km) – glgA2 and pMTL8S141 (Km) – phaC1. The second homologue of *phaC* construct could not be created. Plasmids were confirmed using diagnostic digest and sanger sequencing as previously described.

5.4.4.2 Validation of SacB

Plasmid pMTL8S141 (Km) – pBBR1 containing *SacB* and the pBBR1 replicon that is functional in both isolates was transformed into Isolate 14 and 6 via conjugation using the conjugal donor *E. coli* S17-1. Resulting transformants were inoculated into 2.5 ml of NMS with 15 or 30 µg/ml kanamycin and cultured in a 60 ml serum bottle with 25 ml methane overpressured at respective temperatures at 200 rpm. After 3 days growth was observed and cultures were centrifuged at 8000 rpm for 5 minutes and resuspended in 2ml NMS, this was repeated a further two times. Serial dilutions of 10^{-1}, 10^{-2}, and 10^{-3} were made and 100 µl was plated onto separate NMS plates containing 0, 1, 3 and 5 % sucrose. These were cultured in a methane:air atmosphere of approximately 1:1 at respective temperatures. Once significant growth was observed 50 colonies from each plate (NMS with 0, 1, 3 and 5 % sucrose) were patched simultaneously onto a plate containing only NMS and a plate containing NMS

with 15/30 µg/ml kanamycin. Growth seen on the NMS plate and not on the NMS plate containing kanamycin was indicative of plasmid loss due to stress from the sucrose catalysed by a functional sacB gene. Colony PCR was performed on patched colonies to confirm retention/loss of the plasmid.

5.4.4.3 Allelic Exchange using SacB as a negative selection marker

Isolate 14 with *M. capsulatus* (Bath) was transformed via conjugation with pMTL8S141 (Km) – glgA1 and pMTL8S141 (Km) – glgA2. Isolate 6 was transformed with pMTL8S141 (Km) – phaC. Transformants presumed to be single crossover mutants were assessed using colony PCR and inoculated into 1 ml NMS media in a 60 ml serum bottle with 25 ml methane overpressure then subcultured into 9 ml NMS and grown for 48 hours. After incubation period 300 µl plated on 2, 3, 4 and 5% NMS sucrose plates and incubated in a methane atmosphere as previously described.

5.5 Results

5.5.1 *Transformation of methanotrophs*

Conjugation – The plasmids pMTL80651, pMTL71401, pBBR1-2USER-NPR03-EYFP and pMHA199 attempted to be conjugated into Isolates 14 and 6. Transconjugant colonies for isolate 6 included all plasmids but pBBR1-2USER-NPR03-EYFP. Isolate 14 produced transconjugants for all but pMTL80651. The EYFP fluorescence was seen under blue light in isolate 14.

Across both isolates kanamycin proved to be a suitable selection marker. The pBBR1 replicon was proven to work in isolate 6 on the pMTL71401 plasmid and isolate 14 could also use pBBR1 as a replicon as seen in both pMTL71401 and pBBR1-2USER-

NPR03-EYFP. Similarly both isolates used ColE1 as a replicon in the form of pMHA199. Conjugation can be successfully propagated by *E. coli* S17-1 for both isolates and EYFP along with the trp/lacUV5 promoter is functional in Isolate 14.

Electroporation – Of the electroporation methods attempted, none of the plasmids could be transformed into either of the methanotrophic isolates.

5.5.2 *Alternate markers*

Upon transformation being established in the isolates the number of available antibiotic selection markers was expanded. Of isolate 14 a small amount of growth was seen on 5 µg/ml for chloramphenicol and ampicillin but not erythromycin though colonies did not grow upon restreaking. No growth was seen on higher concentrations for all antibiotics possibly suggesting plasmid did not transform as previously seen for isolate 14.

Isolate 6 transformants initially grew on all antibiotics on all concentrations and the presence of the plasmid was confirmed using colony PCR. Further restreaking compared to the wildtype isolate 6 showed that Ery and Amp could be used as selection markers due to the wildtype sensitivity and transconjugant resistance to the antibiotic as seen in Table 18. Unexpectedly no growth was seen on 0 µg/ml wild type and transconjugant agar plate so Cml cannot be concluded.

Table 18 – Growth observed between wildtype isolate 6 and transconjugant isolate 6 containing pMTL90882 with marker selections of either: Cm (chloramphenicol), Ery (erythromycin), Amp (ampicillin). +, Growth seen on agar plates. -, No growth seen on agar plates.

	Antibiotic (µg/ml)	Wildtype	Transconjugant
Cml	0	-	-
	25	-	-
	50	-	-
	100	-	-
Ery	0	+	+
	25	-	+
	50	-	+
	100	-	+
Amp	0	+	+
	25	-	+
	50	-	+
	100	-	+

5.5.3 *Promoter validation*

5.5.3.1 Constitutive promoter

Promoter strength was quantified by fusing the transcriptional control of the promoter with the fluorescent reporter EYFP. When the EYFP is excited at 495 nm the protein emits fluorescence with a shifted wavelength of 530 nm which intensity is proportional to the amount of protein produced which represents the promoter's strength to express the reporter gene.

The promoters were considered as modular units which included -35, -10, RBS and start codon were compared as one complete unit and do not take into consideration variation in RBS between promoters. As a result in the following section when the promoter strength (level of expression) is referred to the reference is being made to the promoter sequence and its respective RBS.

The promoters selected were based on previous successful modularisation of promoters in methanotrophs, other promoters involved in central carbon metabolism

and other commonly used promoters. The promoter probe vectors were first tested for activity in the conjugal donor *E. coli* S17-1. As seen in Figure 46 all promoters were able to express EYFP which was detected by normalised fluorescence values greater than auto-fluorescence of the media and the promoterless promoter probe vector in *E. coli* S17-1. Pfae was the strongest promoter with a value of 6394. Pmxaf, PphaC, and Ptac all had similar strengths of expression just below ~4000 and Ppyk had low relative expression of 370.

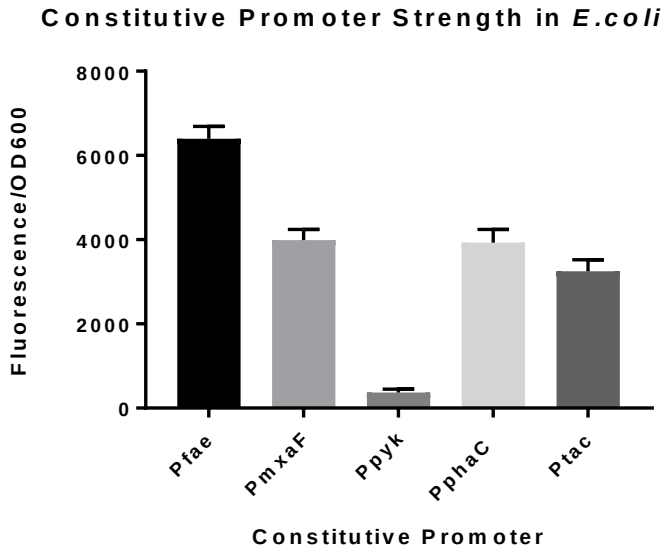


Figure 46 - Expression strength of a variety of constitutive promoters in a promoter probe fusion with enhanced yellow fluorescent protein expressed within *E. coli* S17-1. Pfae, formaldehyde activating enzyme promoter from *M. capsulatus* (Bath) genome. Pmxaf, methanol dehydrogenase promoter from *M. capsulatus* (Bath) genome. Ppyk, pyruvate kinase promoter. Pphac polyhydroxyalkonate synthase promoter from *Cupriavidus necator*. Ptac, constitutive promoter derived from the trp and lac UV5 promoters [131].

The constitutive promoter probe vectors were transferred from the conjugal host *E. coli* S17-1 to Isolate 14 and the relative strength was assessed. The Ptac promoter conjugation failed and so is not included in Figure 47. The methanol dehydrogenase promoter, Pmxaf, exhibited the strongest expression with a value of 80577. The non-native PphaC promoter showed the second highest level of expression though this was

~4x less than the Pmxaf promoter. Pfae and Ppyk had the lowest with 5912 and 1131 respectively.

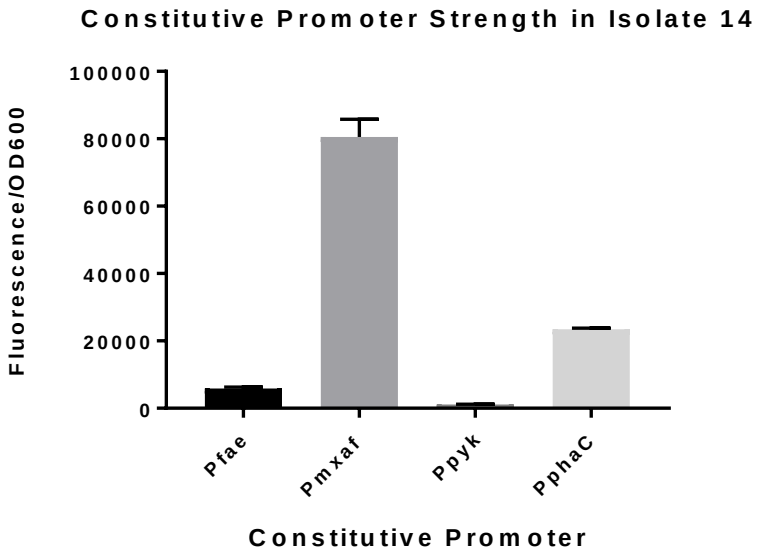


Figure 47 – Expression strength of a variety of constitutive promoters in a promoter probe fusion with enhanced yellow fluorescent protein expressed within isolate 14. Pmxaf, methanol dehydrogenase promoter from *M. capsulatus* (Bath) genome. Pfae, formaldehyde activating enzyme promoter from *M. capsulatus* (Bath) genome. Ppyk, pyruvate kinase promoter. Pphac, polyhydroxyalkonate synthase promoter from *Cupriavidus necator*.

The constitutive promoter strength in isolate 6 greatly varied to isolate 14. Pphac was the strongest promoter with a high value of 33202. Ptac and Pfae had medium relative strength at 22127 and 11438 respectively. Pmxaf and Pyk had lower expression strength at 5071 and 3790 respectively.

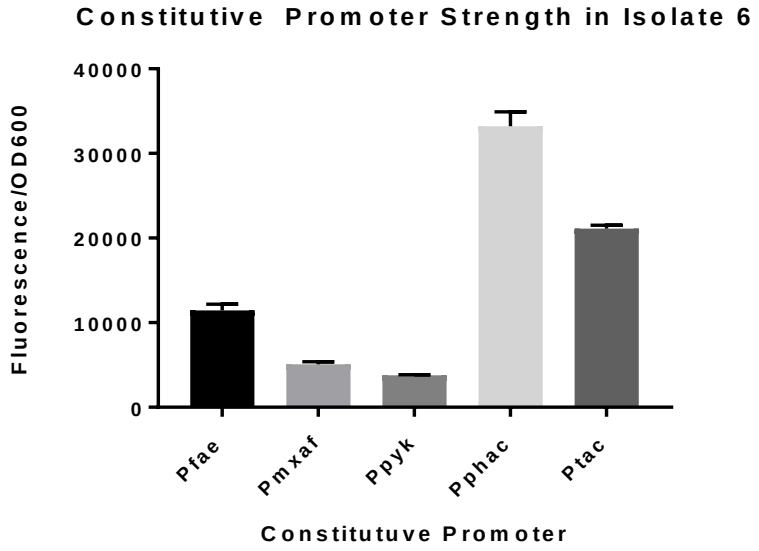


Figure 48 - Expression strength of a variety of constitutive promoters in a promoter probe fusion with enhanced yellow fluorescent protein expressed within *E. coli* S17-1. Pfae, formaldehyde activating enzyme promoter from *M. capsulatus* (Bath) genome.Pmxaf, methanol dehydrogenase promoter from *M. capsulatus* (Bath) genome. Ppyk, pyruvate kinase promoter. Pphac polyhydroxyalkonate synthase promoter from *Cupriavidus necator*. Ptac, constitutive promoter derived from the *trp* and *lac* UV5 promoters [131].

5.5.3.2 Inducible promoters

Inducible promoters can be used to tune gene expression within metabolic networks and so are valuable tools for metabolic engineering. In methanotrophs there is a limited amount of inducible promoters available though one example relevant to metabolic engineering in methanotrophs IPTG includible promoter [65, 121].

5.5.3.2.1 Anhydrotetracycline tolerance

A functional inducible promoter for *M. buryatense* is an anhydrotetracycline (aTc) inducible system. The system is induced with between 0.5-2.0 µg/ml aTc and so the

organism has to grow well with these concentrations of aTc in the media. The assessment of anhydrotetracycline tolerance in isolate 6, as seen in Figure 41, showed it to be very inhibitory to growth and so could not be used as an inducible system for gene expression.

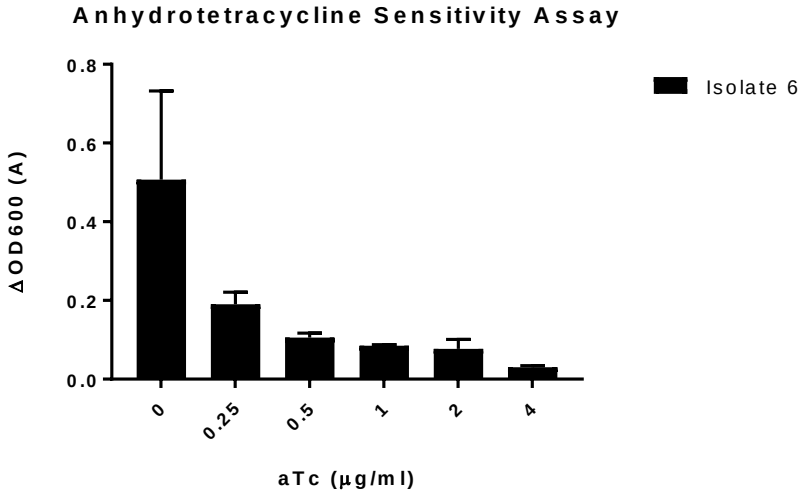


Figure 49 - Anhydrotetracycline sensitivity in isolate 6. Change in OD600 over a 72 hour period.

5.5.3.2.2 Inducible promoters in E. coli S17-1

Inducible promoter probe vectors were assessed to quantify the strength of induced promoters and tight control of when in an uninduced state. Significant expression when the promoter is in an uninduced state is often termed leakiness.

Expression of inducible promoter probe vectors in *E. coli* S17-1 was conducted with the following inducer concentrations with 4 hour incubation: Acetate; 40 mM, acetoin; 10 mM, arabinose; 0.1% (w/v) , benzoate; 1 mM, DAPG(2,4-Diacetylphloroglucinol); 0.1 mM, IPTG (Isopropyl β-D-1-thiogalactopyranoside); 0.5 mM, rhamnose; 0.2% (w/v) and xylose; 1% (w/v). As seen in Figure 50 and Figure 51 EYFP expression was seen in all inducible promoter systems apart from acetoin and IPTG. Acetate was a very strong promoter with normalised fluorescence at 197929 though was also very

leaky with uninduced expression at 68748. Arabinose and xylose inducible systems were medium strength promoters with tight regulation showing no leakiness at 23939 and 27457. Benzoate and DAPG had lower expression at 1576 and 4491 respectively. Xylose showed weak unregulated expression with uninduced at 675 and induced at 121.

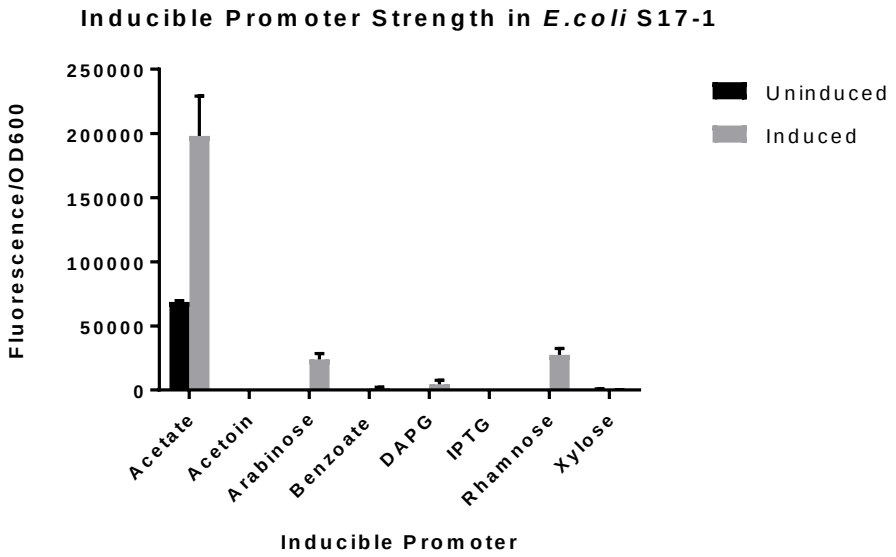


Figure 50 – Expression of EYFP quantified by fluorescence of a variety of inducible systems in *E. coli* S17-1. Inducer concentrations with 4 hour incubation include: Acetate; 40 mM, acetoin; 10 mM, arabinose; 0.1% (w/v) , benzoate; 1 mM, DAPG(2,4-Diacetylphloroglucinol); 0.1 mM, IPTG (Isopropyl β-D-1-thiogalactopyranoside); 0.5 mM, rhamnose; 0.2% (w/v) and xylose; 1% (w/v).

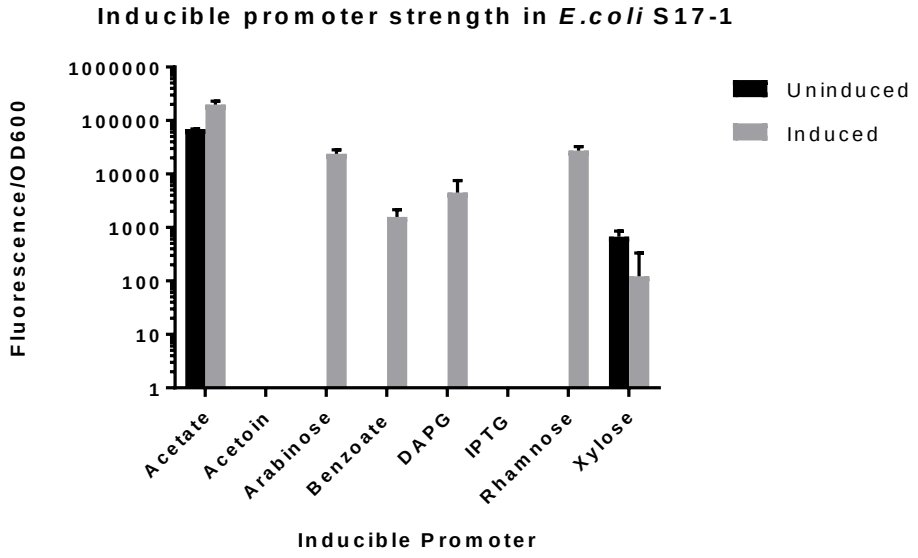


Figure 51 – Figure 5 with a base 10 log scale to show lower comparative expression of inducible promoters.

5.5.3.2.3 Inducible promoters in isolate 6

As the majority of inducible systems showed to be functional in the conjugal donor *E. coli* S17-1, the promoter probe plasmids containing the inducible promoters were conjugated into isolate 6. As seen in Figure 52 all inducible systems showed very low to undetectable levels of fluorescence indicating very poor expression of EYFP with or without inducer. A variety of concentrations up to 8x the concentration of inducer used in *E. coli* were used and still very little expression was observed. The Benzoate inducible promoter showed some increase in relative fluorescence though no dose dependant trend or uninduced regulation was seen. Furthermore for some data points in 1 mM benzoate at 6 hours relative induction is highly varied. This is due to division of OD corrected by media blank being low, this value then divided by the fluorescence value gives an unrepresentatively high fluorescence value.

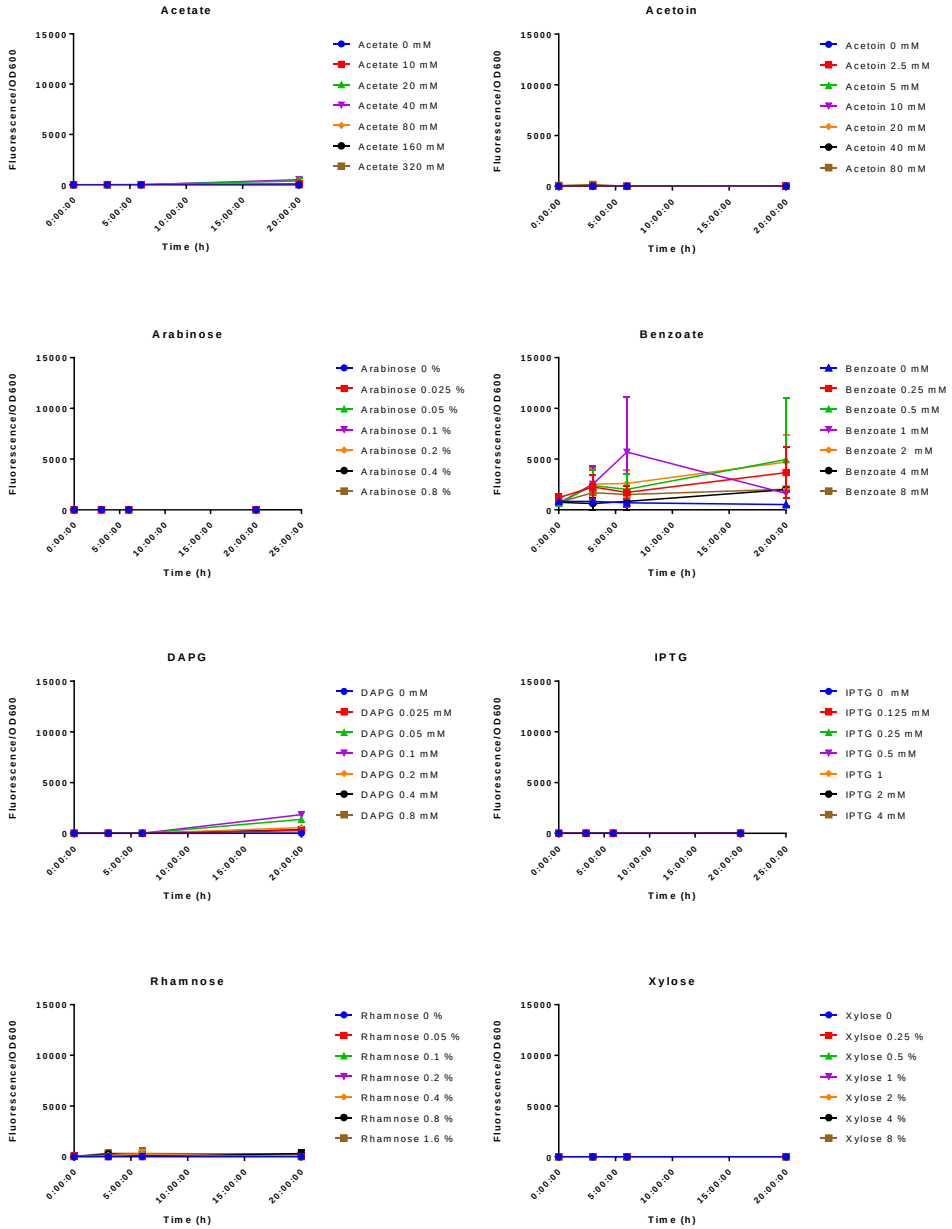


Figure 52 - Expression of EYFP quantified by fluorescence of a variety of inducible systems in Isolate 6 over a 20 hour period of culturing in a 96 well plate with incubation in a methane atmosphere. Inducible systems included: Acetate, acetoin, arabinose, benzoate, DAPG(2,4-Diacetylphloroglucinol), IPTG (Isopropyl β-D-1-thiogalactopyranoside), rhamnose and xylose. % (w/v)

To troubleshoot the data obtained in Figure 52, 8 transformant colonies containing the above inducible promoter probe vectors were re-streaked onto solid NMS agar containing kanamycin and respective inducer (inducer concentrations the same as used in Figure 50 for *E. coli* S17-1). On NMS agar with no inducer and NMS with inducer

benzoate showed fluorescence and none of the other transformants showed fluorescence under blue light further confirming the data obtained in Figure 52. The fluorescence of benzoate is seen in Figure 53, it is worth noting that colony 1 and 8 have low fluorescence unlike the other colonies possibly suggesting heterogeneity within the transformant colonies. On the inducer plates PCR was performed on 3 colonies to amplify the promoter and partial EYFP region for each inducible system and was subsequently sequenced and confirmed as correct.

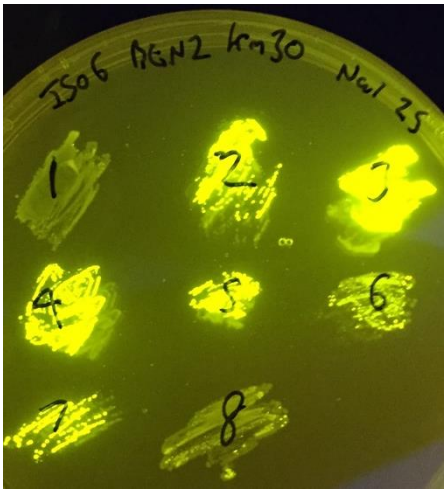


Figure 53 – Visible fluorescence of EYFP under blue light in isolate 6 harbouring the benzoate inducible plasmid with no inducer added. EYFP expression is varied among the patches. Patch 1 and 8 show much less evidence of EYFP expression; this may be due to accumulation of SNPs inactivating expression due to the metabolic burden EYFP may place on the cell.

As a positive control to assess whether error was located in the experimental procedure constitutive promoters were assessed alongside the arabinose inducible system. The same protocol to Figure 7 was performed in serum bottle and the fluorescence was compared within the same experiment, these results are seen in Figure 54. This suggests that the protocol is correct though the inducible systems are more likely to be non-functional.

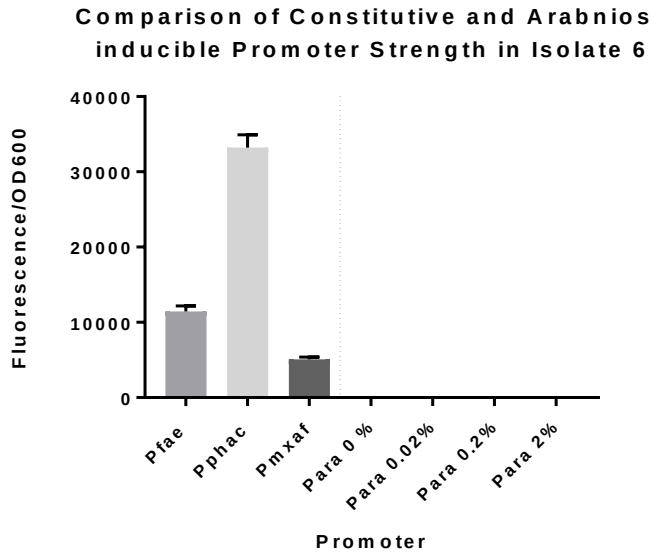


Figure 54 – Expression of constitutive promoter probes and the inducible arabinose promoter probe vector using a range of arabinose concentrations (w/v %).

5.5.3.2.4 Inducible promoters in isolate 14

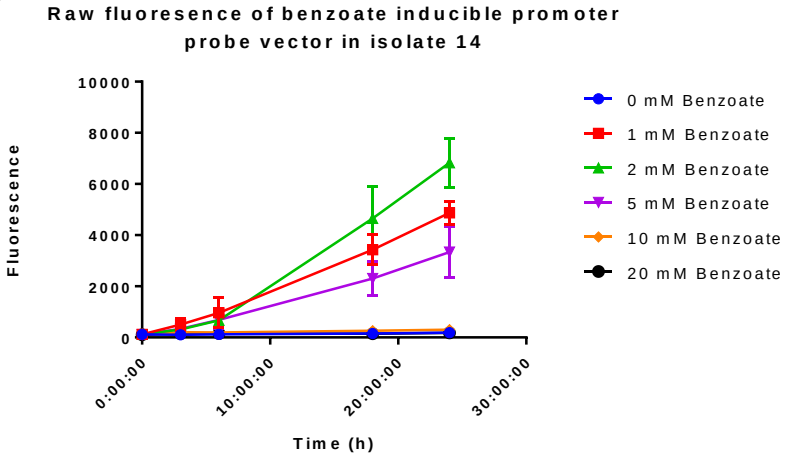
Transformation of isolate 14 was done with limited success and only the benzoate transformation proved successful. Assessing the benzoate inducible system in the EYFP based promoter probe vector is seen in Figure 55. After previous OD measurements proved to be unreliable and gave unrepresentative relative expression in the final analysis all three graphs were plotted to show a more comprehensive view of fluorescence and OD600.

As seen in Figure 55A raw fluorescence shows a clear dose dependant fluorescence with tight transcriptional regulation at 0 mM benzoate with subsequent increase in fluorescence over time to 1 mM and the highest recorded fluorescence observed at 2 mM. Further increasing the concentration to 5 mM drops the fluorescence values and then 10 and 20 mM no fluorescence is seen.

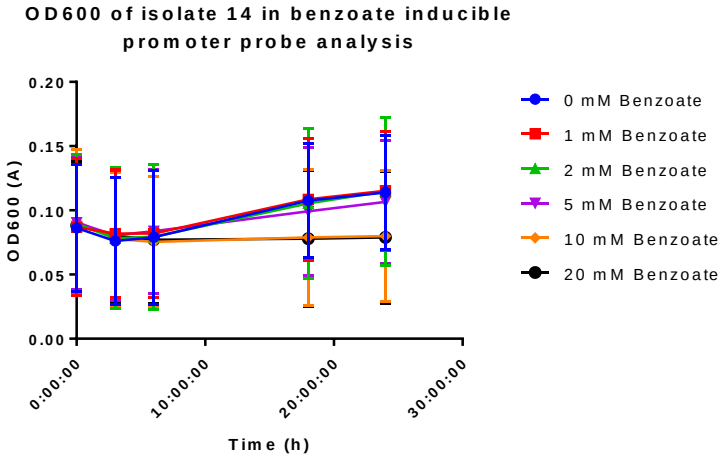
The data values recorded for OD which should be 0.1 as normalised before inoculation into plate are consistent with the average value taken though the standard

deviation is very high and subsequently causes the calculated fluorescence/OD600 to be less accurate. A solution for this as previously described is to take multiple reads per well centred away from the well wall.

A



B



C

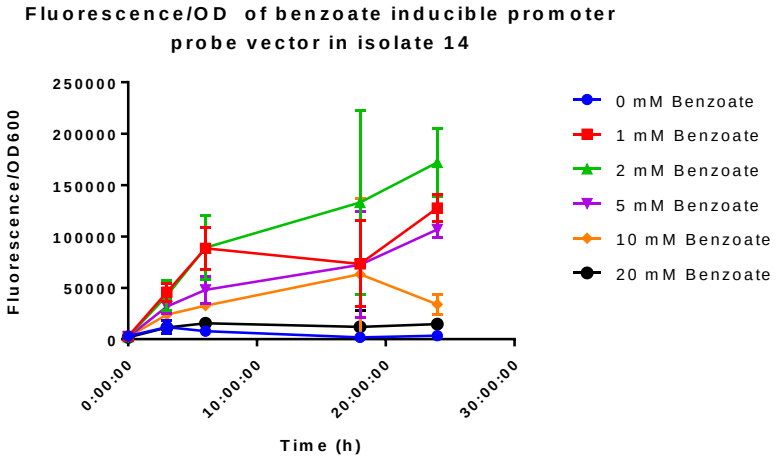


Figure 55 – inducible promoter probe experiment with isolate 14 transformant containing the benzoate inducible promoter probe vector. Concentration of benzoate was varied. (A) Raw fluorescence (B) OD600 (C) Fluorescence/OD600.

5.5.3.2.5 Inducible promoters in isolate 03

No inducible promoters were used successfully in Isolate 6 so an attempt was made to test the promoter probe vectors in a distinct type II methanotroph. Type II methanotroph isolate 03 which was previously shown to be phylogenetically distinct from isolate 6 was transformed with the previously described inducible promoter probe vectors (though the transformation of arabinose and IPTG failed) and fluorescence was analysed as previously described. To reduce error caused by poor OD600 readings 9 points were taken per well with a border of 2200 µM to get a more reliable value and prevent refraction of the laser. Also the starting OD600 was increased to 0.2.

The improved OD600 measuring method was seen in a comparison between the OD600 values in Figure 55B and Figure 56 where isolate 14's OD600 values have a large standard deviation compared with isolate 03's OD600 values with the improved method. This improvement makes the final calculated fluorescence/OD600 values more valid.

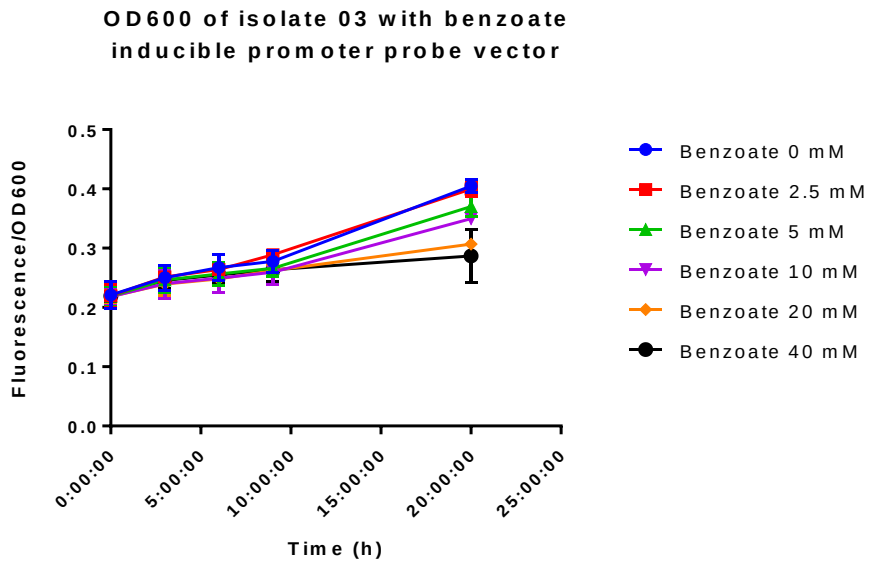


Figure 56 – Improved OD600 measurements employing 9 well reads per well with a border of 2200 µM. This data was taken from the experiment described by Figure 57.

The assessment of promoter probe constructs in isolate 03 show, similar to isolate 6, that there are no suitable inducible systems. All systems aside from acetoin and benzoate inducible promoters showed little fluorescence. Acetoin and benzoate showed medium constitutive expression with no regulation or effect with changing concentration of inducer. Figure 57

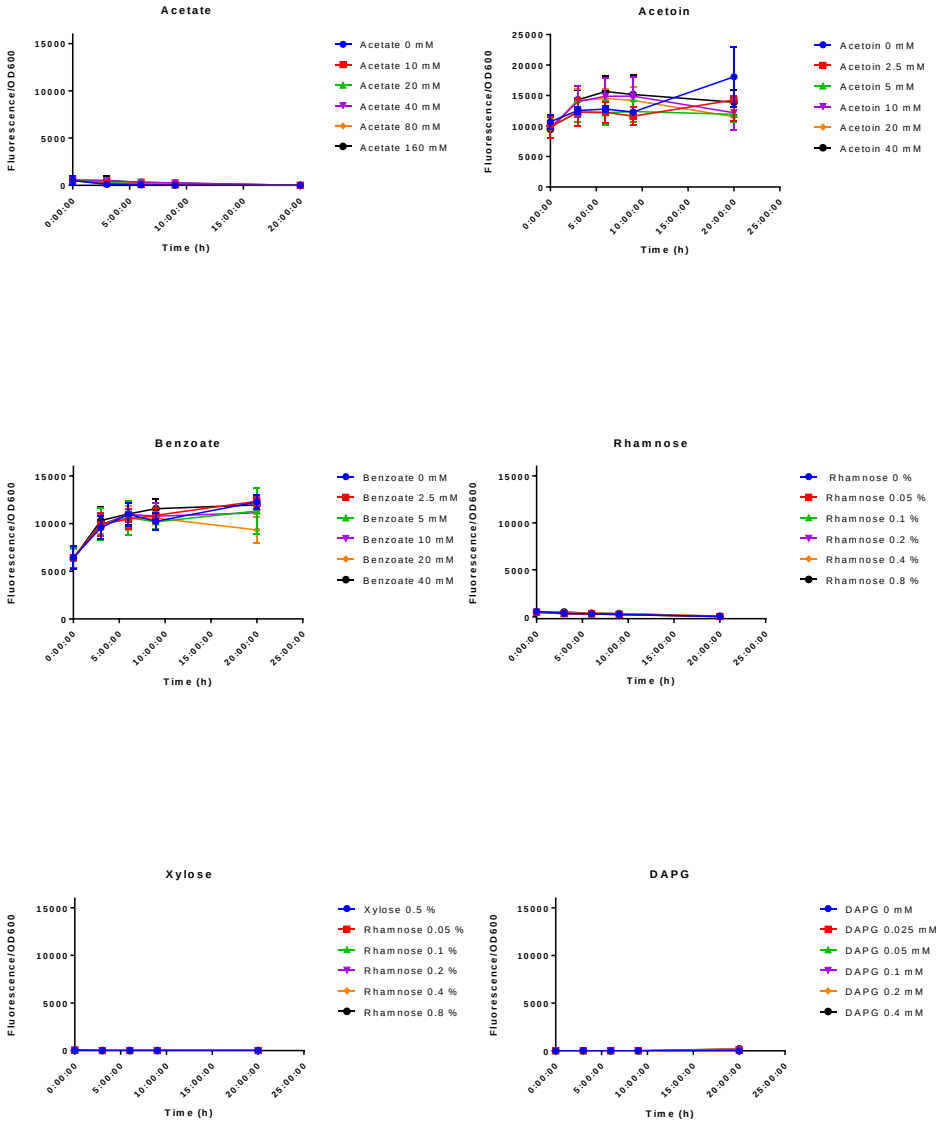


Figure 57 - Expression of EYFP quantified by fluorescence of a variety of inducible systems in Isolate 03 over a 20 hour period of culturing in a 96 well plate with incubation in a methane atmosphere. Inducible systems included: Acetate, acetoin, benzoate, DAPG(2,4-Diacetylphloroglucinol), rhamnose and xylose. % (w/v)

5.5.4 *Allelic exchange using SacB as a negative selection marker*

5.5.4.1 Validation of SacB

Transformants were observed for isolate 6 though not isolate 14. After growth on NMS with sucrose and subsequent patching of 50 colonies onto both NMS and NMS containing 30 µg/ml kanamycin the following growth of colonies is seen in Table 19.

Table 19 suggests that all restreaked colonies aside from one colony grew on NMS plates alone. The addition of kanamycin showed that with an increase in sucrose concentration the amount of colonies that grew on kanamycin decreased due to suspected plasmid loss from the sacB gene conveying the toxic phenotype in the transformants.

Table 19 – Growth of Isolate 6 patches containing the plasmid pMTL8S141 (Km) – phaC on NMS and NMS containing 30 µg/ml kanamycin.

	Number of patches that grew	
Surcose (%)	**NMS**	**NMS Km30**
0	50	44
1	50	40
3	49	33
5	50	26

Further colony PCR of the patches using primers spanning from the MCS to the selection marker could not confirm the presence of the plasmid conclusively.

5.5.4.2 Glycogen Knockout

Multiple attempts to transform pMTL8S141 (Km) – glgA1 and pMTL8S141 (Km) – glgA2 into isolate 14 and *M. capsulatus* (Bath) failed.

5.5.4.3 Poly-3-hydroxybutyrate Knockout

After conjugation of pMTL8S141 (Km) – PhaC1 in isolate 6 possible transformant colonies were obtained on kanamycin selection plates that confirmed the presence of pMMO though single crossover events could not be confirmed via PCR.

5.6 Discussion

Overall a minimum tool set was established for both isolates. The minimum toolkit will allow for the metabolic engineering of the methanotrophic isolates 14 and 6.

5.6.1 *Genetic tractability*

Genetic transformation by conjugation was established based of pre-existing protocols from the literature though isolate 14 proved unreliable to transform which hampered further tool validation. Difficulty in transforming isolate 14 led to the data on isolate 6 becoming more comprehensive than isolate 14. In spite of this pBBR1 and Col E1 were shown to be functional in isolate 14 and 6. Both replicons pBBR1 and Col E1 are known to be functional in *M. capsulatus* [61, 138].

The alternative, electroporation, is common transformation technique in type II methanotrophs though few type I electroporation methods exists. The one existing protocol in *M. buryatense* [130] failed to transform either isolate and so the conjugation method that allowed both isolates to be transformed simultaneously was used as the primary means of transformation. The electroporation method failing may be due to RMS. Restriction modification systems (RMS) methylate or cleave DNA to protect bacterial cells from invasion by foreign DNA e.g. bacteriophage. RMS use methylation of specific sequences to protect their own DNA from cleavage by endonucleases that cleave the unmethylated DNA typically found in foreign DNA. Dam (DNA adenine methyltransferase) and Dcm (DNA cytosine methyltransferase) minus strains of *E. coli* do not possess the corresponding methyltransferase. Within the data described by [130] plasmids from *E. coli*, strains with and without Dam/Dcm, were not able to be transformed into *M. buryatense* and all positive electroporations were established using plasmids extracted from *M. buryatense*. Furthermore if the harvested plasmid was transformed into *E. coli*, purified and transformed back into *M.*

buryatense, no Transformants were seen. This was further verified by [139] where *Methylomicrobium alcaliphilum* 20Z was only able to be effectively transformed with a plasmid harvested from a transconjugant *M. alcaliphilum*. This suggests RMS may be preventing the transfer of the plasmids into the host organism.

5.6.2 *Molecular tools*

The proven usability of markers to now include Ery and Amp expands the molecular tool box for isolate 6 for allowing for broader application for example multiple plasmid transformation. Further to this promoter probe vectors were shown to be functional in *E. coli* then subsequently isolate 14 and 6 which each gave differing strengths. A strong promoter in isolate 14 was obtained from the endogenous methanol dehydrogenase promoter ideal for metabolic engineering. The same promoter in isolate 6 though not endogenous was surprisingly weaker than the *phaC* promoter from *C.necator*. It was the PphaC that would be used for future metabolic engineering applications. It was also surprising that in both isolates Pfae and Ppyk exhibited such low expression though considering the promoterless EYFP control was implemented and expression was seen it can be concluded that the specified promoter was present within the extracted sequence.

To improve tunablilty of metabolic networks a variety of inducible promoter probe constructs were made. Acetate, arabinose, benzoate, DAPG and rhamnose were shown to be functional in the conjugal donor *E. coli* S17-1. IPTG and acetoin inducible systems were seen to be non-functional in *E. coli* S17-1. IPTG is a commonly used inducible system in *E. coli* and sequencing of the plasmid showed the presence of the lacIq promoter, LacI, CAP binding site, lac promoter, lac operator and EYFP. Some SNPs were present such as in the LacI though no SNPs were seen that would cause no expression to be seen under uninduced and induced states. Functionally the presence

of glucose from LB media may have caused cyclic adenosine monophosphate (cAMP) to become low in the cell reducing the formations of cAMP CAP complexes. The camp CAP complexes bind to the CAP binding site (as seen in Figure 58) and promote the binding of RNA polymerase by positive regulation leading, as a result glucose could lead to no expression of EYFP. Acetoin inducible system was also sequenced and no SNPs were seen though function was still not present.

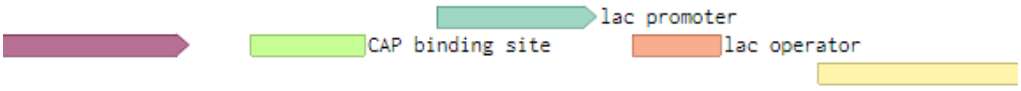


Figure 58 – IPTG inducible system in pMTL71401-IPTG-EYFP. Purple, LacI. Green, Catabolite activator protein binding site. Cyan, Lac promoter. Orange, Lac operator binding site. Yellow, EYFP.

Aside from acetate and xylose inducible systems which had unregulated expression many inducible systems showed no uninduced fluorescence in *E. coli*. This is unexpected as most inducible promoters typically exhibit small amounts of uninduced expression for example in the paper identifying the rhamnose inducible promoter [135] the plasmid was expressed in a promoter probe fasion with GFP in *E. coli*. After 4 hours incubation comparing induced and non-induced in LB media small relative induction is seen which is not seen when using pMTL71401-Benzoate-EYFP. The comparison data used a similar promoterless GFP containing plasmid to remove fluorescence caused by background signal, this may imply that the plasmids used from pMTL71401-EYFP may exhibit residual/baseline transcriptional overlap from upstream sequences. Steps were made to reduce the chances of this by flanking the promoter probe element with terminators though this does not guarantee transcriptional overlap.

Functionality of benzoate inducible promoter in isolate 14 has widened the available molecular toolbox to control expression of heterologous genes in isolate 14. Findings in a patent describing tools for methanotrophs corroborated this finding in *M. capsulatus* (Bath) [140]. Both isolate 6 and isolate 03 exhibited either no expression at all or constitutive dose independent fluorescence. There are a variety of explanations for this behaviour. Firstly, the key elements for recognition of a promoter sequence is the σ factor binding site (known as -10 and -35 region) that facilitates the binding of the RNA polymerase to the respective site upstream of the open reading frame initiating transcription. Most bacteria have distinct σ factor binding sites that vary in sequence. A divergence in σ factor binding sites in methanotrophic isolate could reduce or prevent binding of the RNA polymerase and as a result transcription would be reduced or silenced. This mechanism when applied to the promoter driving expression of EYFP would account for no expression. Constitutive dose independent expression can be explained by the correct function of the promoter driving EYFP and the non-functional transcription of the repressor gene though this is not seen in any of the negatively expressed systems suggesting the problem lies with the promoter of EYFP or both. In isolate 03; acetoin and benzoate, and isolate 6 only benzoate, is constitutively expressed. These systems are positively controlled and so this behaviour could arise from either a leaky promoter driving EYFP, and as no dose independent expression is seen, also no transcription of the activator. Another explanation is that intracellular build-up of benzoate, or molecule with a similar binding capacity, binds to the activator therefore saturating the expression of EYFP.

A possible general mechanism inhibiting the function of all inducible systems is the ability of the inducer molecule to pass the hosts membrane to reach the activator or repressor. *E. coli* has a comparatively diverse metabolism which may imply more

membrane transporters are present which enable the passage of inducer from the extracellular to intracellular space to allow the inducible system to function. Finally, the ribosome binding site consensus sequence varies between organisms, a divergent sequence to this would reduce the rate of translation also inhibiting the amount of EYFP produced and therefore fluorescence. Future work could include the construction of synthetic operon with functional constitutive promoters previously identified and using the *benR* repressor as seen in isolate 14 it has the ability to pass to the intercellular space.

5.6.3 *Genome editing*

Attempts to delete genes involved in carbon storage proved unsuccessful. Isolate 14 is likely to have failed due to poor transformation which has been previously seen. Isolate 6 transformation produced transformants though single crossovers could not be confirmed conclusively by PCR and due to time constraints no further repeats were attempted.

5.7 Conclusion

Overall the genetic toolbox for both isolates was expanded enough to begin the heterologous expression of the pathway to isobutanol production.

6 *Metabolic Engineering of Isobutanol Biosynthesis in Methanotrophic Bacteria*

6.1 Chapter Introduction

With successful characterisation of methanotrophic isolates and molecular tools, the next step for methane to isobutanol bio-conversion is the plasmid based expression of exogenous genes to isobutanol. As summarised in chapter 1.6 there are a variety of successful examples of the exploitation of the keto-acid based valine biosynthesis pathway for the production of isobutanol.

To suggest an improved pathway for expression within a methanotrophic host it is essential to look beyond methanotroph and yeast enzymes used in the previously described engineered pathway in *M. capsulatus* . The enzymes required for conversion of key metabolic pyruvate to isobutanol are described in Figure 59. As a variety of isozymes from different hosts exist a rational comparison of possible enzymes for each step from pyruvate to isobutanol is as follows.

Acetolactate synthase which catalyses the formation of 2-acetolactate from pyruvate is encoded by the overexpression of endogenous *M. capsulatus ilvK* which, similar to other acetohydroxy acid synthases [141], may be subject to feedback inhibition via branch chain amino acids [142] which are likely to be in greater abundance due to the overexpression of *ilvK*, *ilvC* and *ilvD*. An approach to circumvent possible feedback inhibition is to use *AlsS* from *Bacillus subtilis* which is an acetolactate synthase that catalyses the reaction with high activity and has no feedback inhibition [143].

Ketol-acid reductoisomerase and dihydroxy-acid dehydratase catalyses 2-acetolactate to 2,3-dihydroxy-isovalerate and then 2,3-dihydroxy-isovalerate onto 2 keto-

isovalerate respectively. In the previously described production of isobutanol in *M. capsulatus* the optimum yield produced on methane was using endogenous *ilvC* and *ilvD*. A similar approach was taken by Atsumi *et al* [72] in using endogenous *ilvC* and *ilvD* genes, amongst others, to produce high amounts of isobutanol from glucose in *E. coli*. Lin *et al* [78] also used native *ilvC* and *ilvD* enzymes in *G.thermoglucosidaius* based on the rationale that native enzymes were more likely to be heat stable during thermopillic growth at 50°C. In contrast Higashide *et al* utilised non-native *E. coli ilvCD* in C.cellulolyticum for the production of isobutanol from cellulose which yielded 0.64 g/L. As no direct comparison exists of *ilvCD*s with similar enzymes simultaneously over-expressed in a non-native host it is difficult to compare and predict the effectiveness of each strategy.

Keto-acid decarboxylase catalyses 2-ketoisovalerate to isobutyraldehyde. Keto-acid decarboxylase, *KivD*, from *L. lactis* within a *Geobacillus* species which was grown on glucose at 50°C with other enzymes overexpressed yielded 2.8 g/L of isobutanol [78]. This demonstrates good thermostability at *M. capsulatus '* optimum growth temperature. In addition to this it has been used in multiple organisms to produce a yield much higher than that of *M. capsulatus* .

The final step is catalysed by alcohol dehydrogenase from isobutyraldehyde to isobutanol. The highest titres of isobutanol seen in the literature utilised *adh*A from *Lactococcus lactis* which may have not been selected for use in *M. capsulatus* due to its low thermostability at 37°C relative to lower temperatures (optimum 30°C) [71] (considering *M. capsulatus* optimum growth is at 45°C). A comparison of alcohol dehydrogenases was done which showed overexpression of *yqhD* or *adhA* from *E.*

coli outperformed ADH2 from *S. cerevisiae* for isobutanol production in *E. coli* making it a potential candidate for overexpresison [144].

To conclude, there are a number of alternatives that can be rationally selected with the initial aim of improving the pathway though to optimise the pathway a high-throughput combinatorial approach would clearly be needed. The above can provide a basis for an initial (Seen in Figure 59) pathway that can be tuned and modified with further investigation.

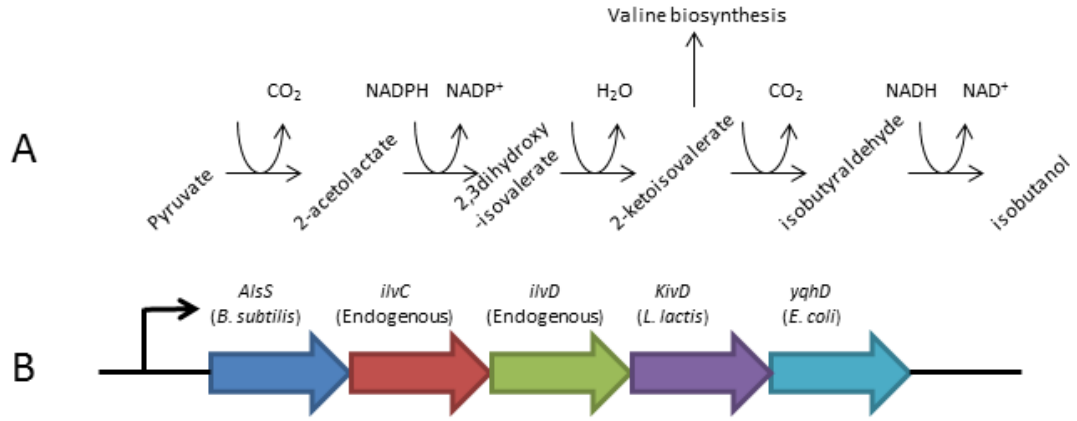


Figure 59 - (A) Metabolic pathway for the production of isobutanol from pyruvate. (B) Schematic of the operon encoding the genes for the metabolic pathway from pyruvate to isobutanol.

The genes to be tested are seen in Figure 59 and it was decided to test them by first expressing the exogenous two step pathway including keto-acid decarboxylase and then the alcohol dehydrogenase which catalyses the conversion of endogenous 2-ketoisovalerate to isobutyraldehyde then isobutanol. For a comparison to type strains *Methylococcus capsulatus* and isolate 14 would be tested using the genes proposed above and the genes used in [65]. This should allow a comparison of the selected enzymes to the existing data on isobutanol production in methanotrophs. After this the

upstream acetolactate synthase, ketol-acid reductoisomerase and dihydroxy-acid dehydratase could be also co-expressed to overexpress the complete pathway from pyruvate to isobutanol. Plasmids were be constructed using the pMTL series format and the expression of the respective genes were expressed using strong constitutive promoters Pmxaf and PphaC seen in 5.5.3.1.

6.2 Strains and Plasmids

The *E. coli* strains strains and methanotrophic organisms used in the following chapter are detailed below in Table 20.

Table 20 - Strains used in this study.

Strain	Description	Reference
Escherichia coli		
DH5α	*F' endA1 hsdR17*(r_k^-,m_k^-) *supE44 thi-1 recA1 gyrA* (Nal^r) relA1Δ(*lacIZYA-argF*) U169 *deo*R (φ *80*d*Lac* Δ(*lacZ*)*M15*)	[128]
S17-1	*Thi, pro hsdR*$^-$*, hsdμ*$^+$*, recA; integrated plasmid RP4-Tc::Mu-Kn::Tn7*	[129]
XL1-BLUE	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIq ZΔM15 Tn10 (Tetr)	(NEB)
Methanotrophs		
Isolate 14	Methanotroph isolated from the Roman Bath in Bath	This study
Isolate 6	methanotroph isolated from University of Nottingham lake	This study
Methylococcus capsulatus (Bath)	*Methylococcus capsulatus* (Bath) kindly donated by Dr Andrew Crombie	[28]
Methylocystis parvus	NCIMB 11129	

The plasmids used in the following chapter are detailed in Table 21 as seen below.

Table 21 - Plasmids used in this study. SBRC CC, Synthetic Biology Research Centre Culture Collection.

Plasmid	Description	Reference
pMTL71401-Pmxaf-EYFP	PmxaF DNA template for construction of pMTL90882-IsoB15 and	This study

	pMTL90882-IsoB17	
pMTL82254-PtcdB_KIVD_yq	DNA template for pMTL90882-IsoB15 and pMTL90882-IsoB16	This study (SBRC CC)
pMTL71401-Pphac-EYFP	PphaC DNA template for construction of pMTL90882-IsoB16	This study
pMTL82254-PtcdB_alsS_yq	DNA template for pMTL90882-IsoB15 and pMTL90882-IsoB16	This study (SBRC CC)
pMTL90882	Backbone for pMTL90882-IsoB plasmids	This study
pMTL90882-IsoB15	pMTL90882 with *KivD* and *yqhD* with expression driver by PmxaF	This study (SBRC CC)
pMTL90882-IsoB16	pMTL90882 with *KivD* and *yqhD* with expression driver by PphaC	This study (SBRC CC)
pMTL90882-IsoB17	pMTL90882 with ilvD and *ADH6* with expression driver by PmxaF	This study (SBRC CC)

6.3 Primers

The primers used in the following chapter are seen in Table 22.

Table 22 - Cloning primers used in this study

Name	**Sequence**	**Description**
61-pMTL71401-Mxaf-EYFP FWD/ 62-pMTL71401-Mxaf-EYFP REV	F - aaacagctatgaccgcggccggccgcGAGGTTCAGGCGAA R - TAGTCGCCCACGGTGTACATtgTCTCCTCCACTACTAGCGCTAAGA	PCR amplification of PmxaF for Hifi assembly of pMTL90882-IsoB15.
63-KivD_*L. lactis*_(Codon Optimized+ HisTag) FWD/ 64-KivD_*L. lactis*_(Codon Optimized+ HisTag) REV	F - TAGTAGTGGAGGAGAcaATGTACACCGTGGGCGACTACCT R - CATGGGTGGTTTCCTCGCTGCGCTAGCTTATCACGACTTGTTCT	PCR amplification of *KivD* for Hifi assembly of pMTL90882-IsoB15.
65-pMTL82254-PtcdB_KIVD_yq FWD/ 66-pMTL82254-PtcdB_KIVD_yq REV	F - GCAGCGAGGAAACCACCCATGAACAACTTTAATCTGCACACCC R - CTATCAACAGGAGTCgctagTTAGCGGGCGGCTTCGTATA	PCR amplification of *yqhD* for Hifi assembly of pMTL90882-IsoB15.

67-pMTL71401-Pphac-EYFP FWD/ 68-pMTL71401-Pphac-EYFP REV	F - aaacagctatgaccgcggccTTGACAGCGCGTGCGTTGCA R - TAGTCGCCCACGGTGTACATTGTTGATTGTCTCTCTGCCGTCAC	PCR amplification of PphaC for Hifi assembly of pMTL90882-IsoB16.
69-KivD_*L. lactis*_(Codon Optimized+ HisTag) FWD/ 70-KivD_*L. lactis*_(Codon Optimized+ HisTag) REV	F - CAGAGAGACAATCAACAATGTACACCGTGGGCGACTACCT R - CATCGACGAGTCCTCCTGCAACCGCTAGCTTATCACGACTTGTTCT	PCR amplification of *KivD* for Hifi assembly of pMTL90882-IsoB16.
71-pMTL82254-PtcdB_alsS_yq FWD/ 72-pMTL82254-PtcdB_alsS_yq REV	F - GGTTGCAGGAGGACTCGTCGATGAACAACTTTAATCTGCACACCCC R - CTATCAACAGGAGTCgctagTTAGCGGGCGGCTTCGTATA	PCR amplification of *yqhD* for Hifi assembly of pMTL90882-IsoB16.
73-pMTL71401-Mxaf-EYFP FWD 74-pMTL71401-Mxaf-EYFP REV	F - aaacagctatgaccgcggccggccgcGAGGTTCAGGCGAA R - CGGGGGTGCTTGTCGGTCATtgTCTCCTCCACTACTAGCGCTAAGA	PCR amplification of PmxaF for Hifi assembly of pMTL90882-IsoB17.
75-Mc.Bath_KDC_F FWD/ 76-Mc.Bath_KDC_F REV	F - CGCTAGTAGTGGAGGAGAcaATGACCGACAAGCACCCCCG R - CATGGGTGGTTTCCTCGCTGCTCAGAGGCCGTCGTCGGTGAC	PCR amplification of ilvD for Hifi assembly of pMTL90882-IsoB17.
77-*ADH6_S. cerevisiae*_(Codon Optimized+ HisTag) FWD/ 78-*ADH6_S. cerevisiae*_(Codon Optimized+ HisTag) REV	F - GCAGCGAGGAAACCACCCATGTCGTACCCGGAGAAGTTCGAG R - CTATCAACAGGAGTCgctagGCTAGCTTATCAGTCCGAGA	PCR amplification of *ADH6* for Hifi assembly of pMTL90882-IsoB17.
Cole1_F1/Kan_R1	F – ACGGTTCCTGGCCTTTTGCT R – TCCAGATAGCCCAGTAGCTGAC	Sequencing primers reading into the MCS site from the Col E1 replicon and KmR.

6.4 Methods used in Chapter

6.4.1 *Plasmid construction*

Plasmids containing keto-acid decarboxylase and alcohol dehydrogenase were created in the pMTL9 series vector and expression was driven by PphaC for *Methylocystis* species and PmxaF for *Methylococcus* species. The enzymes chosen for each operon were keto-acid decarboxylase (*ilvD*) from *M. capsulatus* with alcohol dehydrogenase (*ADH6*) from *S. cerevisiae* and a separate operon containing keto-acid decarboxylase (*KivD*) from *L. lactis* and alcohol dehydrogenase (*yqhD*) from *E. coli*. Plasmids were constructed by first codon optimising the genes *KivD* and *ADH6* using the online tool Jcat [145] using *M. capsulatus* as the organism for codon optimisation. Genes were synthesised by Twist Bioscience, other genes were obtained from the SBRC culture collection. As seen in Table 22 insert genes were PCR amplified using (the primer with the prefixes) 61/62, 63/64 and 65/66 to amplify PmxaF, *KivD* and *yqhD* respectively. Primers 67/68, 69/70 and 71/72 were used to amplify PphaC, *KivD* and *yqhD*. The final construct used primers 73/74, 75/76 and 77/78 to amplify Pmxaf, *KivD* and *ADH6*. During the design of these primers alternative RBS were added to the second gene to prevent recombination. These included MsRBS (GGTTGCAGGAGGACTCGTCGATG from Methylocystis SC2 Mxaf gene) and McRBS1 (GCAGCGAGGAAACCACCCATG from cytochrome p460 protein [146]). All PCR amplifications were performed using Q5 ® High-Fidelity 2X Master Mix. The backbone was taken from restriction digest of pMTL90882 using *NheI* and *NotI* (NEB). PCR and digestion products were purified using gel electrophoresis and Monarch ® DNA Gel Extraction Kit. The fragments were assembled using NEBuilder ® Hifi DNA Assembly Master Mix as previously described. The assembly mixture was then transformed into chemically competent *E. coli* XL1 Blue and S17-1 using heat-shock transformation and constructs were confirmed using Sanger sequencing. The plasmids created were named pMTL90882-IsoB15, pMTL90882-IsoB16 and

pMTL90882-IsoB17. pMTL90882-IsoB18 containing PphaC promoter *KivD* (*M. capsulatus*) and *ADH6* (*S. cerevisiae*) could not be constructed. Constructs with PmxaF promoters were conjugated, as previously described, into *M. capsulatus* (Bath) and isolate 14 and constructs with PphaC promoters conjugated into isolate 6 and *M. parvus*.

Table 23 – Components of isobutanol producing operon in plasmids pMTL90882-Isob15, pMTL90882-Isob16 and pMTL90882-Isob17.

Plasmid	Promoter	Gene 1 (origin)	Gene 2 (origin)
pMTL90882-Isob15	PmxaF	*KivD* (*L. lactis*)	*yqhD* (*E. coli*)
pMTL90882-Isob16	PphaC	*KivD* (*L. lactis*)	*yqhD* (*E. coli*)
pMTL90882-Isob17	PmxaF	*KivD/ilvD* (*M. capsulatus*)	*ADH6* (*S. cerevisiae*)

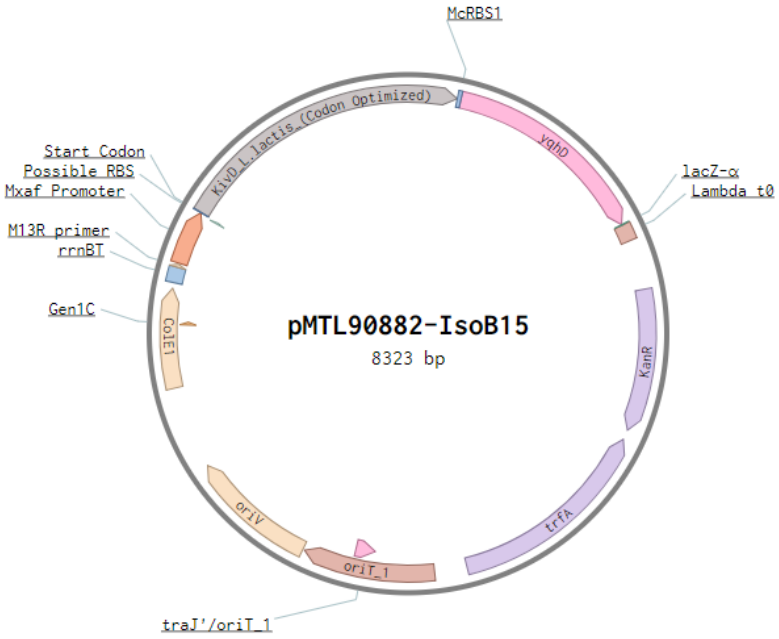


Figure 60 – Genetic elements of pMTL90882-Isob plasmid. *OriV*, Replication origin. *OriT*, Origin of transfer nick site. *trfA*, replication initiation protein. KanR, kanamycin resistance gene. Lambda T0, Terminator sequence. *rrnBT*, Terminator sequence. *KivD*, keto-acid decarboxylase from *Lactococcus lactis*. *yqhD*, alcohol dehydrogenase from *E. coli*.

6.4.2 *Isobutanol production using methanotrophic bacteria*

6.4.2.1 2-ketoisovalerate feeding methanotrophs for isobutanol production

Stationary phase liquid cultures of methanotrophic strains harbouring pMTL90882-Isob15, pMTL90882-Isob16 and pMTL90882-Isob17 were inoculated to 0.05 OD600 into 10 ml NMS media containing 15 or 30 µg/ml kanamycin (*Methylococcus* or *Methylocystis* strains respectively) within a 60 ml serum bottle. The precursor of the heterologous pathway, 2-ketoisovalerate, was introduced into the culture at 0, 1 and 2 g/L. All cultures were made in triplicate, with 25 ml methane added to overpressure and cultured at respective temperature (30°C for *Methylocystis* species or 45°C for *Methylococcus* species) at 200 rpm for 72 hours. Sampling time-points were performed by extracting 1 ml and centrifuged at 13,000 rpm for 1 minute to extract the supernatant for GC-MS analysis of isobutanol. Final time points were taken by storing the culture at 4°C for 2 hours and then removing 1 ml of culture. Remaining liquid culture was span down and stored at -20°C for subsequent SDS-PAGE analysis.

6.4.2.2 SDS-PAGE of heterologous enzymes

Sonication - Previously obtained triplicate cell pellets were pooled and re-suspended in 1 ml of ice cold lysis buffer composed of 50 mM Tris and 5 mM $MgCl_2$ adjusted to a pH of 7.5. The samples were kept on ice throughout. Samples were corrected to an OD600 of 4 and then sonicated for 15 seconds at 9 µm wavelength then placed on ice for 1 minute. The sonication step was cycled a further 9 times. Resulting lysate was centrifuged at 13,300 rpm for 1 minute at 4 °C and the soluble fraction was removed.

SDS-PAGE – Of the cell lysate 10 µl of the soluble and insoluble fraction was added to 10 µl of sample buffer (containing 5 µl LDS Sample Buffer (NuPAGE), 2 µl Sample Reducing Agent (NuPAGE) and 3 µl dH_20). Samples were then touch span down and boiled for 10 minutes at 98°C. Samples were then ran on a 4-12% gel in

MES buffer for 1 hour at 150v alongside a protein ladder. The gel was washed three times for 5 minutes in deionised water then stained with Simply Blue safe stain for 1 hour with gentle shaking. After staining the stain was poured off and washed with deionised water for 15 minutes then further washed over night with gentle shaking. The resulting gel was imaged using a Bio-Rad Gel Doc imager on the Commassie pre-set.

6.4.2.3 Quantification of isobutanol from recombinant isolates

Of the supernatant extracted from the 2-ketoisovalerate feeding experiments, 300 µl was added to 700 µl of acetone containing 0.5M toluene within a capped GC vial. GC-MS analysis was performed by Matthew Abbott from SBRC analytics. The column used was a HP-INNOWAX measuring 20 m x 0.18 mm x 0.18 µm using hydrogen as a carrier gas at 1.1 ml/min flowrate. The oven temperature was held at 40°C for 2 minutes then ramped to 210°C at 50°C/minute then held at 210°C for 1 minute. The inlet was 250°C with a split ratio of 10.

6.4.2.4 Colony PCR of pMTL90882

During and after the feeding experiments to check for the retention of the pMTL90882-IsoB plasmids colony PCR was performed. Colony PCR was performed by centrifugation of 1ml per time point or total cell culture at the end of the 2-ketoisovalerate feeding experiment. Of the cell pellet 10 µl was boiled at 98°C for 10 minutes and 2 µl was used as a template for colony PCR using DreamTaq Green PCR Master Mix (2X) with Col E1_F1/Kan_R1 primers.

6.5 Results

6.5.1 *Isobutanol production using methanotrophic bacteria*

Both type I and type II methanotrophs including Isolate 6, *M. parvus*, isolate 14 and *M. capsulatus* were originally used as hosts for metabolic engineering to produce isobutanol. Plasmids possessing the isobutanol operon (pMTL90882-Isob15/16/17) were transformed into all strains successfully aside from isolate 14 which failed to transform. During the growth of these strains harbouring the isobutanol operon with negative control wildtype strains the change in OD600 was recorded to view inhibition of growth by the pre-curser, 2-ketoisovalerate, and production of isobutanol was recorded simultaneously after extended 72 hour incubation. As seen in Figure 61 growth is consistent with no added 2-ketoisovalerate precursor. Adding 2-ketoisovalerate decreases the change of growth in OD600 greatly in the *Methylocystis* strains both harbouring the isobutanol operon and not harbouring the operon to below an average of 0.038. In both cases the strain harbouring the isobutanol operon grew slightly better in 1 g/L than the wildtype. *Methylococcus capsulatus* (Bath) growth was also significantly affected by the presence of 2-ketoisovalerate though to a lesser extent of the Methylocystis species. The wildtype strain grew better than the strain containing pMTL90882-IsoB15 and worse than pMTL90882-IsoB15 with no 2-ketoisovalerate present though with 1 g/L present pMTL90882-IsoB15 grew the most with greater consistency and under 2 g/L pMTL90882-IsoB17 followed by pMTL90882-IsoB15 showed the most change in growth.

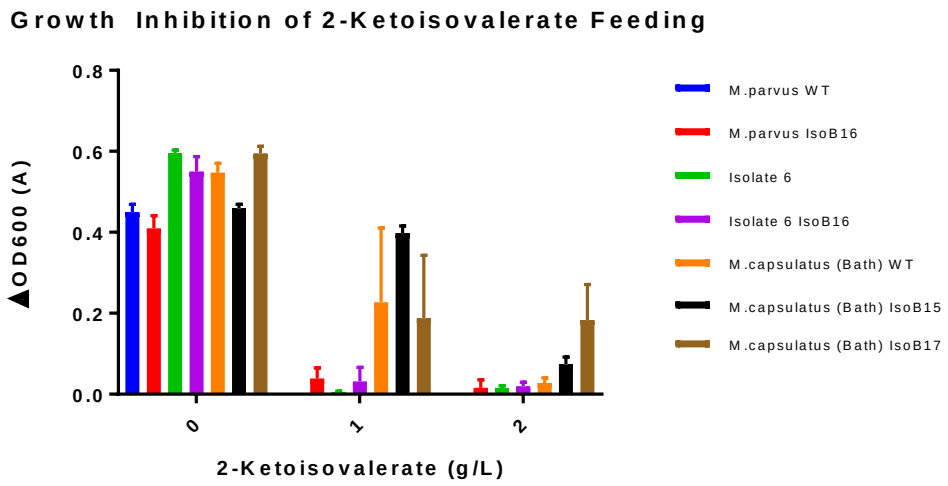


Figure 61 – The effect of 2-ketoisovalerate inhibition on growth between *M. parvus*, isolate 6, and *M. capsulatus* (Bath) with and without plasmids for the heterologous pathway for isobutanol production over a 72 hour incubation. IsoB15, pMTL90882-isoB15 plasmid which contains *KivD* and *yqhD* expressed by PmxaF. IsoB16, pMTL90882-IsoB16 plasmid which contains *KivD* and *yqhD* expressed by PphaC. IsoB17, pMTL90882-IsoB17 plasmid which contains *ilvD* and *ADH6* expressed by PmxaF. WT, Wild Type.

Aside from change in OD600 isobutanol production was analysed in the supernatant of the culture media as seen in Figure 62. *M. parvus* wildtype produced no isobutanol in any condition though whilst harbouring pMTL90882-IsoB16 produced an average of 0.39 mM and 0.53 mM respectively with 1 and 2 g/L 2-ketoisovalerate feeding. No isobutanol was seen in unfed samples. Isolate 6 produced no isobutanol in all conditions and with and without pMTL90882-IsoB15. *M. capsulatus* (Bath) wildtype produced no isobutanol in any condition though could produce isobutanol with 1 g/L feeding of 2-ketoisovalerate harbouring pMTL90882-IsoB15 and pMTL90882-IsoB17. The respective concentration of isobutanol produced was 0.077 mM and 0.117 mM of which pMTL90882-IsoB17 outperformed pMTL90882-IsoB15.

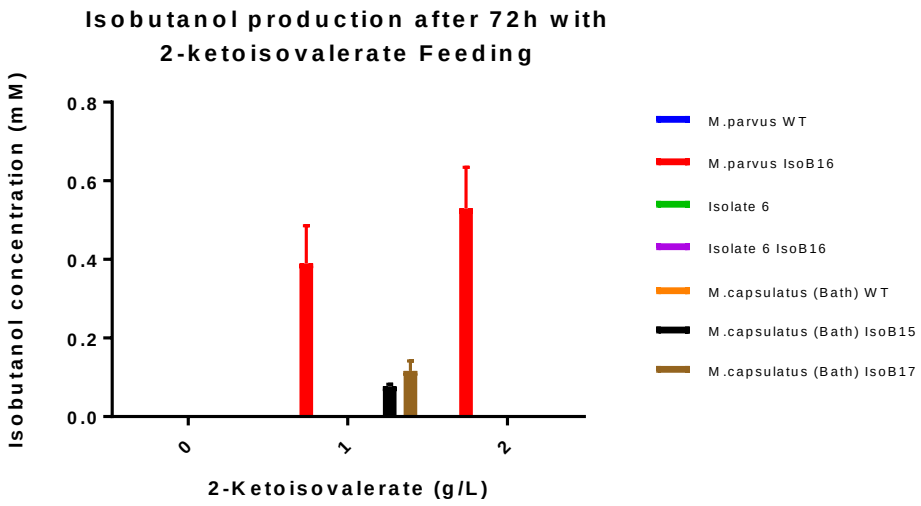


Figure 62 – Isobutanol productions of *M. parvus*, isolate 6, and *M. capsulatus* (Bath) both harbouring and not harbouring plasmids for the heterologous pathway for isobutanol production with 2-ketoisovalerate feeding. IsoB15, pMTL90882-isoB15 plasmid which contains *KivD* and *yqhD* expressed by PmxaF . IsoB16, pMTL90882-IsoB16 plasmid which contains *KivD* and *yqhD* expressed by PphaC. IsoB17, pMTL90882-IsoB17 plasmid which contains *ilvD* and *ADH6* expressed by PmxaF. WT, Wild Type.

To confirm the expression of the heterologous enzymes associated with isobutanol production SDS-PAGE analysis was performed on cell lysates from the cultures in Figure 62. The SDS-PAGE analysis, seen in Figure 63, shows presence of heterologous enzymes not expressed by the wildtype strain. Additional proteins expressed include *KivD* (60.95 kDA), *yqhD* (42.10 kDA), *ilvD* (58.85 kDA) and *ADH6* (39.62 kDA). For *M. parvus* expressing the heterologous pathway a clear band is seen around 42 kDA that is not seen in the wildtype which represents *yqhD* from pMTL90882-IsoB16, no band can be seen at around 61 kDa that is expressed in the plasmid carrying *M. parvus* and not in the wildtype though this may be due to overlap from an endogenous band already present around that size. Similarly in *M. capsulatus* (Bath) *KivD* cannot be seen though *yqhD* can in the respective plasmid carrying strain and non-transformed strain. Isolate 6 harbouring pMTL90882-IsoB16 does not express genes the size of either KivD or *yqhD*. For *M. capsulatus* (Bath) expressing

pMTL90882-IsoB17 there is a large band at around 40 kDA where the expected *ADH6* would be though is also present in the wildtype, also *ilvD* cannot be seen at around 59 kDA.

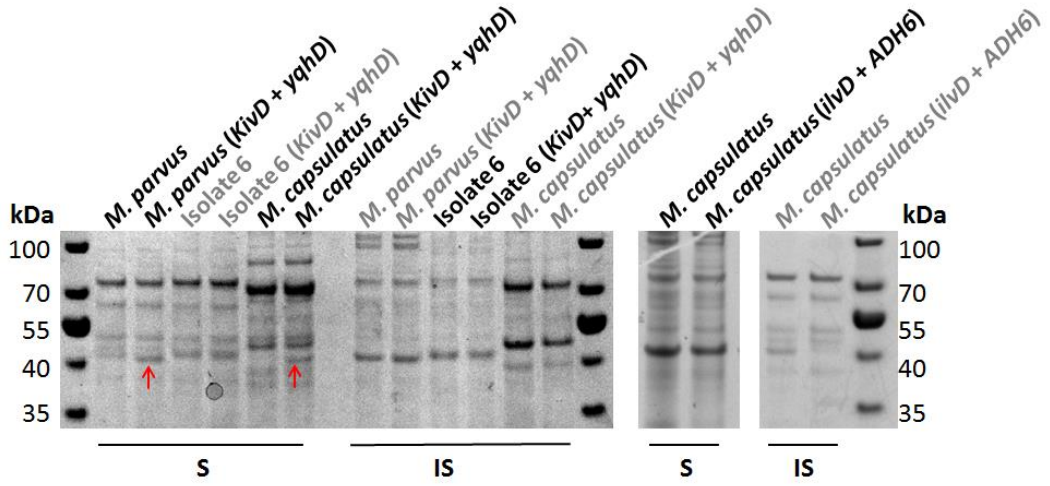


Figure 63 - SDS-PAGE of cell lysate of methanotrophic bacteria expressing heterologous isobutanol operon. S, Soluble fraction. IS, Insoluble fraction. pMTL90882-IsoB16 (Recombinant genes include *KivD* (60.95 kDA) and *yqhD* (42.10 kDA)). pMTL90882-IsoB15 (Recombinant genes include *KivD* (60.95 kDA) and *yqhD* (42.10 kDA)). pMTL90882-IsoB17 (Recombinant genes include *ilvD* (58.85 kDA) and *ADH6* (39.62 kDA)). Red arrow, heterologous yqhD expression.

Figure 64 shows that after the 72 hour feeding experiment PCR amplification of the 3.487 Kb fragment of the isobutanol operon could no longer be detected from isolate 6 suggesting the pMTL90882-IsoB16 plasmid was lost or the PCR binding sites no longer amplify product due to a possible recombination event.

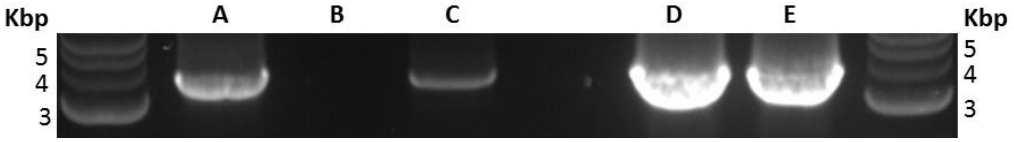


Figure 64 – Colony PCR of cell pellet obtained from the feeding experiment sample. A, *M. parvus* pMTL90882-IsoB16. B, isolate 6 pMTL90882-IsoB16. C, *M. capsulatus* (Bath) pMTL90882-IsoB15. D, pMTL90882-IsoB16 plasmid only. E, pMTL90882-IsoB16 plasmid only.

Further experimentation analysing isobutanol production over time and further assessing if isolate 6 could produce isobutanol was performed. As seen in Figure 65, *M. parvus* harbouring pMTL90882-IsoB16, was assessed for isobutanol production at multiple time points over 72 hours. It can be seen that isobutanol accumulates in the

media overtime and the concentrations is dose dependant increasing up to 1 g/L 2-ketoisovalerate and decreases to 1.5 and 2 g/L in contrast to Figure 62.

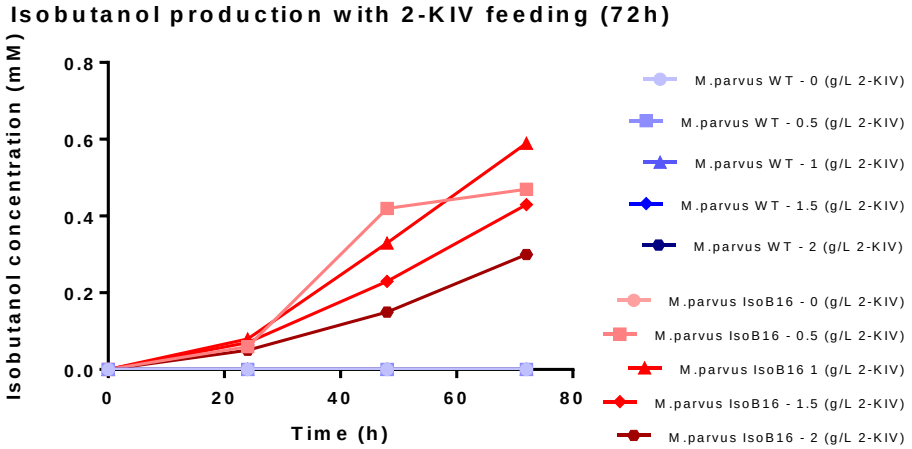


Figure 65 - Isobutanol productions of *M. parvus* harbouring and not harbouring plasmids for the heterologous pathway for isobutanol production with 2-ketoisovalerate feeding. IsoB16, pMTL90882-IsoB16 plasmid which contains *KivD* and *yqhD* expressed by PphaC. WT, Wild Type.

Isolate 6 was repeated in a similar manner to Figure 65 and in contrast to Figure 62 isobutanol was produced in all fed cultures with isolate 6 containing pMTL90882-IsoB16 though the strain not harbouring the plasmid did not. No clear dose dependant effect can be observed with the feeding and all aside from the 0 g/L 2-ketoisovalerate feeding had similar rates of isobutanol production with isolate 6 harbouring the plasmid.

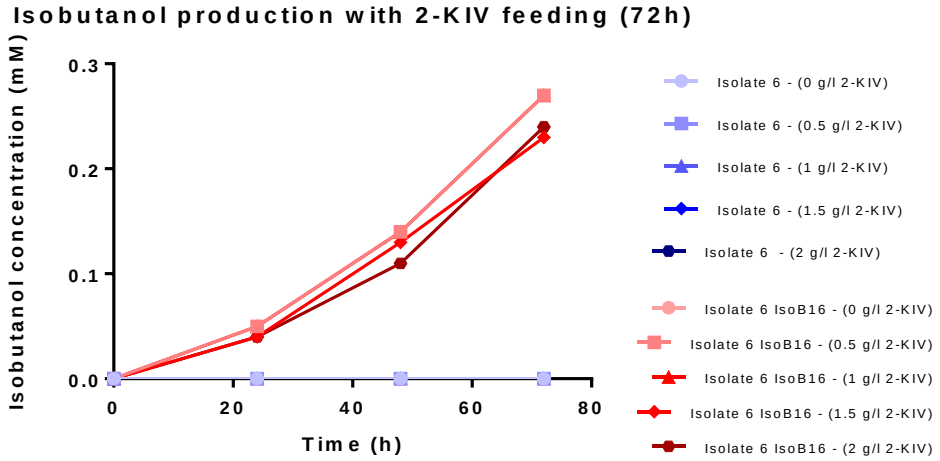


Figure 66 - Isobutanol productions of isolate harbouring and not harbouring plasmids for the heterologous pathway for isobutanol production with 2-ketoisovalerate feeding. IsoB16, pMTL90882-IsoB16 plasmid which contains *KivD* and *yqhD* expressed by PphaC. WT, Wild Type.

As seen previously in Figure 64 colony PCR indicated that isolate 6 lost the plasmid for the production of isobutanol. In the repeated feeding experiment seen in Figure 66, Figure 67 shows that the plasmid was retained from 24 hours to 72 hours in both *M. parvus* and isolate 6. The negative results at 0 hours is likely due to the low biomass available for PCR due to the OD600 being set to 0.05.

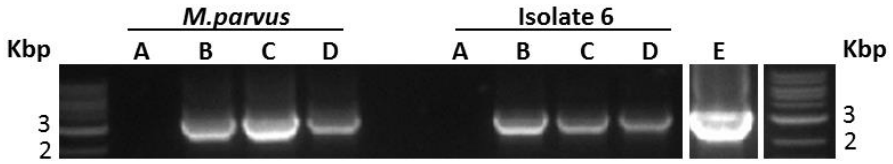


Figure 67 – Colony PCR to assess the retention of pMTL90882-IsoB16 of spun down cell pellet from the 2-ketoisovalerate feeding experiment taken at various time points. A, 0 hours. B, 24 hours. C, 48 hours. D, 72 hours. E, pMTL90882-IsoB16 plasmid.

6.6 Discussion

6.6.1 *Isobutanol production*

Overall isobutanol was produced in both established type strains and an isolate strain. Isobutanol production was undetectable using methane alone though with the addition of the precursor 2-ketoisovalerate isobutanol was detected.

The addition of 2-ketoisovalerate strongly inhibited the growth of all methanotrophs. The isobutanol precursor 2-ketoisovalerate is also a precursor of the valine biosynthesis pathway. One explanation to why the precursor is so inhibitory is that, as seen in *E. coli* K-12, valine inhibits Acetolactate synthase (as seen in Figure 68) in order to control levels of valine and which also catalyses the conversion of α-Ketobutyrate to α-Acetohydroxybutyrate. This feedback inhibition of Acetolactate synthase (specifically *ilvBN* and *ilvH*) accumulates α-Ketobutyrate to toxic levels within the cell which exerts a toxic effect [147]. Either the high concentration of 2-ketoisovalerate alone or cytoplasmic accumulation of α-Ketobutyrate are the most likely causes of growth inhibition [148].

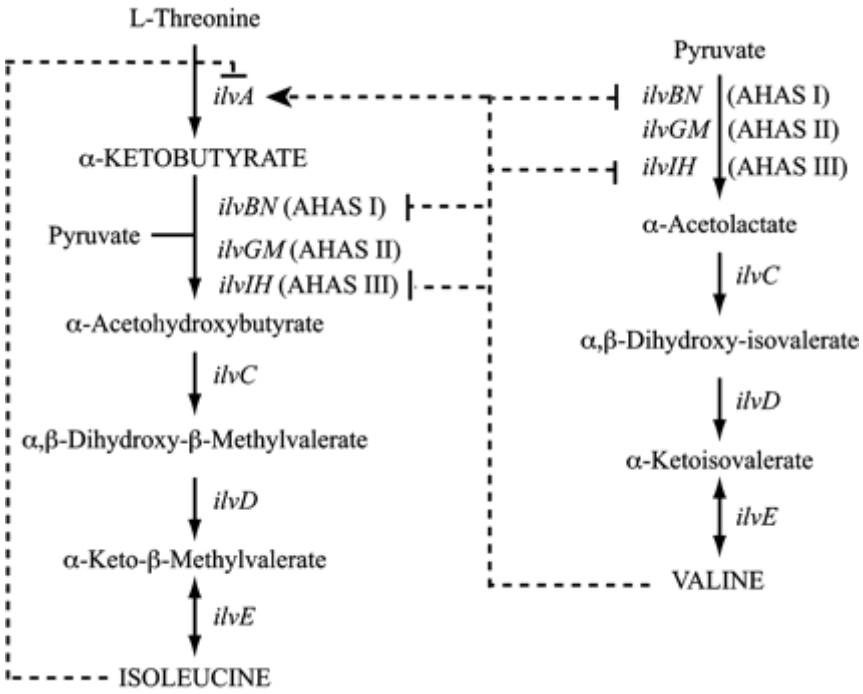


Figure 68 - Isoleucine and valine biosynthesis pathways. (A) *ilvBN*, *ilvGM* and *ilvIH* code for the heterodimeric acetohydroxy acid synthase isozymes AHAS I, AHAS II, and AHAS III, respectively. AHAS I and AHAS III are inhibited in the presence of high amounts of valine, whereas isozyme AHAS II is valine insensitive. Regulations by valine and isoleucine are shown by dashed lines terminated by an arrow for activation or by a vertical line for inhibition [149].

No wildtype strains produced isobutanol within the feeding experiment which suggests that the endogenous keto-acid decarboxylase and alcohol dehydrogenase of each isolate cannot catalyse the reaction to isobutanol (or at least to detectable levels). This implies either native expression is too low or the specific product profile does not produce isobutanol. Differing product profile of isozymes, shown by Atsumi *et al* [65], is seen in a comparison of keto-acid decarboxylases heterologously expressed in *E. coli*. Along with ADH2, It is seen that overexpression of a number of keto-acid decarboxylases including: KivD (*Lactococcus lactus*), Aro10 (*Sacchromyces cerivisiae*), Pdc6 (*Sacchromyces cerevisiae*), and Pdc (*Clostridium acetobutylicum*) produced 5242, 2094, 260 and 75 µM of isobutanol respectively as some keto acid decarboxylases preferentially produced isobutanol as opposed to other compounds [72].

Lack of isobutanol production in the wildtype feeding experiment and the production of isobutanol within the strains overexpressing heterologous enzymes suggests that the two overexpressed enzymes, keto-acid decarboxylase and alcohol dehydrogenase, are expressed and responsible for the isobutanol production. This cannot be confirmed though due to the SDS-PAGE result remaining inconclusive. One possible explanation for poor expression of individual enzymes could be the RBS used though both yqhD enzymes were clearly expressed using the untested RBSs and, in contrast, the RBS responsible for translation of the keto-acid decarboxylase is known to be adequately functional as it was used to translate the EYFP transcript in 5.5.3.1. Further explanation could be codon optimisation of the genes though the codon optimised genes, *KivD* and *ADH6*, were the unconfirmed genes and *yqhD* was non- codon optimised and was clearly expressed.

In the patent described in [65] *M. capsulatus* expressing *KivD* (*M. capsulatus*) and *ADH6* (*S. cerevisiae*) with the addition of 2 g/L ketoisovalerate cultured in 125 ml shake flasks produced 1.8 mM isobutanol. Compared to this study in which *M. capsulatus* harbouring pMTL90882-IsoB17 that produced no isobutanol at 2 g/L and 0.117 mM at 1 g/L feeding with the same enzymes. The difference between these experiments was that a weak *E. coli* promoter named J23115 (though it is not known what relative strength it operates at in *M. capsulatus*) was used in the patent. Specific levels of expression may play a critical role in the level of isobutanol produced or this could maybe further suggest the keto-acid decarboxylase was not sufficiently expressed as indicated by SDS-PAGE. A way to confirm expression would be to histidine tagging to purify the enzyme from the proteome or possibly use anti-His-tag antibodies for detection. Comparison of pMTL90882-isoB15 containing *kivD* (*L. lactis*) and *yqhd* (*E. coli*) and pMTL90882-IsoB17 in *M. capsulatus* , from this data, suggests IsoB17 works slightly better. There are many reasons for this including enzyme kinetics, protein folding, and thermostability at 45°C.

It was initially seen that isolate 6 didn't produce isobutanol though PCR suggests the plasmid was lost and a subsequent repeat of the experiment showed that isolate 6 did produce isobutanol when harbouring pMTL90882-IsoB16. Comparatively *M. parvus* containing pMTL90882-isoB15 containing *kivD* (*L. lactis*) and *yqhd* (*E. coli*) performed better than *M. capsulatus* harbouring either plasmids. It is difficult to draw a comparison due to variation between native flux in host organisms, difference in promoter strengths, and difference in growth temperatures.

6.6.2 *Future work*

Initial metabolic engineering confirms isobutanol can be produced if sufficient ketoic acids are supplied by the valine biosynthesis pathway. In order to do this further

engineering is required in which overexpression of acetolactate synthase, keto-acid reductoisomerase and dihydroxy-acid synthase need to be overexpressed. This will theoretically improve the flux from the key metabolite pyruvate to 2-ketoisovalerate and then, as described above, isobutanol. To further confirm the correct expression of all heterologous enzymes future enzymes will be histidine tagged so expression can be more clearly observed.

7 *Discussion*

The viability of the conversion of waste methane to liquid transportation fuel using environmentally isolated methanotrophs was explored. The approach taken was to isolate methane consuming stains from ecosystems known to produce high amounts of methane, characterisation of the resulting strains and molecular tools for metabolic engineering was developed for biosynthesis of isobutanol.

7.1 Isolation of methanotrophic bacteria

Initially to isolate pure methanotrophic cultures a classic dilution plating method adapted from [41] and miniaturised extinction dilution from [38] was used. This approach was taken as the classic dilution plating method has many examples of successful isolations of methanotrophs [16, 28, 106, 150], in particular the method described by Auman *et al*, isolated multiple species which was the desired outcome. Initially limited success was seen due to the tight syntrophic bonds of methanotrophs and heterotrophs when cultivated on agar plates. To circumvent this issue the method described by Hoefman *et al* [38] was used as the study described a miniaturized method that when performed in parallel with traditional dilution plating, achieved 22 isolates compared to 0 from traditional plating methods. Upon using the improved method, the success of the publication was not repeated as no pure cultures were obtained. Following this the removal of vitamins and passaging from liquid to solid was seen to be a very effective means of obtaining pure cultures. Removing vitamin stock in the enrichment though was not necessarily shown to aid purification. General observations such as dNMS being a more reliable media to support growth (in freshwater samples) and the effective use of PCR for screening were observed.

For future experiments, now a reliable purification protocol has been established, enrichment conditions would be altered as to aid the selection of beneficial industrial traits. Industrial traits such as tolerance to inhibitory compounds found in the waste methane such as H_2S found in both natural gas (known as sour gas, containing 2~70% H_2S [151]) and bio-gas (50-10,000 ppm [152]). Other traits such as salt tolerance can provide opportunities to use sea water for fermentation [153] and also selects for ectoine [119], a high value compound, produced by halotolerant methanotrophs.

7.2 Characterisation of methanotrophic bacteria

The genera of each isolate was identified by 16S rRNA sequencing suggesting five *Methylocystis*, one *Methylocaldum* and one *Methylococcus* species had been obtained. The methods described by J.P Bowman [43] for description of novel methanotrophic bacteria was used as a framework for characterisation. Overall most traits fit well with existing taxonomic nomenclature. Interesting novel traits such as fimbriae were observed in isolate 3* which have not been characterised in the genus and phenotypic characterisation is rare. Isolate 01, 03 and 6 were seen to possess facultative methanotrophic metabolism being able to grow on ethanol and acetate seen in species within the genus such as *M.bryophila*. Isolate 10 on a phenotypic basis was highly novel to the Methylocaldum genus. On a genotypic basis the 16S rRNA sequence was 99.93% homologous in a pairwise alignment to *M.gracile* which differs phenotypically by lack of motility, contains carbon storage granules, is able to use glutamine and ornithine nitrogen sources, and does not exhibit a thin rod morphology. Most isolates obtained did not identically conform to a specific species definition. For example isolate 01 and 03 identified as *M.echinoides* had the differentiating characteristic of PHB production as *M.echinoides* does not produce any whereas isolate 01 and 03 produced 0.28 and 1.63% CDW PHB. Isolate 3* and 6 with identical

16S rRNA to *M. rosea* could grow on ethanol which *M. rosea* cannot utilise as a sole carbon source. Overall the species were identified as: Isolate 01; *Methylocystis* sp. , isolate 03; *Methylocystis* sp. , isolate 3*; Methylocstis nov sp., isolate 6; *Methylocystis* SB2, isolate 10; *Methylocaldum* nov sp. , isolate 12; *Methylocystis parvus* and isolate 14; *Methylococcus capsulatus*.

Simultaneous cultivation of all 9 isolates was practically challenging as inoculating from plate into liquid for characterisation was not consistent so some experiments were performed on selected isolates. In addition to this fungus would occasionally contaminate agar plates during long term storage. Fungi contamination was reduced by sterilisation of anaerobic jars using 70% industrially methylated spirits and drying condensation regularly. Some experiments were performed specific for certain isolates to find novel traits not seen in the Genus, for example motility in isolate 10. To improve high-throughput analysis multiwall plate cultivation such as the method used for assessing growth on a variety of nitrogen sources proved to be successful.

Phenotypic traits with implications on metabolic engineering such as production of intracellular carbon storage granules and carbon assimilation pathways were characterised. Further essential traits for metabolic engineering such as end product tolerance of isobutanol were also characterised.

Overall adequate characterisation was performed and novel phenotypes were observed and a fundamental understanding of each isolate was obtained to use as a microbial chassis for metabolic engineering.

7.3 Characterisation of molecular tools for methanotrophic isolates

The expansion of the basic molecular toolbox for the isolates followed existing literature including use of plasmid replicons, promoters and allelic exchange. Use of broad host range vectors with replicons shown to be functional in methanotrophs such as pBBR1 and Col E1 were tested for functionality. Initial conjugations using a variety of established methods such as use of nitrocellulose paper [138], 1:5 donor:recipient ratios [154] and a variety of alternative carbon sources for mating were used to limited success (Data not shown). The use of 1:2 donor:recipient ratios, 24 hours mating period and yeast extract resulted the most reliable transformations. The clear function of EYFP facilitated the characterisation of a number of promoters which provided a minimal toolkit for metabolic engineering.

Inducible promoters proved to be less successful than constitutive promoters as only isolate 14 had a functional inducible promoter. The genome sequence of both isolates was assessed for possible inducible promoters but no suitable candidates were observed. Using endogenous inducible systems can lead to consumption of effector molecule which can often cause a drop induction. Future approaches would be to construct a synthetic inducible system using established constitutive promoters driving the expression of EYFP and the repressor/activator and incorporate a respective operator region within the promoter controlling expression of EYFP.

The attempt to establish allelic exchange was not successful and was discontinued due to time constraints.

7.4 Metabolic engineering of isobutanol biosynthesis in methanotrophic bacteria

The method used rationally select enzymes to compare existing literature and experiment with potentially superior enzymes for the production of isobutanol. This approach showed that with feeding of 2-ketoisovalerate isobutanol could be produced in established and isolate strains. The keto-acid decarboxylase and alcohol dehydrogenase used in comparison with existing data present in [65] showed a lower titre than predicted. The other gene combination used was rationally selected due to the slow pipeline of cloning and transforming the methanotrophic strains. Ideally a combinatorial approach of the whole pathway would be done to establish optimum expression strength.

Combinatorial approaches require high-throughput techniques to implement the vast possible combinations of genes and promoters. Following this high throughput analytical methods are needed to assess the resulting strains. Some end products favour this approach as the yield can be quantified colorimetrically (as seen in the carotenoid example [54]) or increasingly biosensors have been employed [155], both methods are not compatible with isobutanol. A possible solution to this is to employ high-throughput methods of cloning, transformation and analytical methods relevant to methanotrophs and isobutanol. Current methods used in this study including multi-fragment assembly followed by conjugation, screening, cultivation and finally quantification of desired product.

A recent example of a more high-throughput modular approach using methanotrophs was seen in [156] in the production of lactate using *M. buryatense*. This approach used constructed a combination of promoter RBS combinations by taking a tetracycline inducible promoter, a cumate inducible promoter and a constitutive methanol

dehydrogenase promoter each separately fused to one of 4 RBS sequences generated using an RBS calculator. All 12 combinations were used to express lactate dehydrogenase (*Lhldh*) to produce lactate. Varying the genetic parts and induction concentrations outlined the difficult to predict nature of molecular tools within the host. It was seen that increase in inducer concentration of tetracycline from 0.1 to 0.5 and finally 1.0 µg/ml had little effect on increasing the lactate titre. The variation in RBS had a significant impact on the overall lactate production though the predicted highest strength RBS did not result in the highest lactate formation. In addition to this a particular RBS that would perform well with a certain promoter would perform poorly with a different one.

The large array of strains was able to be characterised by the use of a small scale high-throughput batch culturing system. This system used 27 ml Hungate tubes with 2-6 ml cultures that increased the growth rate of *M. buryatense* most likely due to high headspace to culture ratios and more efficient gas to liquid mass transfer. The Hungate tube is much more space efficient than the traditional serum bottle and so is more practical for a high-throughput approach.

This approach taken in *M. buryatense* was successful in improving the yield of lactate generated via lactate dehydrogenase. Using a multi-step heterologous pathway adds another layer of complexity as bottlenecks within the metabolic flux in the pathway could cause low titre and cytoplasmic toxicity. To balance the flux throughout the pathway fine tuning of individual steps in order to balance flux [157, 158]

7.5 Future direction

The primary focus of future investigation is to improve the yield of isobutanol. To forgo the need of pre-cursor feeding so isobutanol is derived from methane overexpression of the endogenous pathway to 2-ketoisovalerate including acetolactate synthase, ketol-acid reductoisomerase and dihydroxy-acid dehydratase would be attempted. To improve the expression of the heterologous pathway a variety of RBS will be used in conjunction with the isobutanol biosynthetic pathway. Genetic knockout manipulations would also be revisited to modify native flux to further improve isobutanol titre.

A recent approach used a combinatorial approach to express a four step pathway catalysed by three heterologously expressed enzymes to produce crotonic acid in *M. buryatense* [159]. The approach taken was a rational selection of enzymes expressed with a methanol dehydrogenase promoter and a variety of synthetic RBS (generated using an RBS calculator). Four combinations of orders from transcriptional start site using a fixed RBS order was tested to see the variation of transcription and interference in translation. Using cell lysate of the organism expressing the four combinations of heterologous expression enzyme assays were performed on each step of the reaction by adding precursor and monitoring substrate/product concentrations using absorbance measurements. Enzyme kinetics varied greatly from 0.05-0.1 to 20 µmol/mg/min between each enzyme though within each arrangement the change in activity varied to a maximum of 2-fold. Through all combinations titres were consistent ranging from 60-70 mg/L of crotonic acid production. Further improvement of the titre was achieved with overexpression of acetyl-CoA synthase that recycled acetate back to acetyl-CoA which improved crotonic acid production. The results suggest specific enzymes orders with activity orders of magnitude lower than other

enzymes, such as 3-hydroxybutyryl-CoA dehydrogenase, were rate limiting steps. Efforts to replace the rate limiting enzyme with an isozyme to improve kinetics resulted in a similar titre. This approach allowed a combination of medium throughput combinatorial approach of a multi-step pathway with an analytical method for guided pathway de-bottlnecking that could be applied to the production of isobutanol in methanotrophs.

7.6 Overall outcome

Overall all project aims were successfully achieved. A number of methanotrophic microbial chassis were obtained. The isolates characterised showed to have diverse and novel characteristics and originated form a range of genera. Molecular tools were established and a heterologous isobutanol biosynthetic pathway was expressed in a methanotrophic organism. Isobutanol was produced in an isolate and established strains with pre-curser feeding.

As seen in this study end to end metabolic engineering of methanotrophic isolates is achievable albeit time consuming. General limitations include slow growth and transformability though effective means to circumvent these problems such as multi-well plate cultivation, seen in this study, and growth and transformation methods such as use of Hungate tubes and electroporation are feasible for improving the pipeline. Bioconversion of methane to liquid transportation fuel remains a promising approach for fuel production and methane mitigation though significant strain engineering and chassis optimisation are required.

8 *References*

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Supplementary Material

Colony screening during enrichment isolation of methanotrophic bacteria

PCR was used to identify potential methanotrophic colonies during enrichment isolation experiments. The identified colonies can be seen below.

Table 24 – Blast (NCBI) identification of methane enriched colonies based on 16S RNA sequence. Samples include: Cow manure fresh (CMF), cow manure old (CMO), surface slurry (SS). Numbers after each sample denote replicate environmental samples. Enrichment conditions are: 50:50 methane:air 10µM $CuSO_4$ (A), 80:20 methane:air 10 $CuSO_4$ (B), 50:50 methane air 0 µM $CuSO_4$ (C), and 80:20 methane:air 0 µM $CuSO_4$ (D).

Sample	Condition	BLAST search of predicted organism	Query cover	Identification
CMF 1	*A*	*Mucilaginibacter paludis* DSM 18603	93%	93%
CMF1	*C*	*Mucilaginibacter paludis* DSM 18603	93%	92%
CMF 2	*A*	*Sphingobium japonicum* UT26S	93%	95%
CMF 2	*B*	*Acinetobacter calcoaceticus* PHEA-2	73%	98%
CMF 3	*C*	*Mucilaginibacter paludis* DSM 18603	95%	92%
CMF 3	*D*	*Pedobacter saltans* DSM 12145	90%	92%
CMO	*C*	*Mucilaginibacter paludis* DSM 18603	96%	91%

Sample	Condition	BLAST search of predicted organism	Query cover	Identification
CMO	*C*	*Mucilaginibacter paludis* DSM 18603	93%	92%
SS	*C*	*Pedobacter saltans* DSM 12145	92%	92%
SS 2	*B*	*Mucilaginibacter paludis* DSM 18603	96%	92%
BS	*C*	*Acinetobacter baumannii* ATCC 17978	94%	97%
BS	*A*	*Mucilaginibacter paludis* DSM 18603	91%	92%
FS	*B*	*Brevundimonas subvibrioides* ATCC 15264	93%	98%
ADS 1	*B*	*Bacillus* sp. 1NLA3E	93%	98%
ADS 1	*D*	*Streptomyces albus* J1074	95%	99%
ADS 2	*B*	*Brevibacillus brevis* NBRC 100599,	92%	99%
ADS 2	*D*	*Sphingobium* sp. SYK-6,	95%	93%
ADS 3	*D*	*Brevibacillus brevis* NBRC 100599,	93%	99%
ADS 3	*A*	*Brevibacillus brevis* NBRC 100599,	93%	99%

Table 25 – Blast (NCBI) identification of methane enriched colonies based on 16S RNA sequence. Samples include: Cow manure fresh (CMF), cow manure old (CMO), surface slurry (SS). Numbers after each sample denote replicate environmental samples. Enrichment conditions are: 50:50 methane:air 10µM $CuSO_4$ (A), 80:20 methane:air 10 $CuSO_4$ (B), 50:50 methane air 0 µM $CuSO_4$ (C), and 80:20 methane:air 0 µM $CuSO_4$ (D). The presence (+) or lack (-) of pMMO and sMMO are also included which was assessed by colony PCR.

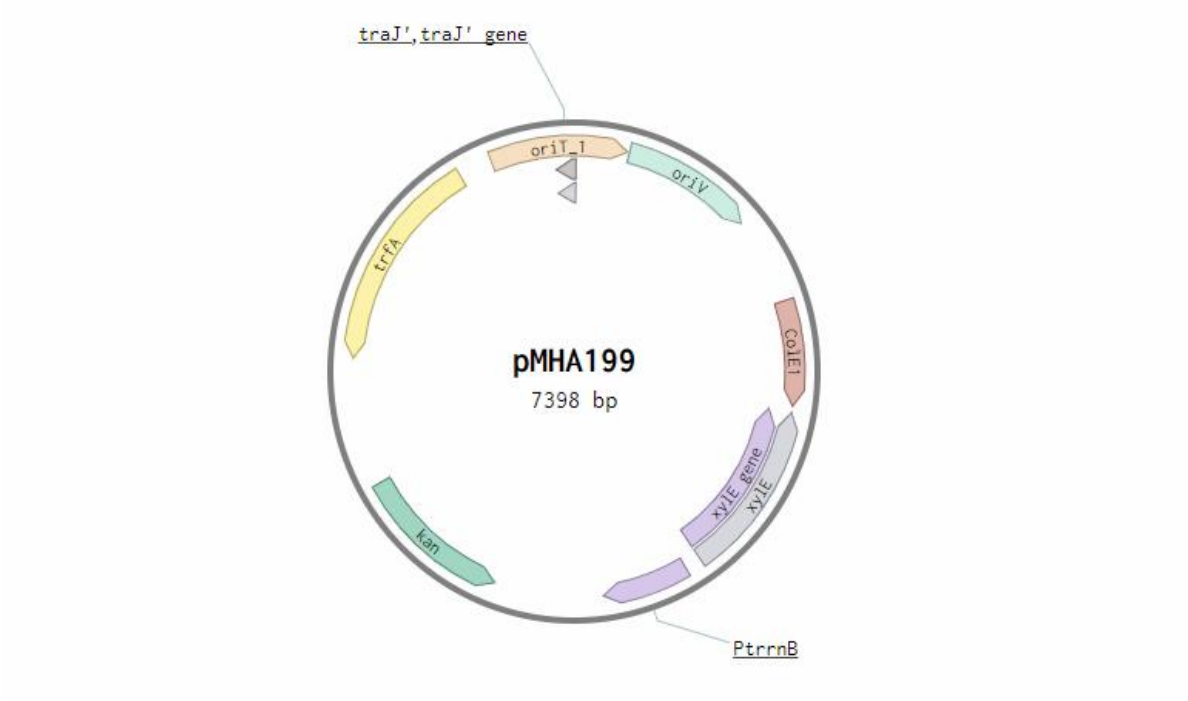
Sample	Condition	Organism	pMMO	sMMO	Query cover	Identification
ADS	D	*Brevibacillus brevis*	-	-	98%	99%
CMO	C	*Pedobacter saltans* DSM 12145	+	-	98%	88%
CMO	A	*Pedobacter saltans* DSM 12145	-	-	97%	87%
CMO	A	*Pseudoxanthomonas suwonensis*	-	-	97%	97%
BS	D	*Niastella koreensis* GR20-10,		+	96%	95%
CMO	B	*Riemerella anatipestifer* DSM 15868	+	+	88%	88%
BS	C	*Niastella koreensis* GR20-10,	+	-	97%	94%
BS	C	*Niastella koreensis* GR20-10,	-	-	97%	94%
CMF1	B	*Mucilaginibacter paludis* DSM 18603		-	97%	92%
CMF1	B	*Mucilaginibacter paludis* DSM 18603	+	-	99%	91%
SS	C	*Pedobacter saltans* DSM 12145	+	-	96%	92%
SS	C	*Niastella koreensis* GR20-10,	-	-	96%	94%
SS	B	*Niastella koreensis* GR20-10,	-	-	78%	84%
SS	B	*Niastella koreensis* GR20-10,	+	+	99%	95%
SS	D	*Niastella koreensis* GR20-10,	-	-	97%	93%
SS	D	*Niastella koreensis* GR20-10,	-	-	97%	85%
BS	A	*Stenotrophomonas maltophilia* K279a	+	-	96%	99%
BS	A	*Mucilaginibacter paludis* DSM 18603	-	-	81%	84%

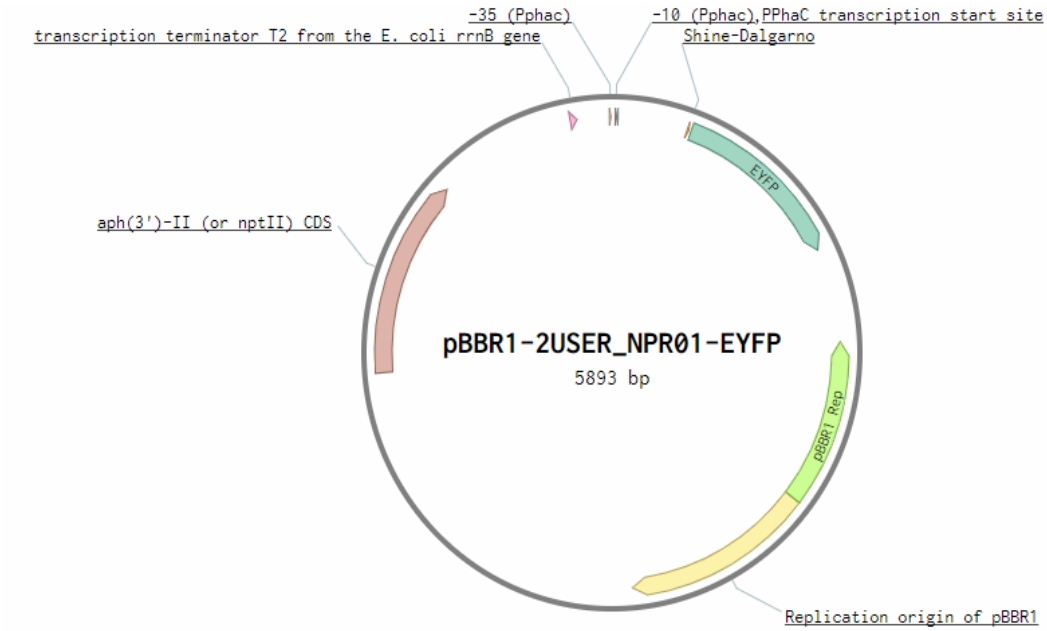
Sample	Condition	Organism	pMMO	sMMO	Query cover	Identification
CMF1	C	*Mucilaginibacter paludis* DSM 18603	-	-	97%	92%
CMF1	C	*Chitinophaga pinensis* DSM 2588	+	-	96%	97%
SS	A	*Niastella koreensis* GR20-10,	-	+	98%	93%
SS	A	*Rhodoferax ferrireducens* T118,	-	-	96%	96%

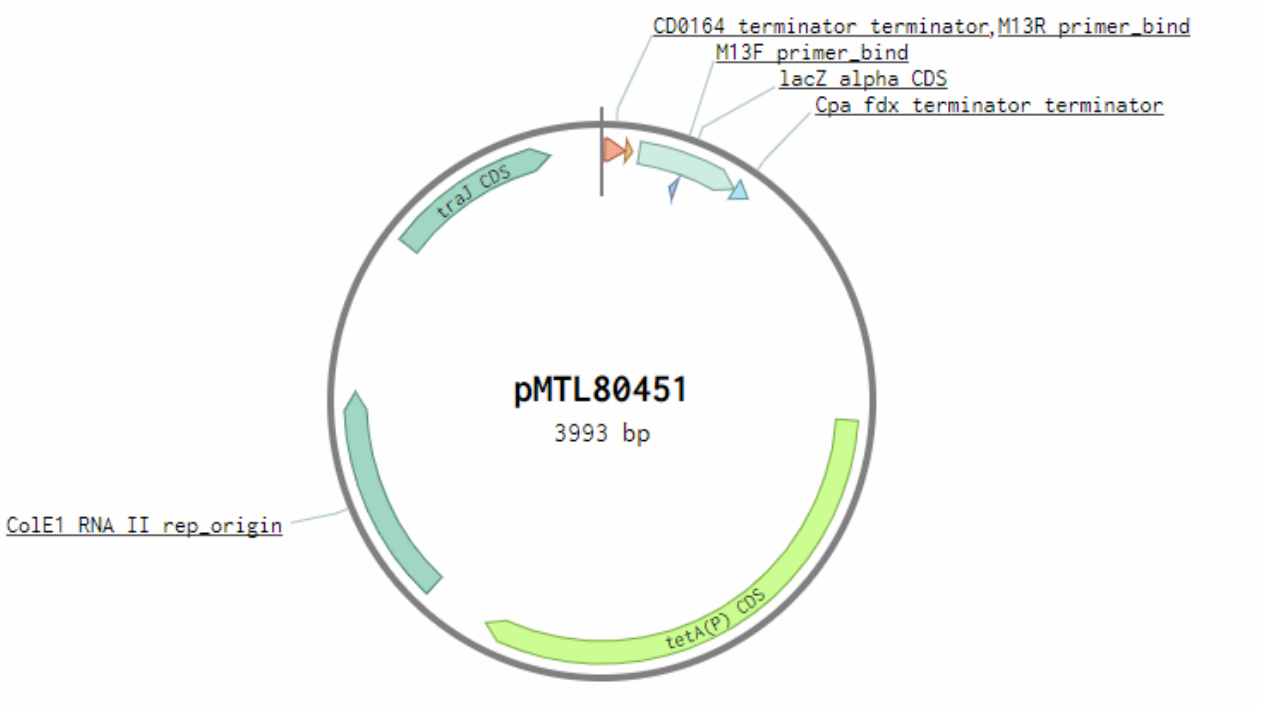
Table 26 - Blast (NCBI) identification of methane enriched colonies based on 16S RNA and pMMO sequence. Samples include: surface slurry (SS), bottom slurry (BS). Numbers after each sample denote replicate environmental samples. Enrichment conditions are: 50:50 methane:air 10µM $CuSO_4$ (A), 80:20 methane:air 10 $CuSO_4$ (B), 50:50 methane air 0 µM $CuSO_4$ (C), and 80:20 methane:air 0 µM $CuSO_4$ (D).

Sample	Condition	Primer	Blast Description	Query cover	Identification
SS1.	A	pMMO	*Methylococcus capsulatus* str. Bath	94%	83%
		16S Universal	*Haliscomenobacter hydrossis* DSM 1100	79%	84%
SS1	B	pMMO	*Methylocystis* sp. SC2	95%	95%
		16S Universal	*Niastella koreensis* GR20-10	92%	94%
SS1	C	pMMO	*Methylocystis* sp. SC2	91%	93%
		16S Universal	*Methylovorus* sp. MP688	89%	76%
SS1	A	pMMO	*Methylococcus capsulatus* str. Bath	95%	83%
		16S Universal	*Methylococcus capsulatus* str. Bath	96%	91%
BS1	D	pMMO	*Methylocystis* sp. SC2	91%	92%
		16S Universal	*Niastella koreensis* GR20-10	96%	93%

Key plasmid schematics



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M13F primer_bind
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Cpa fdx terminator terminator
traJ CDS
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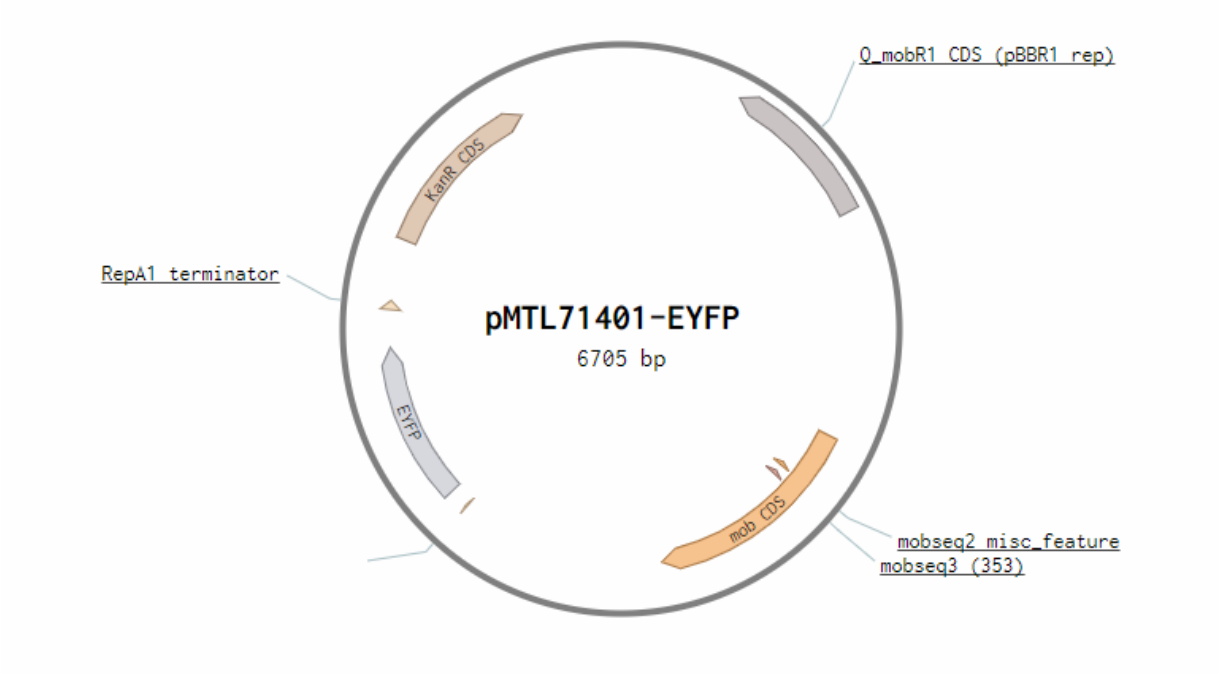
tetA(P) CDS


Q_mobR1 CDS (pBBR1 rep)
KanR CDS
RepA1 terminator
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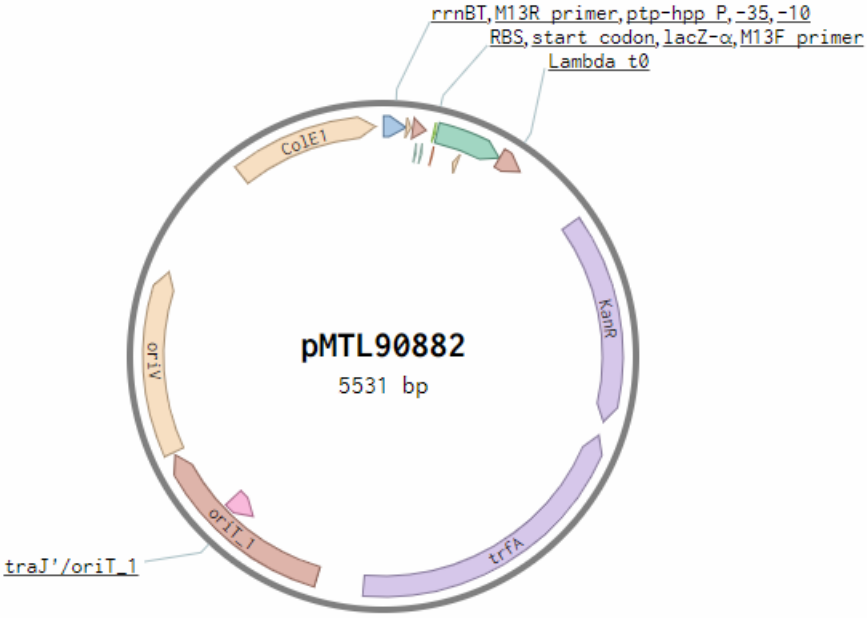
6705 bp
EYFP
mob CDS
mobseq2 misc_feature
mobseq3 (353)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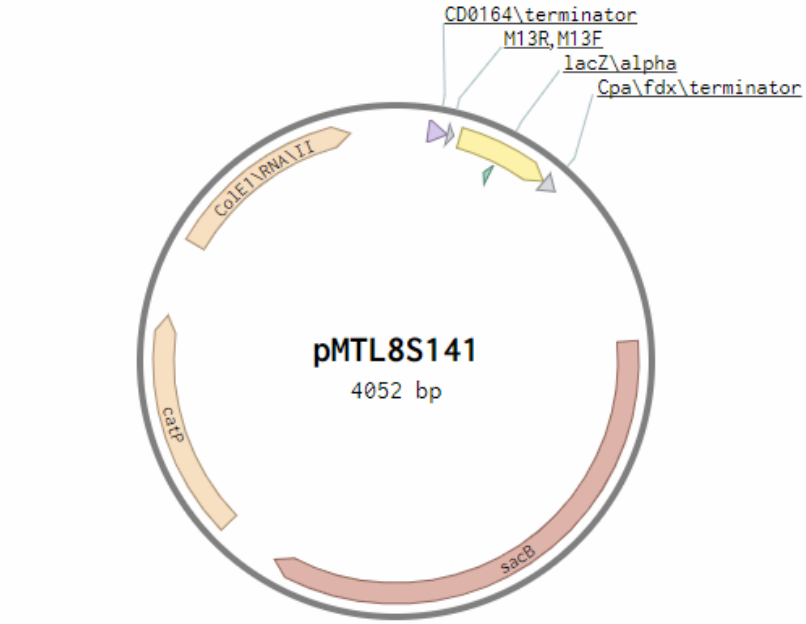
traJ'/oriT_1
trfA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Cpa\fdx\terminator
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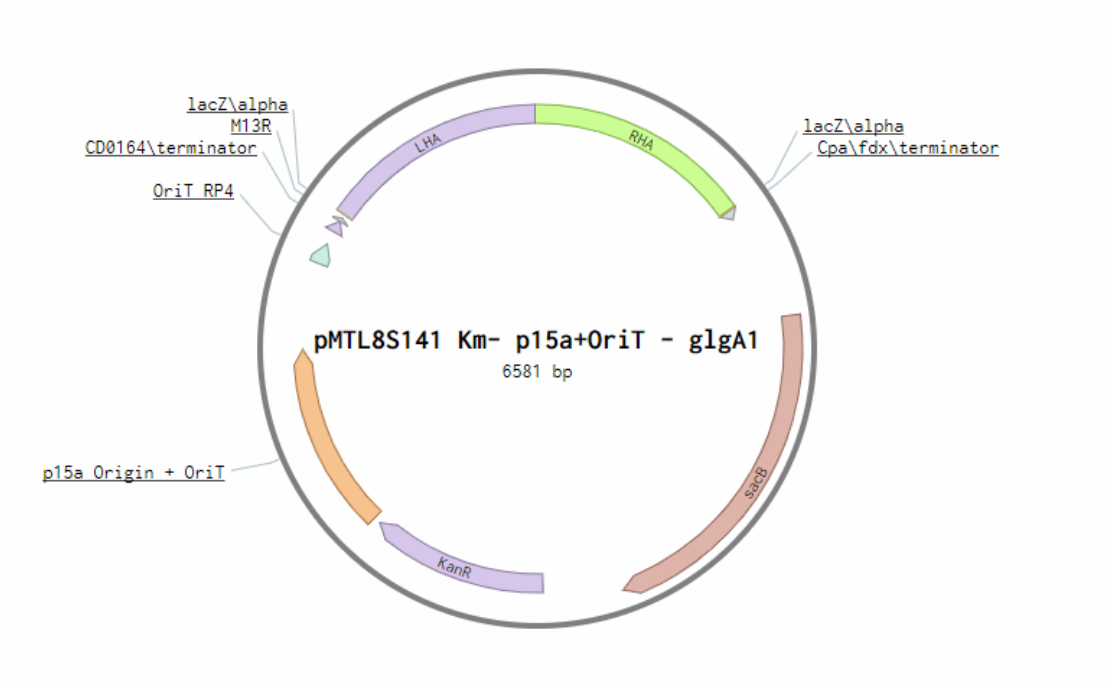
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Cpa\fdx\terminator
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sacB
KanR