

Isolation, characterisation and development of genetic tools for methane-oxidising bacteria

Bashir Lawal Rumah, MSc

Thesis submitted to the University of Nottingham for the degree of Doctor of
Philosophy

31st of October, 2018

For Mummy, ok and Daddy

Declaration

The work presented in this thesis is my own. No part has been submitted for another degree in the University of Nottingham or any other institute of learning. Except Isolate 7 (*Methylomonas* species); Isolate 9 (*Methylococcus capsulatus*) and Isolate 14 (*Methylococcus capsulatus*), isolation of all other methanotrophs in this study was carried out together with Chris Stead. The Phase Contrast Microscopy and Transmission Electron Microscopy imaging was also carried out together with Chris Stead. Finally the antibiotic minimum inhibitory concentration (MIC) tests were carried out with Chris Stead.

Abstract

Methane is an important greenhouse gas (GHG) and a source of energy for heating, lighting, generation of electricity and growth of methanotrophs. In addition to balancing CH₄ levels in the environment, methanotrophs can be used to produce platform chemicals like lactate; biopolymers such as poly-3-hydroxybutyrate (PHB) and animal feed in the form of single cell proteins. To harness their industrial and environmental potential, this study was carried out to understand their physiology, biochemistry and to develop the much needed forward and reverse genetic tools in methane fixing bacteria. Nine methanotrophs were isolated using dilution to extinction methods and streaking on dilute Nitrate Mineral Salt (dNMS) agar plates. They include Isolate 01 (*Methylocystis species*) isolated from Wollaton lake; Isolate 03 (*Methylocystis species*) also isolated from Wollaton lake; Isolate 3* (*Methylocystis species*) isolated from a bog in Moseley; Isolate 6 (*Methylocystis species*) isolated from University Of Nottingham lake, Isolate 7 (*Methylococcus species*) isolated from Nottingham landfill; Isolate 9 (*Methylococcus capsulatus*) isolated from a landfill in Nigeria; Isolate 10 (*Methylocaldum species*) isolated from cow manure; Isolate 12 (*Methylocystis species*) also isolated from a bog in Moseley; and Isolate 14 (*Methylococcus capsulatus*) isolate from Roman baths in Bath. Isolate 6 and Isolate 10 are type I; Isolate 9 and 14 are type X; while Isolate 01, 03, 3*, 6 and 12 are type II methanotrophs. All the isolates were able to grow both on CH₄ and biogas. Only the type II methanotrophs were capable of accumulating PHB under nitrogen limiting conditions with Isolate 12 accumulating approximately 10 times more PHB (25.05% of dry cell weight) when grown with biogas compared to CH₄. The important characteristics, availability of PacBio genome sequence and good conjugation efficiency of Isolate 12 qualified it as the best candidate for the development of transposon random mutagenesis in methanotrophs. A Tn5 plasmid derivative (miniTn5Kan_R6K) was used to deliver the transposon, and Inverse PCR identified 36 different insertion locations of transposon suggesting unbiased transposition insertions. A suicide plasmid replicon (R6K) was used for the plasmid and its non-replicative nature was demonstrated in Isolate 12. It was the first time random transposon mutagenesis was reported in type II methanotrophs. To enable more efficient genome editing, CRISPR-Cas9 gene knockout system was attempted on four methanotrophs (Isolate 3*, 10, 12 and *M. parvus* OBBP). Gene knockout was achieved in Isolate 12 and *M. parvus* (OBBP) when DNA ligase IV gene (*ligD*) was targeted using a plasmid with guide RNA under the transcriptional control of a promoter with moderate strength and *Streptococcus pyogenes* Cas9 under the transcriptional control of a weak promoter. Up to 80% editing efficiency was achieved in Isolate 12. This is the first demonstration of CRISPR genome editing in methanotrophs.

Acknowledgements

I would first of all like to thank God for guiding me towards this path of knowledge. I would also like to thank my parents especially my mum who is the strongest person I know. All my love goes to my beautiful siblings Rahama, Jamila, Aisha, Sa'adatu, Farida, Yazid, Zayyad and Abdullahi. They make me feel blessed all the time. My gratitude goes to my supervisor Professor Nigel Minton for his guidance and insight during the course of my project. I would like to especially thank my primary supervisor Dr Ying Zhang for her supervision, support, advice and confidence she placed on me. I could not ask for a better supervisor. The Petroleum Technology Development Fund (PTDF) Nigeria played an immense role in my postgraduate studies, sponsoring my MSc and PhD degree. My appreciation goes to their management and the Nigerian government. The Isolation part of this project was conducted in collaboration with Chris Stead and Emily Harding who I both feel indebted to. I particularly want to appreciate Chris Stead who has been solidly behind and beside me exploring and unravelling the mysteries, hardship and reward of working with methanotrophs. The broad nature of this study necessitated cross disciplinary collaborations which saw me working or seeking guidance from a lot of people. Special thanks goes to Naglis Malys for his support and contribution with promoters and Erik Hanko, a very good friend who helped a lot with understanding expression systems in bacteria. Marco Garavaglia was very instrumental in my understanding of transposons and TraDIS. Craig Woods was also very helpful, especially when it came to understanding TraDIS and library assembly. I also consulted Alex Grosse-Honebrink and Rubab and Martina Pasini. Gareth Little was of immense help in analysing the Illumina and PacBio genome sequence. For my CRISPR work, I was guided by Pete Rowe, Sophie Vaud and Ines Canadas. I also want to acknowledge members of my office C51 and my lab B67, especially Dr Sam Bryan, Kathlyn Kovacs and Raj Patel who sets the lab alive. They made my PhD years very rewarding. I will use this opportunity to mention a few friends who have supported me including Bashir Mohammed, Ibrahim Ahmed, Jamal Bibinu, Abubakar Shaaban, Ahmed Wada, Hamid Tukur, Aliyu Magaji, Aminu Hayatudeen, Mohammed Ramalan and others. I would also like to mention Rupert Norman, Jen Cooke, Lorna Finch and Linzy Furness.

Table of Contents

Title.....	I
Dedication.....	II
Declaration.....	III
Abstract.....	IV
Acknowledgement.....	V
Table of content.....	VI-XI
List of figures.....	XII-XIX
List of tables.....	XX-XXII
Abbreviation.....	23
1 Chapter 1: Introduction	25
1.1 Methanotrophs and the use of methane (CH ₄) as a feedstock.....	25
1.1.1 Chemical conversion of CH ₄ such as GTL	25
1.1.2 Biological conversion of CH ₄ by methanotrophs	26
1.2 Overview of methanotrophs	29
1.2.1 History of methanotrophs.....	29
1.2.2 Phylogeny and categorisation	30
1.2.3 Isolation of methanotrophs	33
1.2.4 Methane monooxygenase	35
1.2.5 Characterisation of methanotrophs	40
1.3 Genetic tools in methanotrophs	41
1.3.1 CRISPR	45
1.3.2 Transposons	55
1.4 Aims and Objectives of study	62
2 Chapter 2: Materials and Methods.....	63

2.1	<i>E. coli</i> strains and culture conditions:.....	63
2.2	Preparation of chemically competent <i>E. coli</i> cells	64
2.3	Transforming chemically competent <i>E. coli</i> cells	65
2.4	DNA Extraction and purification.....	65
2.4.1	Plasmid miniprep	65
2.4.2	DNA recovery from gel.....	66
2.4.3	DNA purification.....	66
2.4.4	Genomic DNA extraction	66
2.4.4.1	Genomic DNA extraction using kits.....	66
2.5	Analysis of DNA	68
2.5.1	Polymerase Chain Reaction (PCR)	68
2.6	Cloning Techniques	70
2.6.1	Restriction digest of DNA	70
2.6.2	DNA blunting using Klenow fragment.....	70
2.6.3	DNA dephosphorylation using Antarctic Phosphatase.....	70
2.6.4	Agarose gel electrophoresis.....	70
2.6.5	T4 ligation.....	71
2.6.6	NEB HiFi plasmid assembly	71
2.7	DNA sequencing	71
2.7.1	Sanger sequencing	71
2.7.2	Illumina sequencing	72
2.7.3	PacBio sequencing	72
2.8	Methanotroph culture conditions.....	72
2.8.1	Methanotroph Media	72
2.9	Methanotroph growth	74
2.9.1	Culturing freeze-dried methanotrophs.....	74
2.9.2	Culturing methanotrophs from freezer stock.....	74

2.9.3	Subculturing and restreaking methanotrophs.....	74
3	Chapter 3: Isolation of methanotrophs	76
3.1	Introduction.....	76
3.2	Specific Research Aims	78
3.3	Hypothesis	78
3.4	Materials and methods	79
3.4.1	Primers	79
3.4.2	Enrichment 1	79
3.4.3	Enrichment 2	80
3.4.4	Enrichment 3	82
3.4.5	Enrichment 4	82
3.4.6	Enrichment 5	83
3.4.7	Enrichment 6	84
3.5	Results of methanotroph isolation experiments	85
3.5.1	Enrichment 1	85
3.5.2	Enrichment 2	91
3.5.3	Enrichment 3	95
3.5.4	Enrichment 4	99
3.5.5	Enrichment 5	101
3.5.6	Enrichment 6	102
3.6	Discussion	105
3.7	Conclusion	110
4	Chapter 4: Characterisation of Methanotrophs	111
4.1	Introduction.....	111
4.2	Specific Research Aims	114
4.3	Material and methods.....	115
4.3.1	Primers	115
4.3.2	Strains.....	115

4.3.3	Plasmids	115
4.3.4	Conjugation of methanotrophs.....	117
4.3.5	Determination of conjugation efficiency in methanotrophs	118
4.3.6	Electroporation of methanotrophs.....	119
4.3.7	Phylogeny of Isolates	120
4.3.8	Microscopy	120
4.3.9	Antibiotic minimum inhibitory concentration (MIC) test	121
4.3.10	Growth on alternative carbon sources	121
4.3.11	Growth on methanol.....	122
4.3.12	Tolerance test.....	122
4.3.13	Growth of methanotrophs in biogas.....	122
4.3.14	Headspace analysis	123
4.3.15	PHB analysis	123
4.4	Results	125
4.4.1	Morphology of methanotrophs	125
4.4.2	Conjugation Efficiency of Methanotrophs.....	133
4.4.3	Electroporation of methanotrophs.....	134
4.4.4	Phylogeny	135
4.4.5	Antibiotic Minimum Inhibitory Concentration (MIC) test	136
4.4.6	Growth on alternative carbon sources	137
4.4.7	Genomic DNA sequencing and analyses	138
4.4.8	Chemical tolerance for methanotrophs.....	143

4.4.9	Effect of NMS components on growth of Isolate 12	145
4.4.10	Biogas as a source of methane for methanotrophs.....	149
4.5	Discussion	153
5	Chapter 5: Transposon mutagenesis in Methanotrophs.....	162
5.1	Chapter Introduction.....	162
5.2	Specific Research Aims	165
5.3	Hypotheses.....	166
5.4	Materials and methods	167
5.4.1	Strains.....	167
5.4.2	Primers	168
5.4.3	Plasmids	169
5.4.4	Conjugation of Isolate 12 with transposon plasmid	170
5.4.5	Inverse PCR of transposon mutants.....	171
5.4.6	Efficiency of transposition.....	172
5.4.7	Mutant library preparation	172
5.4.8	Library processing	173
5.4.9	Mutant library growth conditions.....	173
5.5	Results	174
5.5.1	Transposon insertion and plasmid loss.....	174
5.5.2	Inverse PCR.....	175
5.5.3	Mutant library preparation	179
5.6	Discussion	184
5.7	Conclusion	188
6	Chapter 6: CRISPR genome editing in methanotrophs.....	189
6.1	Chapter Introduction.....	189
6.2	Specific Research Aims	193
6.3	Hypothesis.....	193

6.4	Materials and methods	194
6.4.1	Promoter probe assays	194
6.4.2	Promoters.....	195
6.4.3	Strains.....	196
6.4.4	Methanotroph strains	196
6.4.5	Primers	197
6.4.6	Plasmid design.....	199
6.4.7	M. parvus (OBBP) and Isolate 12 CRISPR-Cas9 plasmids.....	202
6.4.8	Plasmid sets for Isolate 3*	206
6.4.9	Plasmid sets for Isolate 10	206
6.4.10	Conjugation of CRISPR plasmids into methanotrophs.....	207
6.5	Results	208
6.5.1	Finding promoter candidates for CRISPR-Cas9 plasmid construction.....	208
6.5.2	Conjugation of methanotrophs with CRISPR plasmids	212
6.5.3	Isolate 3* CRISPR-Cas9 plasmid conjugation	213
6.5.4	Isolate 10 plasmid	216
6.5.5	M. parvus plasmid (P6 and P11)	217
6.6	Discussion	228
6.7	Conclusion	233
7	Chapter 7: Final Discussion	234
7.1	Isolation of methanotrophs.....	234
7.2	Random transposon mutagenesis in methanotrophs.....	239
7.3	CRISPR-Cas genome editing in methanotrophs	242
8	Conclusion.....	245
9	References	247

10	Appendix	269
10.1	pMTL90882 plasmid sequence.....	269
10.2	pMTL71401 Plasmid sequence.....	270
10.3	pMTL90882_EYFP-P3 plasmid sequence.....	270
10.4	pBBR1-2USER_NPR3-EYFP	271
10.5	Minitn5Kan_R6K plasmid sequence	272
10.6	pMTL90882_12L11 plasmid sequence	273
10.7	<i>ligD</i> gene knockout plates for <i>M.parvus</i> (OBBP)	275
10.8	<i>ligD</i> knockout sequencing	276
10.9	Materials and equipment list	277
10.1	Materials used in this study	277

List of figures

Figure 1: Appearance of Intracytoplasmic Membrane (ICM) in methanotrophs.....	27
Figure 2: Methane monooxygenase	36
Figure 3: Regulation of CH ₄ oxidation under low and high copper concentrations.....	37
Figure 4: pMMO showing its iron and Copper centres, and orientation across ICM.....	38
Figure 5: The soluble methane monooxygenase gene cluster	39
Figure 6: Simplified map of the central metabolic pathways in Group I methanotrophic.	43
Figure 7: Gene editing is promoted by DSB induced by Cas9 which can be repaired either by NHEJ or HDR.	46
Figure 8: 61 base pairs of direct repeat sequences flanking the 3'-end of <i>iap</i>	46
Figure 9: Functional classification of Cas proteins	50
Figure 10: An update of class 2 CRISPR-Cas systems including type VI which consists of Cas 13a, b and c.	51
Figure 11: Mechanism of cut and paste transposition	58
Figure 12: The Tn5 transposon.	58
Figure 13: Methods of massively parallel genome sequencing	61
Figure 14: Electrophoresis gel image of PCR products using 16S rRNA primers	85
Figure 15: Bands from PCR products for <i>pmoA</i> and <i>mmoX</i>	87
Figure 16: Fresh cow manure restreak consisting of cream and brown colonies.....	89
Figure 17: Brown colonies of CMF sample after restreaking	90
Figure 18: Appearance of pure Isolate 10 colonies	90

Figure 19: PCR of methanotroph-specific genes in gDNA extracted for growth culture of methanotrophs	92
Figure 20: Appearance of pure Isolate 6 colonies on BA plates - Nottingham lake sample.....	93
Figure 21: <i>pmoA</i> (left) and <i>mmoX</i> (right) bands for samples obtained from Wollaton lake	96
Figure 22: Appearance of Isolate 01 and Isolate 03 colonies	98
Figure 23: Appearance of pure Isolate 7 colonies isolated from landfill.....	99
Figure 24: Isolate 3* was Moseley bog gravel sediment first grown on dNMS gellan gum plates and transferred to dNMS BA plates.	100
Figure 25: Appearance of pure colonies of Isolate 14.....	102
Figure 26: <i>mmoX</i> and <i>pmoA</i> PCR screen of colonies from KS Moss colonies growing on dNMS BA no copper no vitamins plates	102
Figure 27: PCR confirmation of Isolate 9 showing it possess <i>pmoA</i> and <i>mmoX</i> genes	103
Figure 28: Appearance of pure Isolate 9 colonies	103
Figure 29: pMHA199 plasmid assembled by digesting components of pBSK1, pBSK2 pJET 1.2 plasmids.	116
Figure 30: pMTL80451 plasmid with kanamycin resistant cassette and modular restriction sites available in SBRC Nottingham culture collection.	116
Figure 31: pMTL90882 assembled from pMHA199 and pMTL80451.	117
Figure 32: Colony morphology of Isolate 01 (left) and Phase Contrast Microscopy image (right).	125
Figure 33: Colony morphology of Isolate 03 (left) and Phase Contrast Microscopy image (right).	126
Figure 34: Colony morphology of Isolate 3* and Phase Contrast Microscopy image..	126

Figure 35: TEM images of Isolate 3*	127
Figure 36: Colony morphology of Isolate and Phase Contrast Microscopy image	128
Figure 37: TEM images of Isolate 6	128
Figure 38: Colony morphology of Isolate 7 and Phase Contrast Microscopy image	129
Figure 39: Colony morphology of Isolate 9 and Phase Contrast Microscopy image	129
Figure 40: Colony morphology of Isolate 10 and Phase Contrast Microscopy image.....	130
Figure 41: TEM images of Isolate 10.....	130
Figure 42: Colony morphology of Isolate 12 and Phase Contrast Microscopy image .	131
Figure 43: TEM images of Isolate 12.....	132
Figure 44: Colony morphology of Isolate 14 and Phase Contrast Microscopy image .	132
Figure 45: TEM images of Isolate 14	133
Figure 46: Conjugation efficiency of pMTL90882 and pMTL71401 in Isolate 3*, 10 12 and <i>M. parvus</i> (OB BP). Conjugation efficiency measured as number of transconjugants per donor cell.....	133
Figure 47: Conjugation efficiency of pMTL90882 in Isolate 12 at different mating times	134
Figure 48: Electroporation efficiency in CFU per mL of recovery culture of isolated methanotrophs	135
Figure 49: Molecular phylogenetic analysis of isolated methanotrophs inferred by using the Maximum Likelihood method based on the Tamura-Nei model.....	135
Figure 50: Molecular phylogenetic analysis of isolated methanotrophs inferred by using the Maximum Likelihood method based on the Tamura-Nei model.....	136

Figure 51: Growth of Isolate 3* with CH ₄ , without CH ₄ and with acetate only as carbon source.....	137
Figure 52: Growth of methanotrophs with different CH ₃ OH concentrations. Growth in CH ₄ was used as control.....	138
Figure 53: BioRad gel doc image after running gDNA samples on agarose gel.....	138
Figure 54: Restriction modification system of Isolate 12 on genome and second mega plasmid.....	140
Figure 55: Restriction modification system of Isolate 14. Type I, II and III RM system present.....	141
Figure 56: 3HP tolerance of methanotrophs grown in NMS media.	143
Figure 57: Effect of CH ₃ OH addition on growth of Isolate 12 with CH ₄	144
Figure 58: Effect of isobutanol on growth of methanotrophs in NMS media with CH ₄	144
Figure 60: Effect of Fe-EDTA concentration in NMS on growth of Isolate 12.....	146
Figure 61: Effect of CuSO ₄ ·7H ₂ O addition to NMS on growth of Isolate 12	146
Figure 62: Effect of nitrogen source on growth of Isolate 3*. Total removal of nitrogen stopped growth in Isolate 3*. Isolate 3* grew with ammonia or nitrate acting as nitrogen source.....	147
Figure 63: Effect of CO ₂ on growth of Isolate 3* in dNMS, dAMS and dMS. The addition of CO ₂ did not stop Isolate 3* from growing in either dNMS or dAMS.....	148
Figure 64: Effect of nitrogen and CH ₄ limitation on growth peak of Isolate 3* in dNMS, dAMS and dMS	148
Figure 65 Composition of bulk gases in BG1 and BG2 measured using trace GC.	149
Figure 66: Comparison of methanotrophs grown in CH ₄ and BG1 and comparison between methanotrophs grown in CH ₄ and BG2	150

Figure 67: Percentage O ₂ consumed by methanotrophs grown in CH ₄ and BG2.....	150
Figure 68: Percentage CH ₄ consumed by methanotrophs grown in CH ₄ and BG2.....	151
Figure 69: Percentage CO ₂ produced by methanotrophs grown in CH ₄ and BG2.....	151
Figure 70: Growth of methanotrophs in CH ₄ and BG2	151
Figure 71: Percentage PHB dry cell weight of methanotrophs grown with CH ₄ and %PHB dry cell weight of methanotrophs grown with CH ₄ and biogas.....	152
Figure 72: Comparison of Isolate 3* (a) and <i>Methylocystis rosea</i> (b) morphology. Isolate 3* possesses fimbriae absent in <i>M. rosea</i>	160
Figure 73: Arrangement of transposon and transposase in transposon plasmid used in this study.....	164
Figure 74: Tn5 is composed of two insertion sequences, IS50L and IS50R.....	165
Figure 75: MiniTn5kan-R6K plasmid used for making transposon library	170
Figure 76: Transposon insertion detection using PCR amplification with primer pair Kan_IG_for and Kan_IG_rev and plasmid loss investigation using primer pair Kan_IG_for and TnpaR.....	174
Figure 77: Inverse PCR band size of genomic DNA digested with SacI and Transposon insertion location in the genome of Isolate 12.....	175
Figure 78: Inverse PCR band sizes of genomic DNA digested with EcoRI and transposon insertion locations in the genome of Isolate 12	176
Figure 79: Location of transposon insertion around different regions of Isolate 12 chromosome.....	178
Figure 80: Location of insertion around different regions of Isolate 12 mega plasmid 1.	178
Figure 81: Inverse PCR bands for miniTn5-R6K transconjugants from mating plates incubated for 3 hrs, 6 hrs, 12 hrs, 24 hrs and 48 hrs	178

Figure 82: Contaminants present in transposon libraries as they appear on LB agar with kanamycin 50 µg/mL and nalidixic acid 25 µg/mL.....	181
Figure 83: Pals promoter introduced to control transcription of transposase in new plasmid.....	182
Figure 84: Regulator elements controlling <i>tnp</i> and <i>inh</i> expression	187
Figure 85: pMTL90882 plasmid used as backbone for cloning CRISPR modules	202
Figure 86: pCas9_Empty_+fdx-term carrying Cas9 without guide RNA and HA	202
Figure 87: pMTL90882-Cas9 plasmid carrying Cas9 with synthesized CRISPR module made of Pals promoter for Ca9 and Phps promoter for sgRNA and terminator.	203
Figure 88: pMTL90882_Cas9For3 plasmid with promoters that work in methanotrophs and sgRNA for <i>phaC</i> in Isolate 12 and <i>M. parvus</i> (OBBP).	203
Figure 89: Comparison of promoter expression in <i>M. parvus</i> (OBBP) grown in NMS and NMS + Cu.....	209
Figure 90: Promoter expression in Isolate, 3*, 10, 12 and <i>M. parvus</i> (OBBP) measured by Fluorescence/OD.....	209
Figure 91: Visual observation of fluorescence in Isolate 3*, 10, 12 and <i>M. parvus</i> (OBBP) on NMS BA plates under blue light	210
Figure 92: Promoter expression in <i>E. coli</i> S17 λ pir. P3 was the most expressed promoter while Phik was the least.	210
Figure 93: Screen for <i>phaC</i> knockouts in Isolate 12 after transforming with Cas9For3	212
Figure 94: Screen for <i>phaC</i> knockouts in Isolate 12 after transforming with Cas9For6	213
Figure 95: Plasmid design of pMTL90882_3P6 and pMTL90882_3P11 used for CRISPR_Cas9 genome editing of Isolate 3*	214

Figure 96: Conjugation efficiency of CRISPR-Cas9 plasmids used for Isolate 3* gene knockout..	214
Figure 97: Screen for <i>PhaC</i> knockouts in Isolate 3* after transforming with pMTL90882_3P6 and pMTL90882_3P11.....	215
Figure 98: Plasmids pMTL90882_10E11 with Pmdh and Pals promoter and pMTL90882_10E12 with Phik and P3 promoters used for CRISPR-Cas9 knockout in Isolate 10.....	216
Figure 99: Screen for <i>efe</i> knockouts in Isolate 10 after transforming with pMTL90882_10E11 and pMTL90882_10E12	217
Figure 100: Plasmid design of pMTL90882_12P6 and pMTL90882_12P11 used for CRISPR_Cas9 genome editing of <i>M. parvus</i> (OBBP). With the exception of Cas9 and guide RNA promoters, both plasmids are identical.....	218
Figure 101: Conjugation efficiency of CRISPR-Cas9 plasmids used for <i>M. parvus</i> (OBBP) <i>phaC</i> gene knockout	218
Figure 102: Screen for <i>phaC</i> knockouts in <i>M. parvus</i> (OBBP) after transforming with pMTL90882_12P6 and pMTL90882_12P11.....	219
Figure 103: Plasmids pMTL90882_12P12 with Phik and P3 promoter and pMTL90882_2.12P12 with Phik and P3 promoters, and in addition a different guide RNA used for CRISPR-Cas9 knockout in <i>M. parvus</i> (OBBP).	220
Figure 104: Conjugation efficiency of CRISPR-Cas9 plasmids used for <i>M. parvus</i> (OBBP) gene knockout.	220
Figure 105: Screen for <i>phaC</i> knockouts in <i>M. parvus</i> (OBBP) after transforming with pMTL90882_12P12 and pMTL90882_2.12P12.....	221
Figure 106: Plasmids pMTL90882_C5-12P11 with ColEI replicon, Cas9 and guide RNA but no homology arms and pMTL71E01-Phac with pBBR1 replicon, erythromycin resistance gene and <i>phac</i> homology arms used for CRISPR-Cas9 knockout in <i>M parvus</i> (OBBP).....	222

Figure 107: Screen for <i>phaC</i> knockouts in <i>M. parvus</i> (OBBP) after transforming with pMTL90882_C5-12P11 and pMTL_71E01-Phac	222
Figure 108: Screen for <i>phaC</i> knockouts in <i>M. parvus</i> (OBBP) after transforming with pMTL90882_12P11 in the presence of 100 μ M Scr7	223
Figure 109: Conjugation efficiency of CRISPR-Cas9 plasmids used for <i>M. parvus</i> (OBBP) <i>ligD</i> gene knockout	224
Figure 110: Screen for <i>ligD</i> knockouts in <i>M. parvus</i> (OBBP) after transforming with pMTL90882_12L11	224
Figure 111: Screen for <i>ligD</i> knockouts in Isolate 12 after transforming with pMTL90882_12L11	225
Figure 112: CRISPR-Cas9 plasmid region amplified to investigate mutation of Cas9 plasmid.....	226
Figure 113: PCR of CRISPR-Cas9 transconjugants to amplify Cas9 to guide RNA region to investigate the sequence of Cas9 plasmid after transforming in methanotrophs using primer pair ColEI_F and Fdx_term_rev.....	226

List of tables

Table 1: General characteristics of known families of methanotrophs [46]	32
Table 2: Comparison of PHA properties that determine suitability for use as plastic materials	44
Table 3: Structures, domain architectures, and functions of the core components of CRISPR-Cas systems	48
Table 4: Types of transposons, structural features and mechanisms of movements ...	56
Table 5: <i>E. coli</i> strains used in the study	64
Table 6: List of primers	79
Table 7: Organisms identified on BLAST using 16S rRNA sequence data from colonies growing on dNMS agar plates.....	86
Table 8: Organisms identified on NCBI BLAST using 16S rRNA sequence data obtained from samples growing on dNMS agar plates.....	88
Table 9: 16S rRNA Blast search result for Isolate 10	90
Table 10: Growth of LS samples after 25 days incubation	91
Table 11: Conditions under which growth was seen in the 5 days LS samples were subcultured	92
Table 12: Colonies of LS samples positive for <i>pmoA</i> and <i>mmoX</i> gene	93
Table 13: 16S rRNA BLAST search result of Isolate6.....	94
Table 14: 23S rRNA BLAST search result of Isolate 6	94
Table 15: Growth of LTS samples controls after 16 days	95
Table 16: Growth of Wollaton lake samples in dNMS media after 16 days.....	95
Table 17: Growth of samples obtained from Wollaton lake five days after subculturing	96

Table 18: Colonies of Wollaton lake samples positive for <i>pmoA</i> and <i>mmoX</i>	97
Table 19: 16S rRNA BLAST search result for Isolate 01 and Isolate 03.....	98
Table 20: 16S rRNA BLAST search result for Isolate 7	99
Table 21: 16S rRNA BLAST search result of Isolate 3*	100
Table 22: 16S rRNA BLAST result of Isolate 12	101
Table 23: 16S rRNA BLAST search result of Isolate 14.....	102
Table 24: 16S rRNA BLAST search result of Isolate 9	102
Table 25: Summary of Isolations carried out in this study	104
Table 26: Primers used in Chapter 3.....	115
Table 27: Strains used in Chapter 3	115
Table 28: Minimum inhibitory concentration of kanamycin, tetracycline, nalidixic acid, gentamicin, streptomycin and erythromycin when tested on Isolate 3*, 6, 10, 12 and 14	137
Table 29: Genomic profile of isolated methanotrophs	139
Table 30: Putative Isolate 12 RM systems and predicted recognition sequence.....	140
Table 31: Putative Isolate 14 RM systems and predicted recognition sequence.....	141
Table 32: Comparison of genes shared by isolates sequenced in this study	142
Table 33: Trace gas composition of BG1 and BG2	149
Table 34: Comparison of <i>M. marinum</i> and Isolate 10 characteristics.....	161
Table 35: Strains used in Chapter 4	167
Table 36: Primers used in Chapter 4.....	168
Table 37: Plasmids used in Chapter 4	169
Table 38: Transposition efficiency of Isolate 12 at different mating times.....	176

Table 39: Growth OD ₆₀₀ of transposon mutant library in four successive passages under different growth conditions.....	180
Table 40: Dilution of Transposon mutants grown under different conditions	183
Table 41: Estimated number of transposon mutants growing in 40 mL of NMS media and antibiotic under specified conditions	183
Table 42: List of promoters.....	195
Table 43: <i>E. coli</i> strains.....	196
Table 44: Methanotroph strains	196
Table 45: Primers Used in Chapter 5	197
Table 46: Primers used in Chapter 5 (continued)	198
Table 47: Summary of Isolate 3* and <i>M. parvus</i> (OBBP) CRISPR-Cas9 plasmids, gene targets and conjugation efficiency data	227

Abbreviations

3HP – 3-hydroxypropionic acid
ATP – Adenosine Triphosphate
BA – Bacto Agar
BG – Biogas
CCE – Carbon Conversion Efficiency
CRISPR – Clustered Regularly Interspaced Short Palindromic repeats
DNA – Deoxyribonucleic Acid
DSB – Double Strand Break
EYFP – Enhanced yellow Fluorescent Protein
GG – Gelan Gum
GHG – Greenhouse Gas
GRNA – Guide RNA
GTL – Gas to Liquid
HA – Homology Arm
HDR – Homology Directed Repair
HR – Homologous Recombination
ICM – Intracytoplasmic Membrane
IE – Inner Ends
Inh – Transposon Inhibitor Protein gene
KU – Protein Kinase
LIGD – ATP-Dependent DNA Ligase IV
ME_I – Inner Mosaic End
ME_O – Outer Mosaic End
MMO – Methane Monooxygenase
NAD – Nicotinamide Adenine Dinucleotide
NHEJ – Non-Homologous End Joining
OE – Outer End
PCR – Polymerase chain reaction
PHAC – Poly-(3)-Hydroxyalkanoate Synthase C subunit
PHB – Poly-3-Hydroxybutyrate
PMMO – Particulate Methane Monooxygenase
PLFA – Phospholipid Fatty Acid
RNA – Ribonucleic Acid
RT-PCR – Reverse transcription polymerase chain reaction
sgRNA – Seed Guide RNA
SMMO – Soluble Methane Monooxygenase
SSODNA – Single Stranded Oligo Deoxyribonucleic Acid
TIR – Terminal Inverted Repeat
tnp(A) – Transposase gene

1 Chapter 1: Introduction

1.1 Methanotrophs and the use of methane (CH₄) as a feedstock

Methanotrophs belong to the group of microorganisms known as methylotrophs which metabolise single carbon (C1) compounds such as methane, methanol and formaldehyde among others [2-5]. Methanotrophs are also known for their bioremediation properties as well as their role in balancing methane flux in the environment [6, 7]. This makes them important organisms of study especially with the rising public concern regarding global climate change. Although the effort to tackle climate change is surrounded by a lot of scepticism, it is no longer in contention that atmospheric Greenhouse Gases (GHGs) are rising as a result of anthropogenic activities. What is debatable is the rate and consequent effect on global climate. Amongst these gases, CO₂ attracts a considerable amount of attention. This is not only because it lasts about 100 years in the atmosphere, but because it also has the largest GHG proportion, contributing 81.6% of all U.S. greenhouse gas emissions from human activities in 2016 [8]. CH₄ is another important GHG because of its relative abundance in the atmosphere which contributed 10% of U.S. GHG emissions from human activities in 2016 [8]. Furthermore, the Global Warming Potential (GWP) of CH₄ is 25 times greater than CO₂ over a 100-year period [9].

1.1.1 Chemical conversion of CH₄ such as GTL

With continuing rise in global energy demand, it seems counterproductive to underutilise CH₄ which can be used as an energy source. One vital sector that CH₄ can be used is the transportation industry which accounts for 28% of global energy demand [10]. Although CH₄ in the form of natural gas is a viable transportation energy resource, its use is not yet mature as it supplied less than 3% of global transport energy demand in 2010 [10]. Natural gas which contains CH₄ has a key environmental advantage due to its relatively low CO₂ emission on combustion and is used in either its compressed (Compressed Natural Gas) or super cooled (Liquefied Natural Gas)

form. However, the spread of its use is constrained by a relatively higher vehicle cost and the need to provide separate refuelling infrastructure [11].

CH₄ can also be converted to liquid chemicals and fuels (Gas to Liquid – GTL) using various conversion technologies. This is particularly important in remote natural gas sites where building gas pipelines is not economically viable. Fischer Tropsch (FT) is the more prominent amongst GTL conversion technologies although it has some disadvantages which limit its widespread operation. Among them is the need to establish this technology on a large scale for successful commercialisation [12]. In addition to the technology's low Carbon Conversion Efficiency (CCE) of 25 – 50%, there is requirement for syngas generation which involves a high-temperature steam reforming step [13]. This step is followed by syngas conversion and hydroprocessing which are all energy intensive [14]. These hindrances have necessitated the need for viable and more sustainable alternatives. One area gaining attention is the use of biological systems for GTL conversion.

1.1.2 Biological conversion of CH₄ by methanotrophs

With the advent of biotechnology, microbial processes have been widely used for manufacturing food products, biopharmaceuticals and bio-based chemicals among others. For example, yeast has been used for thousands of years for production of fermented beverages and food [15]. In the pharmaceutical industry, the use of biotechnology resulted in large scale production and launching of insulin from *Escherichia coli* in 1982, making it widely accessible [16]. Bio-based chemicals such as succinic acid, butanol and methanol are widely available today because the metabolic reactions of microorganisms have advanced and accelerated the manufacturing processes.

To utilise biological systems for GTL processes using natural gas, organisms capable of oxidising CH₄ are required. Methanotrophs are Gram-negative bacteria possessing this capability. Although studies are more focused on aerobic species, anaerobes have also been isolated which use sulphates as terminal electron acceptors [17]. Methanotrophs are ubiquitous due to the versatility of their physiology which enables them to survive a wide range of pH, salinity, oxygen concentrations, temperatures, relatively high

heavy metal concentrations and radiation [18-22]. As a result, they have been isolated from various environments including landfills, lake sediments, wetlands and marine environments [23-26].

The distinguishing characteristic that enable methanotrophs to oxidise CH_4 and survive in different environments is their broad-spectrum methane monooxygenase (MMO) enzyme which is exclusive to them [27]. This enzyme is present in two forms, each found in different parts of the cell. The particulate form (pMMO) is membrane-associated while the soluble form (sMMO) is found in the cytoplasm. All known methanotrophs possess pMMO except a *Methylocella* species [28]. However, a few known methanotrophs possess the sMMO enzyme [26, 29]. Some species for example *Methylococcus capsulatus* and *Methylosinus trichosporium* possess both forms of MMO. In methanotrophs with pMMO, methane oxidation takes place in the intracytoplasmic membrane (ICM). Based on the morphology of their internal structures, particularly the ICM, and other characteristics, methanotrophs are divided into type I and type II. In type II, the ICM appears as paired membranes assembled at the cell periphery usually running round the whole cell, while in type I, they resemble disc-shaped vesicles and can be present throughout the cell (Figure 1) [2]. Type X represents a third group phylogenetically related to Type I although it possesses metabolic properties similar to Type II [30].

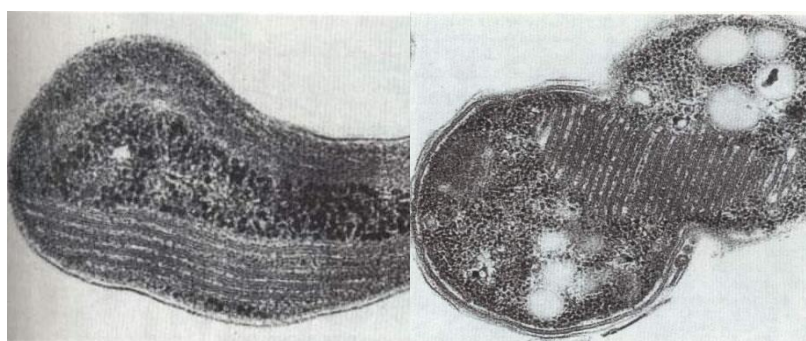


Figure 1: Appearance of Intracytoplasmic Membrane (ICM) in type II (left) running along the cell periphery and type I and X methanotrophs (right) running across the cell [2]

Obligate methanotrophs utilise CH_4 for energy production and carbon assimilation. However, more facultative methanotrophs are continuously being isolated. These utilise compounds including ethanol, methylamine and acetate as carbon sources in

addition to CH₄ [31]. The bioremediation properties of facultative methanotrophs have led to broader research on the physiology of these organisms. However, their facultative properties may be disadvantageous especially when used as industrial strains in processes where the preferred substrate is CH₄. In such processes, obligate methanotrophs will be more beneficial as this will ensure the optimum utilisation of CH₄ which is the substrate of interest.

Numerous procedures have been developed to make methanotrophs amenable to genetic engineering and molecular biology applications. For example, electroporation has been successfully carried out in *Methylocella silvestris* [32]; transposon mutagenesis has been demonstrated in *Methylomonas* species [33]; and expression of heterologous genes for lactate production was shown in *Methylobacterium buryatense* [34]. More recently, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) has been used as a quick and easy-to-design tool for genome editing in eukaryotes and prokaryotes. This has the potential to increase the efficiency and decrease the time required to genetically engineer methanotrophs if successfully applied. This can have significant effects on metabolic engineering outcomes and subsequent production of bio-based chemicals among other benefits. Some important aspects of genetic engineering are alteration of gene function through insertions, deletions and nonsense mutations, processes employed by CRISPR-Cas for genome editing. Before gene alterations are carried out, it is crucial to understand the function(s) of such genes and their essentiality. This can be determined by Transposon-directed Insertion Site (TraDIS) sequencing [35], one of the many tools available for insertion sequencing. Together, TraDIS and CRISPR can be used to carry out informed genome editing. If successfully applied, they will pave the way for metabolic engineering and enhance the use of methanotrophs for production of bio-based chemical and food commodities. It is unlikely that one or both technologies will work on all species of methanotrophs. It is therefore advantageous to have a variety of species to determine which are better suited for utilising these tools.

1.2 Overview of methanotrophs

1.2.1 History of methanotrophs

Bacillus methanica was the first named methanotroph and it was isolated in 1906 by a Dutch microbiologist called Sohngen [36]. Before this event, Sohngen suspected the involvement of microorganisms in CH₄ oxidation as the low amount of CH₄ in the atmosphere was not commensurate with the large amounts released by the environment. Isolation of methane-oxidising bacteria supported his suspicion. *Bacillus methanica* was later renamed *Methanomonas methanica* in 1909 by Orla-Jensen [3]. It was to be more than half a century before other methanotrophs would be isolated. These were *Methanomonas methanoxidans*, *Pseudomonas methanitrificans* and *Methylococcus capsulatus* [37-39]. The turning point was to come in 1970 when Roger Whittenbury and his group at the University of Edinburgh isolated 100 methanotrophs using Plate Microscopy. These isolates were obtained from mud and water of streams, ponds, rivers and ditches, as well as soil samples from Europe, America and Africa. Their method involved viewing microorganisms under the microscope and simultaneously transferring colonies of interest onto agar slopes using straight wires [2].

For a long time, methanotrophs were considered obligate and could only grow on CH₄. This changed when in 1974 Hanson and his colleagues isolated a methanotroph that could grow on malate, acetate, glucose and galactose among other compounds. This organism was later described as *Methylobacterium organophilum* [40]. Other reports of facultative methanotrophic bacteria include *Methylobacterium ethanolicum* isolated around 1980 [41]. However, these claims were later dismissed due to loss of methanotrophic activity. In the case of *M. organophilum*, it was hypothesized that methanotrophic activity was conferred by a plasmid which was later lost along with the methanotrophic characteristic. For *M. ethanolicum*, it was attributed to a syntrophic association between *Xanthobacter* species and a methanotroph [42]. Nonetheless, validated facultative methanotrophic activity has been recently described among *Methylocella* species using rigorous techniques and these organisms are

continuously attracting more attention due to their ability to degrade complex organic compounds [31].

Nitrate Mineral Salt (NMS) media formulated by Whittenbury is among the most common medium used for culturing methanotrophs [2]. This medium has been modified to serve different growth, enrichment and isolation purposes. One important change to this medium that led to a significant discovery was the observation that a high copper concentration to cell biomass ratio promoted the expression of particulate methane monooxygenase (pMMO) while sMMO expression was promoted by low copper concentration to high biomass ratio [43]. This has enabled the selective isolation of either sMMO or pMMO-expressing methanotrophic organisms. *M. capsulatus* (Bath) which is among the most widely studied methanotrophs had its genome sequenced in 2004, the first in this group of organisms [44].

1.2.2 Phylogeny and categorisation

Classification came late in the history of methanotrophs. This can be attributed to the low number of isolates before 1970 when more than 100 methanotrophs were isolated [2]. This explosion in number of known and culturable methanotrophs established the earliest classification which was based on cellular morphology, fine structure of Intracytoplasmic Membranes (ICM) and resting stage formed. Five groups were postulated – *Methylosinus*, *Methylocystis*, *Methylomonas*, *Methylobacter* and *Methylococcus* [2]. Based on pathways for assimilation of carbon (serine pathway or ribulose monophosphate pathway), G-C content and phospholipid fatty acid composition among other characteristics, methanotrophs were later divided into type I composed of *Methylosinus* and *Methylocystis*, and type II composed of *Methylomonas* and *Methylobacter*. Type X was also introduced to represent organisms which shared characteristics between and outside Type I and II [45]. Based on 16S rRNA gene sequence analyses, methanotrophs were grouped either as Gammaproteobacteria or Alphaproteobacteria. Type I methanotrophs fell under Gammaproteobacteria while type II were categorised as Alphaproteobacteria which were further subdivided into *Methylocystaceae* and *Beijerinckaceae* Table 1 [46]. It is worth mentioning that although not included in Table 1, the family of *Beijerinckaceae* include non-

methanotrophs such as *Beijerinckia* and *Rhodoblastus*. However, evidence from Dedysh *et al.* suggests they could be methylotrophs [31]. With regards to the conventional naming of methanotrophs which starts with the prefix of the genus (Methylo), Gammaproteobacteria comprise exceptions - *Crenothrix polyspora* and *Clonothrix fusca*. *C. polyspora* is a filamentous, sheathed, iron-accumulating bacterium shown to possess an unusual *pmoA* gene (u-*pmoA*). Although *C. fusca* has similar structural properties, it has been shown to possess a conventional *pmoA* gene [47]. Another phylum called *Verrucomicrobia* was added to the classification following the independent isolation of three methanotrophs from geothermal regions having high 16S rRNA sequence similarity (>98%). They were assigned the genus *Methylacidiphilum* [47]. Information about 16S rRNA based classification is summarised in Table 1.

Table 1: General characteristics of known families of methanotrophs [46]

Phylum	<i>Gammaproteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Verrucomicrobia</i>
Family	<i>Methylococcaceae</i>	<i>Methylocystaceae</i>	<i>Beijerinckaceae</i>	<i>Methylacidiphilaceae</i>
Genera	<i>Methylobacter</i> , <i>Methylococcus</i> , <i>Methylocaldum</i> , <i>Methylohalobius</i> , <i>Methylomicrobium</i> , <i>Methylomonas</i> , <i>Methylosoma</i> , <i>Methylosarcina</i> , <i>Methylosphaera</i> , <i>Methylothermus</i> , <i>Crenothrix</i> , <i>Clonothrix</i>	<i>Methylosinus</i> , <i>Methylocystis</i>	<i>Methylocapsa</i> , <i>Methylocella</i>	<i>Methylacidiphilum</i>
RuMP pathway	+	–	–	–
Serine pathway	–	+	+	+
Rubisco	Rarely	–	–	+
sMMO	Varies between species	Varies between species	Varies between species	–
pMMO	+	+	Varies between species	+
Nitrogen fixation	Varies between species	Varies between species	+	Varies between species
Intracytoplasmic membrane formation	Bundles of disks perpendicular to cell periphery	Membrane stacks parallel to cell periphery	<i>Methylocapsa</i> – membrane vesicles parallel to long axis on one side of cell membrane. <i>Methylocella</i> – cytoplasmic membrane invaginations	–
Carboxysome-like	–	–	+	+
Major PLFAs	14:0; 16:0; 16:1 ω 5t; 16:1 ω 6c; 16:1 ω 7;	18:1 ω 7c; 18:1 ω 8c	18:1 ω 7c	i14:0; a15:0; 18:0
	16:1 ω 7c; 16:1 ω 8c; 18:1 ω 7			
Resting stages	Varies – cysts or none	Varies – cysts, spores or none	Cysts or spores	None
G+C mol%	43–65	62–67	60–63	41–46
Facultative	–	–	Varies between species	–

1.2.3 Isolation of methanotrophs

The first methanotroph was isolated in 1906 from enrichments of aquatic macrophyte leaves submerged in water [36]. From then till the 1970s, few works on methanotrophs were published. One important factor limiting the study of methanotrophs was the difficulty associated with isolating laboratory strains as many species were unculturable. An important reason why a lot of methanotrophs are unculturable may be the inability to replicate their natural environment in the laboratory. This has been reported to be the reason why a lot of other bacteria are unculturable [48]. Furthermore, there is no one optimum temperature, medium or atmosphere required for isolation of all methanotrophs as a result of the diverse nature of this group [49]. Nitrate Mineral Salt (NMS) media is the most widely used, sometimes with modifications. However, among the most limiting factors hindering the study of methanotrophs, obtaining pure cultures stands out. In nearly all situations, enrichments of methanotrophs are contaminated by non-methanotrophs which usually outgrow and feed on their metabolic by-products. Humidity which accumulates during long incubation periods on agar plates also encourages fungal growth making it harder to obtain pure colonies. This problem can be managed by using alcohol to wipe containers or jars before use. Antifungals such as cycloheximide and nystatin are also effective [50]. The challenge of obtaining pure cultures was almost always attributed to the contaminating bacteria which tend to feed on methanotrophic metabolites such as methanol, formaldehyde and organic acids [51]. Whittenbury in the late 1960s using Plate Microscopy techniques solved this problem as his team was able to select and separately grow species with similar morphology suspected to be methanotrophs [2].

Another method that can be used to obtain pure cultures is the addition of methanol (0.025% v/v) and yeast extract (0.025% w/v) to NMS agar onto which serial dilutions of the enrichment culture are spread. The supplements encourage the separate growth of contaminants into large colony sizes without interfering with methanotrophic growth [52]. The supplements may also stimulate the growth of methanotrophs [53]. Single colonies identified to be methanotrophs can then be grown in liquid media and spread back onto plates. Several passages may need to be

carried out before purity is obtained. Repeated streaking on solid media and inoculating single colonies back into liquid media is also practiced. This may be an effective way of reducing the number of contaminants with each successive passage. Another variable that may be altered to give methanotrophs an advantage is the addition of high salt concentrations which marine isolates are more tolerant to. Temperature sensitivity can also be exploited to select for moderately thermophilic methanotrophs such as *Methylococcus capsulatus* which grows at 37 – 45°C [49].

Other novel techniques have been designed to enhance isolation of pure methanotrophic bacteria. Soil Substrate Membrane System (SSMS) involves spreading different dilutions of enrichments on a special filter paper such as polycarbonate microporous cell culture insert. The filter paper supporting methanotrophs on the top side is placed in a Petri dish containing agricultural soil slurry. The filter paper acting as a barrier between methanotrophs at the top and slurry at the bottom allows the passage of metabolites produced by methanotrophs on the top side of the filter paper. The metabolites then flow through the filter paper and are then consumed by microorganisms in the agricultural soil slurry at the bottom of the filter paper hence starving and inhibiting the growth of contaminants associated with methanotrophs on the top side of the filter paper. Colonies that grow on the top side of the filter papers are then diluted and spread on another filter paper until pure colonies are isolated [54].

A simple and straight forward way of isolating methanotrophs from environmental samples or separating different species of methanotrophs from one another is through the use of Percoll. Percoll is a medium that provides buoyancy to cells according to their densities, thereby separating the cells into different layers in a tube. It involves centrifugation steps that result in cell pellets which are transferred to polycarbonate tubes containing Percoll and then re-centrifuged. This result in cells floating at different levels of the polycarbonate tubes based on their respective densities. A control tube containing Density Marker Beads (DMB) illustrates the density of cells at each level of the tube. Type 1 and X cells have densities ranging from 1.084 to 1.116 g/mL, with an average of 1.093 g/mL. Type II methanotrophs

have a density range of 1.039 to 1.052 g/mL, with an average of 1.047 g/mL. This technique is described in detail by Putzer *et al.* [55].

It is important to confirm purity of methanotrophic isolates. For obligate methanotrophs, the following suggest the absence of contaminating bacteria: indistinct colonies growing on plates; absence of growth after spreading colony of methanotrophs on rich media (LB agar); absence of growth when colonies of methanotrophs are grown in the absence of CH₄; uniform morphology when cells are viewed under the microscope [49]. However facultative methanotrophs require more stringent determination techniques such as quantitative real-time PCR of methanotroph specific genes [31].

1.2.4 Methane monooxygenase

Methanotrophs oxidise CH₄ for energy generation and carbon assimilation. The reaction is catalysed by methane monooxygenase (MMO) which is highly specialised for this immense task considering the highly stable C-H bond of CH₄. The process requires two reducing equivalents from NAD(P)H to split the O-O bond of O₂, with one forming H₂O while the other oxygen reacts with CH₄ to form methanol, a reaction approaching 100% efficiency [56, 57]. Methanol dehydrogenase oxidises methanol to formaldehyde, approximately 50% of which is assimilated as cell carbon [3]. The remaining is oxidised to formate and eventually CO₂. These reactions from formaldehyde to CO₂ produce the reducing power for the initial oxidation of CH₄ and cellular biosynthesis (Figure 2).

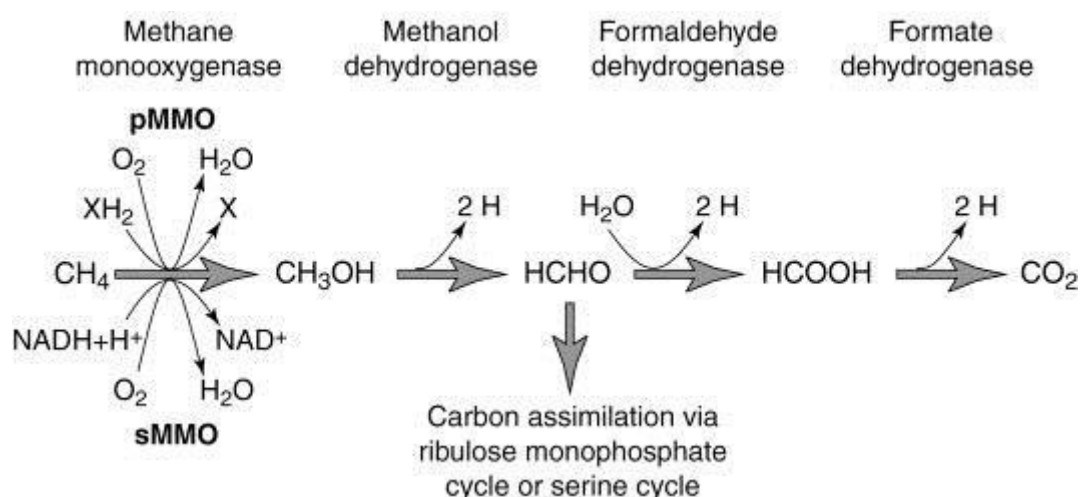


Figure 2: Methane monooxygenase: During methane oxidation, NADH is the electron donor for sMMO while the electron donor for pMMO is unknown. The reducing equivalent generated is represented by 2H [29]

Species such as *M. capsulatus* and *M. trichosporum* possess both pMMO and sMMO [29]. In such species, high copper to cell biomass ratio selects for expression of pMMO while sMMO is expressed when there is a low copper to cell biomass ratio [43]. Methanobactin (mb) is a fluorescent chromopeptide purified from *Methylosinus trichosporium* OB3b. It mediates the acquisition of copper thus stimulating the expression of pMMO in the cell (Figure 3). It may either shuttle copper into the cell or sequester it from growth environment during low Copper bioavailability [58]. Also due to the high toxicity of copper [59], it may also play a defensive role by forming a mb-Cu bond thus preventing copper toxicity [58]. Apart from CH_4 oxidation properties which sMMO and pMMO share, both enzyme systems have striking structural, functional and regulatory differences.

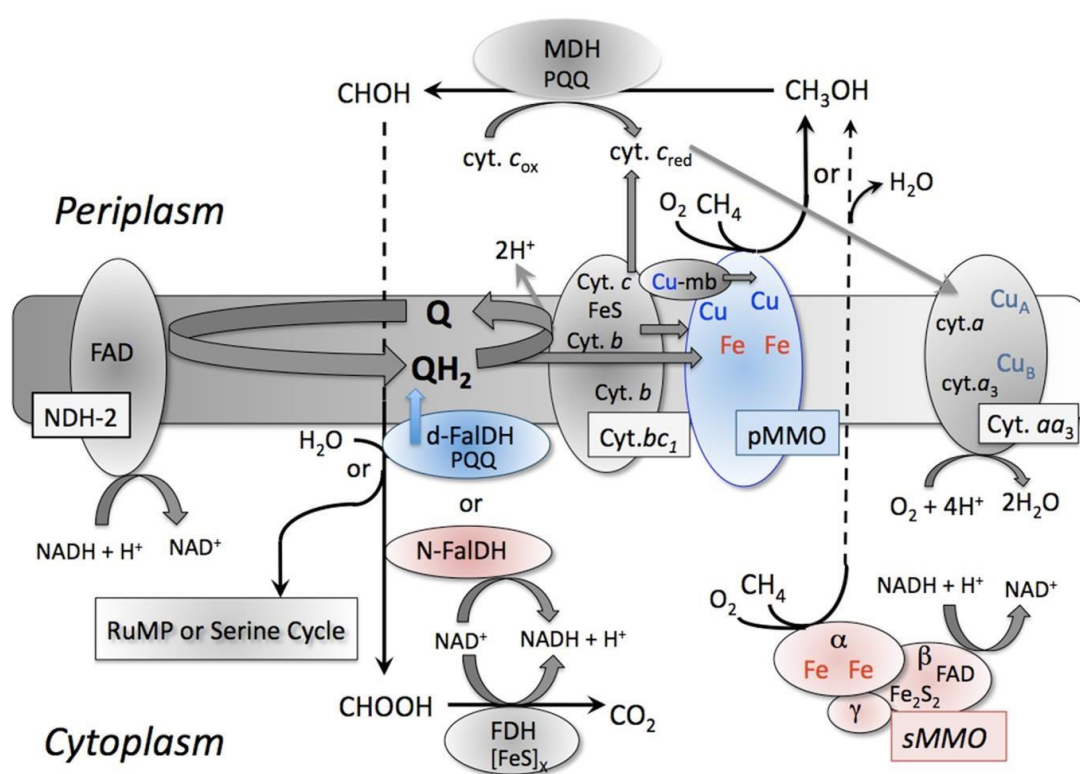


Figure 3: Regulation of CH₄ oxidation under low and high copper concentrations. Proteins that have positive copper regulations are in blue while the red ones have negative effects on Copper regulation. Cyt, cytochrome; pMMO, membrane-associated or particulate methane monooxygenase; sMMO, cytoplasmic or soluble methane monooxygenase; D-FalDH, dye-linked/quinone-linked formaldehyde dehydrogenase; N-FalDH, NAD(P)-linked formaldehyde dehydrogenase; NDH-2, type 2 NADH dehydrogenase; Q, ubiquinone; FDH, formate dehydrogenase; FAD, flavin adenine dinucleotide; MDH, methanol dehydrogenase; ribulose monophosphate PQQ, pyroloquinoline quinone; RuMP [46].

1.2.4.1 Particulate Methane Monooxygenase (pMMO)

In methanotrophs that possess both forms of MMO, pMMO is expressed when copper concentrations in the growth media reach approximately 4 μ M. *M. capsulatus* pMMO for example undergoes a proportional increase relative to copper concentrations up to approximately 60 μ M of copper after which pMMO expression declines [60]. In *M. capsulatus*, pMMO has three subunits – *pmoA*, *pmoB* and *pmoC* approximately 45, 27 and 23 kDa respectively. *pmoB* contains a soluble region with a mononuclear and dinuclear copper subunit. A third membrane bound subunit exists for iron (Figure 4). The dinuclear copper subunit is suspected to be the active site. When the crystal structure of pMMO was resolved in 2005 with a resolution of 2.8Å, it was found to exist as a trimer in an $\alpha_3\beta_3\gamma_3$ arrangement [61]. Although pMMO is widely present in methanotrophs, it has relatively narrow substrate specificity compared to sMMO which can oxidise halogenated aromatic compounds [62].

However, it can oxidise alkanes and alkenes having up to four and five carbons atoms in length respectively [63]. It also has bioremediation properties [64]. The operon *pmoCAB* codes for pMMO. In *Methylococcus capsulatus* Bath, two complete copies have been found, with a third copy found for *pmoC* [65]. The possession of *pmoA* by most methanotrophs has made it an important gene probe, indicating the presence of methanotrophs in the environment. Chromosomal insertions of all seven genes in *M. capsulatus* Bath highlighted their relevance. Null mutants of *pmoC* could not be obtained suggesting it plays an essential role in CH₄ oxidation. Additionally, double null mutants of both copies of *pmoCAB* could not be obtained which also suggests their requirement for cell growth. However, copy 1 mutant lost two-thirds of its methane-oxidising activity while copy 2 lost one-third [65].

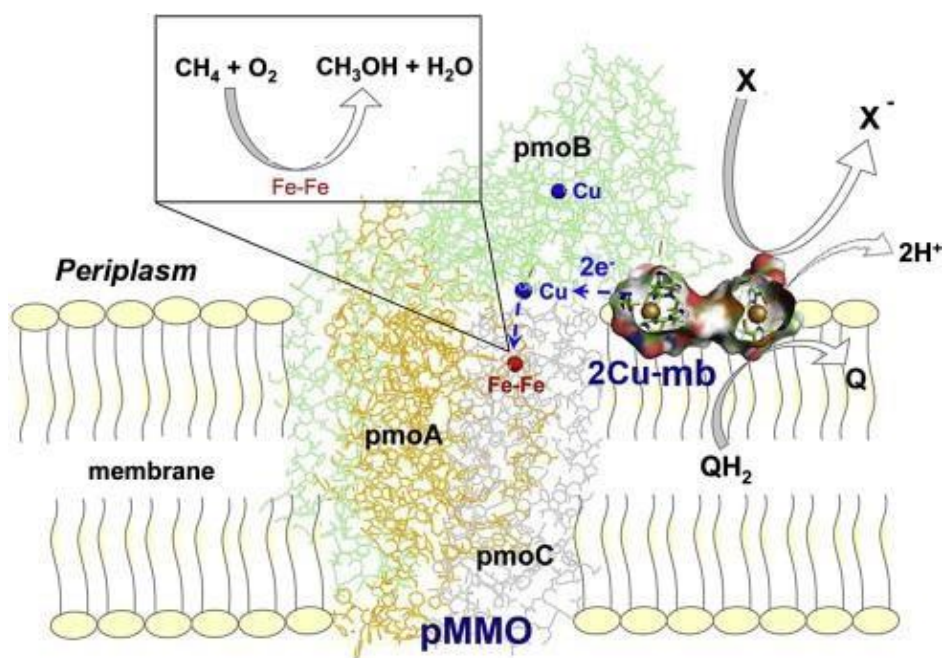


Figure 4: pMMO showing its iron and Cu centres housed in the *pmoB* region, and orientation across ICM. Cu-mb is also illustrated mediating the flow of electrons from respiratory chain to metal centres [60].

1.2.4.2 Soluble Methane Monooxygenase (sMMO)

Unlike pMMO, sMMO is sensitive to copper and expressed at concentrations <0.4 μM and <0.8 μM in *M. trichosporium* and *M. capsulatus* respectively [60, 66]. sMMO has a broad substrate specificity and is known to co-oxidise various alkanes, aromatic compounds and substituted aliphatics which makes it very attractive for bioremediation and biotransformation [67]. With the resolution of its structure in 1978, it was reported to be a non-heme iron complex containing three components –

hydroxylase, Protein B and Protein C. The hydroxylase consists of α , β and γ subunits approximately 60, 45 and 20 kDa, in $\alpha_2\beta_2\gamma_2$ arrangements (Figure 5). The active site which is located in the α subunit houses a di-nuclear iron centre where CH_4 is oxidised [68]. Protein B which is a small single protein subunit plays a strong role in regulating sMMO activity [69]. Protein C is a reductase containing FAD as cofactor and NADH as electron donor. The NADH is said to contain an iron-sulphur core. The mechanism for electron transfer involves the FAD accepting electrons from NADH and transferring them to MMO hydroxylase [70].

The genes *mmoB* and *mmoC* which code for Protein B and C respectively have been successfully expressed in *E. coli*. However, the expression of sMMO hydroxylase genes which code for the active site for methane oxidation was unsuccessful in *E. coli* although methods to improve mutagenesis of sMMO genes and their expression have been reported in *M. trichosporium* [71, 72]. On the other hand, sMMO genes have been successfully expressed in methanotrophs previously lacking them. For example sMMO from *M. trichosporium* OB3b was expressed in *Methylobacterium album* BG8 and *Methylocystis parvus* OBBP [73].

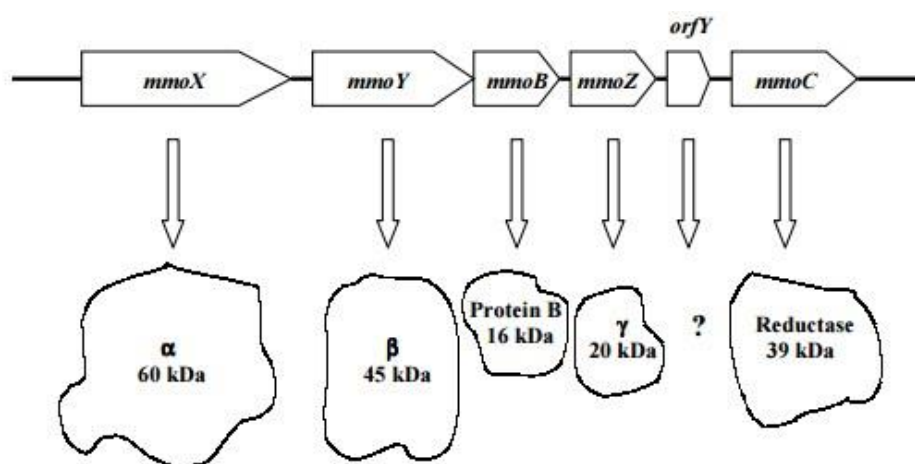


Figure 5: The soluble methane monooxygenase gene cluster. *mmoXYZ* code for the hydroxylase which component which houses the α subunit where the di-nuclear iron centre is located. *mmoB* and *mmoC* code for Protein B and Protein C respectively. Protein B plays a regulatory role while Protein C is a reductase involved in electron transfer during methane oxidation [68, 69, 74].

1.2.5 Characterisation of methanotrophs

In studies involving isolation of microorganisms, characterisation is important to determine the novelty of the species. This will provide the information required to assign the new isolates into appropriate taxa. Notwithstanding, the hierarchical framework laid down by the Bacteriological Code mentions that names given to bacteria are primarily to provide means of referring to them rather than indicate the character or history of the isolates [1]. Although many criteria exist for characterisations, select properties of new isolates have been repeatedly investigated in methanotrophs. These have formed the basis on which new isolates are categorized. One of the most commonly studied properties of new isolates is 16S rRNA and *pmoA* gene sequences. Sequence similarity with other closely related methanotrophs will determine where new isolates are placed in a phylogenetic tree [75, 76]. Another important property examined is physiological conditions under which isolates can grow. This includes experiments that investigate source of carbon for growth which eventually determines if isolates are classed as obligate or facultative [77]. Other physiological studies include source of nitrogen for growth and ability to fix nitrogen; tolerance to salt; growth pH; growth temperature range and PHB production [26, 78-80]. Methanotrophs are made of different lipid contents which forms the basis on which many new isolates are characterised [76]. C₁₆ phospholipid fatty acids (PLFA) are predominantly found in Type I methanotrophs while type II contain mainly C₁₈ PLFA [79]. Another common property that can determine the novelty of an isolate is its morphology. This can be investigated by visualisation of colonies formed, phase contrast microscopy, Gram staining and electron microscopy among others [2, 77, 79]. Biochemical tests such as urease, oxidase and catalase activity are also common. A very common biochemical test is activity assay of the soluble form of the methane monooxygenase (MMO) enzyme using the naphthalene assay. The naphthalene assay involves the development of a colored complex between 1-naphthol, which is formed when sMMO reacts with naphthalene, and o-dianisidine (tetrazotized) [77, 81, 82].

1.3 Genetic tools in methanotrophs

From an environmental and industrial points of view, methanotrophs have capabilities that make them interesting organisms to exploit for pollutant detoxification, climate change mitigation as well as bio-based chemical production [34, 83, 84]. However, their slow growth and poor amenability to DNA transfer in some species have generated scepticism around research in methanotrophs. Nevertheless, biotechnology and molecular biology in methanotrophs have progressed. Electroporation can now be used for introduction of genetic material into methanotrophs [32, 85]. Insertions and deletions are now also common. These are achieved by marker exchange through standard homologous recombination. For example flanking regions of approximately 500 base pairs have been used. pCm184, one of such vectors used, constitutes an antibiotic marker which is flanked by loxP thereby enabling the marker to be removed and reused via Cre recombinase [86]. Mutations into genomes have also been achieved using transposon systems. For example nitrogen fixation mutants were created from *Methylosinus* species strain 6 using a Tn5 system [87]. Genetic tools are widely available for manipulating methanotrophic bacteria. Replicating vectors which have broad host range such as IncP, IncQ and pBBR have been reported. A comprehensive list of genetic tools are available in a public online archive (Methanotroph Commons) [88].

As important as it is to develop genetic tools, it is also crucial to identify realistic metabolic targets especially for applications of bio-based chemical production. Methanotrophs produce pyruvate and acetyl-CoA in high flux in type I and II methanotrophs respectively which can be used as starting points for the biosynthesis of other products. Various enzymes can be targeted along these pathways to influence flux (Figure 6). A similar method can be used for increasing acetyl-CoA flux by eliminating the enzymes that catalyse the production of poly-3-hydroxybutyrate (PHB) [89]. The presence of ICM suggests the metabolism of methanotrophs evolved to cater for high lipid requirement for membrane biosynthesis. This has generated a lot of interest around the use of lipids from methanotrophs for biodiesel production which has been the focus of a \$4M grant by the ARPA-E in the USA [90]. Type I methanotrophs due to their high biomass yield, seem more attractive species to

exploit [89]. The idea of using methanotrophs as chassis for bio-based chemical and food production seems logical as the process involves a cheap and sometimes waste carbon source in the form of methane. Secondly its use mitigates climate change by reducing GHG emissions. Among the patented bio-based chemicals that can be produced by heterologous expression of genes is isobutanol [91]. Isobutanol is an important end product attracting attention which is believed to be a better substitute for ethanol as a gasoline replacement [92]. Using methanotrophs to produce methanol has also been investigated. Among the drivers of this push are the environmentally unfriendly, non-economic and energy intensive chemical conversion processes of CH₄ to methanol [93]. Methanol production reaching 1.1 g/L has been achieved in a bioreactor using *Methylosinus trachosporium* [94]. Methanol production of up to 0.309 g/L has also been reported using *Methylomonas methanica* and *Methylocella tundra* as co-culture using simulated biogas as a source of CH₄ [95]. The ability of methanotrophs to produce vitamin B12 was also shown where a *Methylobacterium* species G-10 strain produced the highest amounts (800 ng/g wet biomass) [96]. Enhanced biosynthesis of desired carotenoids has been achieved in a *Methylomonas* species using the Tn5 transposon [97]. Through heterologous expression of L-lactate dehydrogenase genes, up to 0.8 g/Litre of lactate was achieved in *Methylobacterium buryatense* [34]. The products of methanotroph biosynthesis that are nearing commercial exploitation are poly-3-hydroxybutyrate (PHB) and single cell proteins (SCP). Type II methanotrophs that use the serine pathway for carbon assimilation are known to produce PHB [98]. However some physical properties of PHB such as its brittleness make it less suitable for bioplastic production [99]. Additionally, PHB melts at temperatures ranging from 180 – 200 °C, a range close to the temperature where degradation begins, an undesirable thermal property during shaping of plastic [100]. Hence processes have been developed to modify its properties using different methods such as increasing its molecular weight [101]. Other studies have shown addition of valeric acid as co-substrate for methanotrophs can lead to production of other poly-3-hydroxyalkanoates (PHAs) such as 3-hydroxyvaleric acid (3HV) [102]. Biosynthesis of PHB as a copolymer with other PHAs such as hydroxyvalerate has also been explored to mitigate its disadvantages [103]. Companies like Mango Materials currently use PHB for production of biodegradable

polyester fibres for textiles, as well as biopolymers for plastics such as a plastic bottle caps [104].

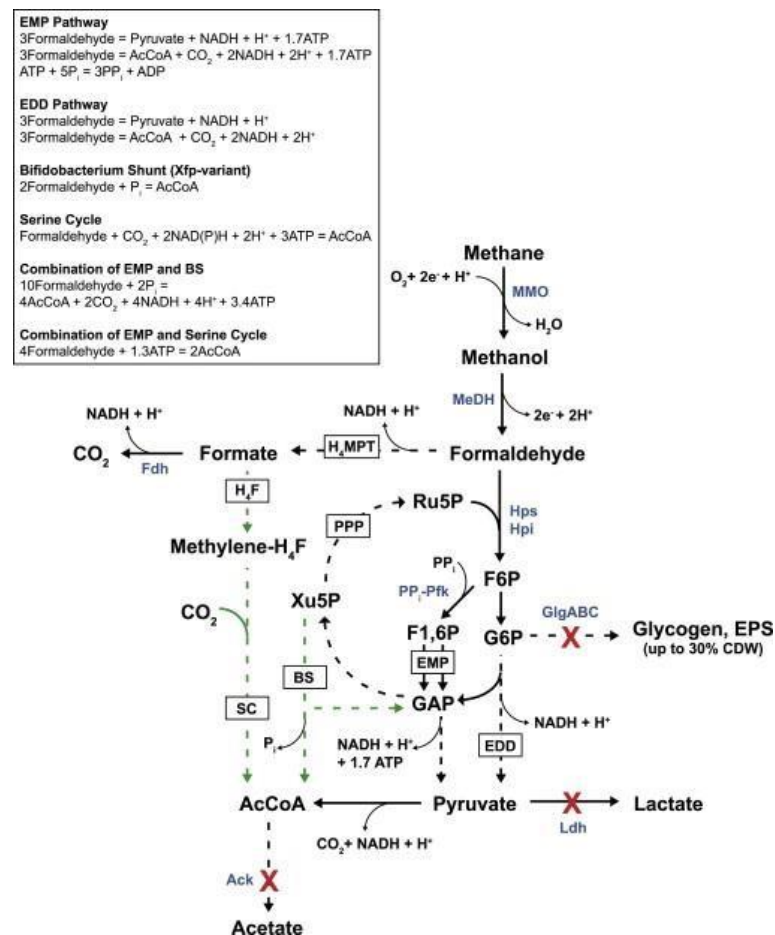


Figure 6: Simplified map of the central metabolic pathways in Group I methanotrophic bacteria, such as *Methylobacterium* species and *Methylobacter* spp. Key enzymes are shown in blue and pathway abbreviations are boxed. Green arrows: predicted metabolic alterations to improve carbon conversion efficiency for production of acetyl-CoA (AcCoA); red Xs: suggested deletions that could lead to increased flux into pyruvate and AcCoA. SC: serine cycle; H4F: tetrahydrofolate pathway; H4MPT: tetrahydromethanopterin pathway; BS: Bifidobacterium shunt; EMP: Embden–Meyerhof–Parnas pathway; EDD: Entner–Doudoroff pathway; PPP: pentose phosphate pathway. Xu5P: xylulose 5-phosphate; F6P: fructose 6-phosphate; F1,6P: fructose 1,6-bisphosphate; G6P: glucose 6-phosphate; GAP: glyceraldehyde 3-phosphate; Pi: inorganic phosphate; EPS: exopolysaccharides. MMO: methane monooxygenase; MeDH: methanol dehydrogenase; Fdh: formate dehydrogenase; Hps: hexulose 6-phosphate synthase; hpi: Hexulose 6-phosphate isomerase; PPI-Pfk: pyrophosphate-dependent 6-phosphofructokinase; GlgABC: glycogen production enzymes; Ldh: Lactate dehydrogenase; ack: acetate kinase [89].

Apart from PHB, sourcing proteins from methanotrophs is attracting a lot of attention with regards to sustainable food sources. SCP as a food source has been produced from *Methylobacterium capsulatus* (Bath) by Norferm Danmark [105]. Currently, Calysta using its loop reactor to grow *Methylobacterium capsulatus* produces commercially representative samples of SCP called FeedKind Protein. This product is available for

testing and will provide a good alternative for fishmeal in the aquaculture industry [106]. The Calysta's patented loop reactor technology has been demonstrated at the 10,000 ton per year scale for biological conversion of methane, and has been successfully deployed and operated at commercial scale. The unique design allows for maximal transfer of multiple gaseous feedstocks into solution to allow rapid and efficient conversion by the microorganisms. The unique feature of Calysta's loop reactor design is the use of rapid liquid flow to drive the gas mixtures downwards against gravity faster than the normal rise rate of the bubbles. This leads to pressurization of the gas in situ, dramatically improving the rate of dissolution of the gas into the liquid. The liquid flow is achieved using pumps which operate at significantly lower energy costs relative to traditional stirred tanks while achieving orders of magnitude increases in mass transfer rates [107].

Table 2: Comparison of PHA properties that determine suitability for use as plastic materials. The high melting temperature of PHB makes it unsuitable because it starts to degrade at such temperatures. Other properties such as its brittleness make it an unsuitable plastic raw material [108]

Polymer	Melting Temperature <i>T_m</i> (°C)	Glass-Transition Temperature <i>T_g</i> (°C)	Young's Modulus (GPa)	Tensile Strength (MPa)	Elongation to Break (%)
PHB	180	4	3.5	40	5
P(3HB-co-20 mol%3HV)	145	-1	0.8	20	50
P(3HB-co-6 mol%3HA) *	133	-8	0.2	17	680
Polypropylene	176	-10	1.7	38	400
Low-density polyethylene	130	-30	0.2	10	620

*HA, mixed

hydroxyalkanoate co-monomer units including 3-hydroxydecanoate (3 mol%), 3-hydroxydodecanoate (3 mol%), 3-hydroxyoctanoate (1 mol%), 3-hydroxy-cis-5-dodecanoate (1 mol%)

1.3.1 CRISPR

In the past decade, genome engineering has been furnished with a plethora of tools that has made it easier to manipulate genes in living cells. The most recent include Transcription activator-like effector nucleases (TALENs) [109], zinc-finger endonucleases (ZFNs) [110] and finally clustered regularly interspaced short palindromic sequences (CRISPR) nuclease system [111] which has received the widest attention due to its efficiency, specificity and ease of design. The CRISPR-Cas system is an RNA-guided nuclease and Cas9 in particular is currently among the most studied and utilised for genome editing. Just like TALENs and ZFN, Cas9 initiates genome editing by creating double-stranded breaks. Key to this action is the presence of two nucleases HNH and RuvC implicated in DNA and RNA interference [112]. Double stranded breaks are lethal which is how the CRISPR-Cas system provides immunity in bacteria and archaea against viruses and bacteriophage [113, 114]. Therefore cells that undergo Cas9 cleavage die unless they are rescued through two pathways: homology directed repair (HDR) which involves a repair template homologous to the region flanking the target site or non-homologous end joining (NHEJ) which occurs in the absence of a repair template (Figure 7). NHEJ is error-prone while HDR takes place with high fidelity. The intervention of both NHEJ and HDR during DNA cleavage, in combination with Cas9 can be manipulated to target and edit specific regions in the genome. This is particularly important for HDR as its efficiency can be increased ~ 1000 fold by creating DSB at the target site [115].

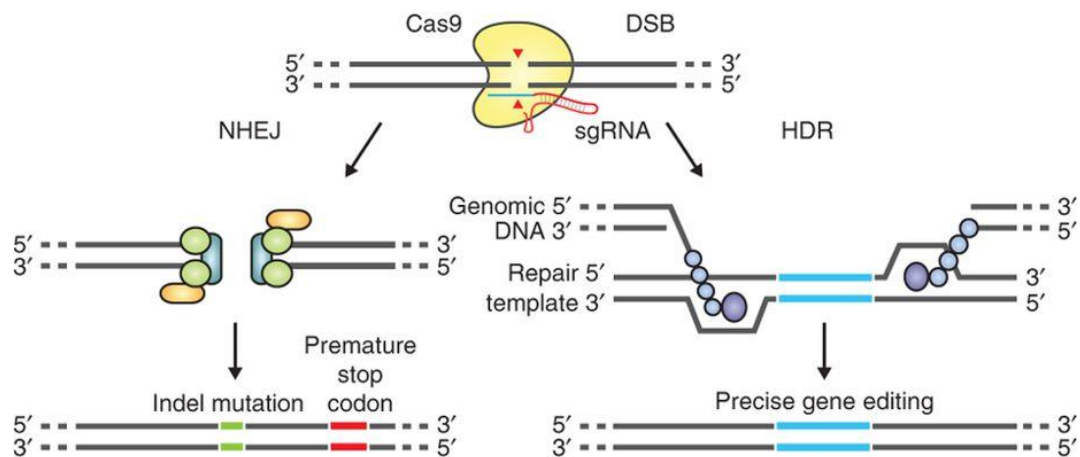


Figure 7: Gene editing is promoted by DSB induced by Cas9 (yellow) which can be repaired either by NHEJ or HDR. The repair by NHEJ is error prone which leads to indel mutations that result in frameshifts and the creation of faulty stop codon thereby causing gene knockout. Repair by HDR is carried out by plasmid or single stranded DNA oligonucleotides which results in precise and high fidelity gene editing [116].

1.3.1.1 CRISPR: A brief history

CRISPR stands for clustered regularly interspaced short palindromic repeats. These DNA repeats are found in the chromosomes of bacteria and archaea. Such repeats interspaced by random nucleotides were first observed in a study investigating *iap*, a gene in *E. coli* that converts alkaline phosphatase into its isoenzyme form. It was observed that the 3' end of this gene was flanked by five highly homologous sequences of 29 nucleotides arranged as direct repeats with 32 nucleotides interspaced between them (Figure 8) [117]. It was to be later reported in Gram-positive bacteria [118] and archaea [119]. Further studies in archaea highlighted involvement of these sequences with replicon partitioning [120].

```

TGAAAATGGGAGGGAGTTCTACCGCAGAGGCGGGGAACTCCAAGTGATATCCATCATCGCATCCAGTGCGC (1,451)
(1,452) CGGTTTATCCCCGCTGATGCGGGGAACACCAGCGTCAGGCGTGAAATCTCACCGTCGTTGC (1,512)
(1,513) CGGTTTATCCCTGCTGGCGCGGGGAACTCTCGGTTCAAGCGTTGCAAACCTGGCTACCGGG (1,573)
(1,574) CGGTTTATCCCCGCTAACGCGGGGAACTCGTAGTCCATCATTCACCTATGTCTGAACTCC (1,634)
(1,635) CGGTTTATCCCCGCTGGCGCGGGGAACTCG (1,664)

consensus: CGGTTTATCCCCGCTGGAACGCGGGGAACTC

```

Figure 8: 61 base pairs of direct repeat sequences flanking the 3'-end of *iap*. There are 29 conserved nucleotides containing a dyad symmetry of 14 base pairs underlined and shown at the bottom. Homologous nucleotide found in at least two DNA segments are in bold [117].

It would take another 10 years before CRISPR was linked to its now well established function – bacterial immunity. The authors of the study showed that the random sequences known as spacers present between the repeats in bacterial genomes pre-existed in DNA sequences of bacteriophages and conjugative plasmids. Furthermore

they observed that these extrachromosomal elements like bacteriophages failed to infect specific spacer-carrier strains, suggesting an antagonistic relationship between organisms that carry these spacers and genetic elements containing these spacer sequences [121]. A clear demonstration of this correlation was later shown in *Streptococcus thermophilus* [122]. By this time these repeats were already referred to as CRISPR. Before CRISPR, these DNA clusters were known as SRSR (short regularly spaced repeats). The term SRSR was coined in a study that used computer programmes to compare repeats in prokaryotic genome sequences [123]. CRISPR was first mentioned in a 2002 paper involving an in silico analysis to study these repeats. The paper also highlighted the association of these repeats to Cas genes [124].

Cas genes transcribe for the proteins associated with CRISPR activity. Notable among them is Cas9 which is an RNA-guided endonuclease [125]. Hence in isolation it is a nuclease and will cut random DNA sequences. Targeting its nuclease activity towards a specific DNA sequence requires the formation of a complex with CRISPR RNA (crRNA) which is transcribed from spacer DNA, and a trans-activating crRNA (TracrRNA). The ability of this complex to target a desired DNA region underpins the current widespread use of CRISPR-Cas as a genome editing tool. CRISPR RNAs were first described in an RNA study of *A. fulgidus* where 22 cDNA clones of small non messenger RNAs (snmRNA) were mapped to three different genomic loci within clusters of CRISPRs [126]. When crRNA are transcribed, they are inactive as they form a sequence of multiple spacer and repeat transcripts. RNA maturation has to occur and this was shown to be carried out by a trans-encoded small RNA called TracrRNA which have 24-nucleotide complementarity to the repeat regions of crRNA precursor transcripts. TracrRNAs were also shown to recruit endogenous RNase III and the CRISPR-associated Csn1 protein for its activity [127]. TracrRNA maturation is however not required by Cas12a, another Cas protein with nuclease activity also known as Cpf1. This protein was shown to carry out its own crRNA maturation [128]. Although CRISPR has been recognised as a system for immunity by acquiring pieces of foreign genetic elements as spacer DNA, the process of spacer acquisition was yet to be addressed. A motif ranging from 3 – 7 DNA base pairs associated with spacer acquisition was first detected in *Streptococcus thermophilus* [129]. These motifs later

Table 3: Structures, domain architectures, and functions of the core components of CRISPR-Cas systems [130]

Family	Biochemical/in silico evidence	Examples of available structures and structural features
Cas1	Metal-dependent deoxyribonuclease; deletion of Cas1 in <i>E. coli</i> results in increased sensitivity to DNA damage and impaired chromosomal segregation.	PDB: 3GOD, 3LFX, 2YZS Unique fold with two domains: N-terminal β -stranded domain and catalytic C-terminal α -helical domain
Cas2	RNase specific to U-rich regions. Double-stranded DNase.	PDB: 2IVY, 2I8E, and 3EXC RRM (ferredoxin) fold
Cas3 (helicase and HD domain)	Single-stranded DNA nuclease (HD domain) and ATP-dependent helicase; required for interference.	
Cas3" stand-alone HD nuclease	Metal-dependent deoxyribonuclease specific for double-stranded oligonucleotides.	PDB: 3S4L and 3SKD
Cas4	PD-(DE)xK superfamily nuclease with three-cysteine C-terminal cluster; possesses 5'-ssDNA exonuclease activity.	PDB: 4IC1
Cas5	Subunit of Cascade complex interacting with large subunit and Cas7 subunit and binding the 5'-handle of crRNA. In the subtype I-C system Cas5 is the ribonuclease that replaces Cas6 function.	PDB: 3KG4; 3VZI; 3VZH Two domains of RRM (ferredoxin) fold, the C-terminal domain is deteriorated in many Cas5 protein of type I; RAMP superfamily
Cas6	Metal-independent endoribonuclease that generates crRNAs.	PDB: 2XLJ, 1WJ9, 3I4H, 4C8Z, 4DZD Two domains of RRM (ferredoxin) fold, RAMP superfamily
Cas7	Subunit of Cascade complexes binding crRNA; often present in Cascade complexes in several copies.	PDB: 3PSO, 4NOL
Cas8abcef (large subunit)	Subunit of Cascade complex, involved in PAM recognition	PDB: 4AN8
Cas10 (large subunit)	Subunit of Cascade (Cmr) complex.	PDB: 3UNG, 4DOZ
Small subunit	Small, mostly alpha helical protein, subunit of Cascade complex.	PDB: 2ZCA (Cse2); 2ZOP, 2OEB (Cmr5); 3ZC4 (Csa5) Cse2 has two alpha helical bundle-like domains; Cmr5 has a domain matching N-terminal domain of Cse1 and Csa5 has a domain matching C-terminal domain of Cse2
Cas9	In type II CRISPR-Cas systems, Cas9 is sufficient both to generate crRNA and to cleave the target DNA, although it requires the help of a housekeeping gene coding for RNase III and a special gene tracrRNA encoded in the respective CRISPR-Cas locus. Both the RuvC and HNH nuclease domains of Cas9 are involved in the cleavage of the target DNA.	PDB: 4OGC, 4OO8, 4CMP Cas9 has several subdomains and adopts a two-lobed general structure. Beyond two catalytic nuclease domain its subdomains do not appear to be similar to other known protein structures

referred to as protospacer adjacent motifs (PAMs) vary depending on the Cas system (5' NGG 3' for *Streptococcus pyogenes* Cas9 [131] and *Francisella tularensis* Cas12a 5'-

TTN [128]), and were shown to be present in arrays of all CRISPR types [132]. PAMs became crucial in designing guide RNAs for programmable genome editing. Perhaps the most notable study which influenced the outlook of CRISPR as a tool for genome editing demonstrated a prerequisite of crRNA and tracrRNA base pairing, as well as the presence of a PAM. The study further showed that Cas9 endonuclease family could be programmed with RNA molecules to cleave target DNA sites resulting in DSBs for genome editing [125].

1.3.1.2 Classification of CRISPR Systems

Understanding regarding CRISPR has evolved rapidly which has had significant impact on the classification of the system. CRISPR-Cas systems are broadly classified based on *cas* gene and Cas protein nomenclature, functionality, architecture as well as phylogeny [129] (Figure 9). When a link was observed between CRISPR repeats and *cas* genes, four names were proposed for the most conserved and abundant CRISPR-Cas systems: Cas1, Cas2, Cas3 and Cas4 [133]. Further bioinformatic analyses of CRISPR containing organisms showed many more distinct sets of orthologous Cas proteins [134]. This eventually led to the discovery and classification of many more Cas proteins. For CRISPR-Cas systems to confer immunity, three main processes are involved: adaptation, expression and interference [135-137]. These processes are carried out by distinguishable genomic CRISPR modules which form the basis for grouping all CRISPR-Cas systems into two broad classes (1 and 2). The two classes are subdivided into six types (I – VI) and numerous subtypes. Class 1 CRISPR–Cas systems are defined by the presence of a multi-subunit crRNA–effector complex and they include type I, III and IV. Class 2 is also made of three types (II, V and VI) which are defined by the presence of a single subunit crRNA–effector module [138]. Type VI is the most recently discovered which includes Cas13a, b and c (Figure 10) [139].

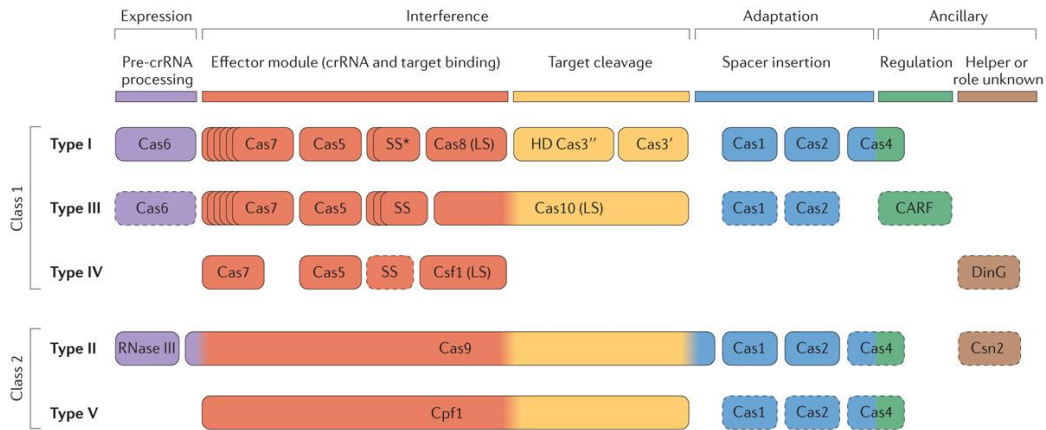


Figure 9: Functional classification of Cas proteins: The asterisk indicate fusion of the putative small subunit (SS) protein with Cas8. Components that are dispensable are indicated by dashed outlines. Cas4 and Cas9 are represented by two and three colours respectively to reflect that these proteins contribute to different stages of the CRISPR-Cas response [140].

During the adaptation process in bacterial or archaeal immunity, fragments of DNA from invading viruses and plasmids known as protospacers are incorporated into CRISPR arrays as new spacers. Cas1 and Cas2 which make up the adaptation module are found in most CRISPR-Cas systems and they form a complex required for insertion of protospacers into CRISPR arrays [141, 142]. Cas4, a restriction endonuclease superfamily enzyme has also been implicated in adaptation [143]. In *Streptococcus thermophiles*, Cas9 was shown to be involved in adaptation of Type II CRISPR systems [144]. Expression and interference on the other hand are carried out by CRISPR-Cas modules that highly vary across CRISPR-Cas systems [145].

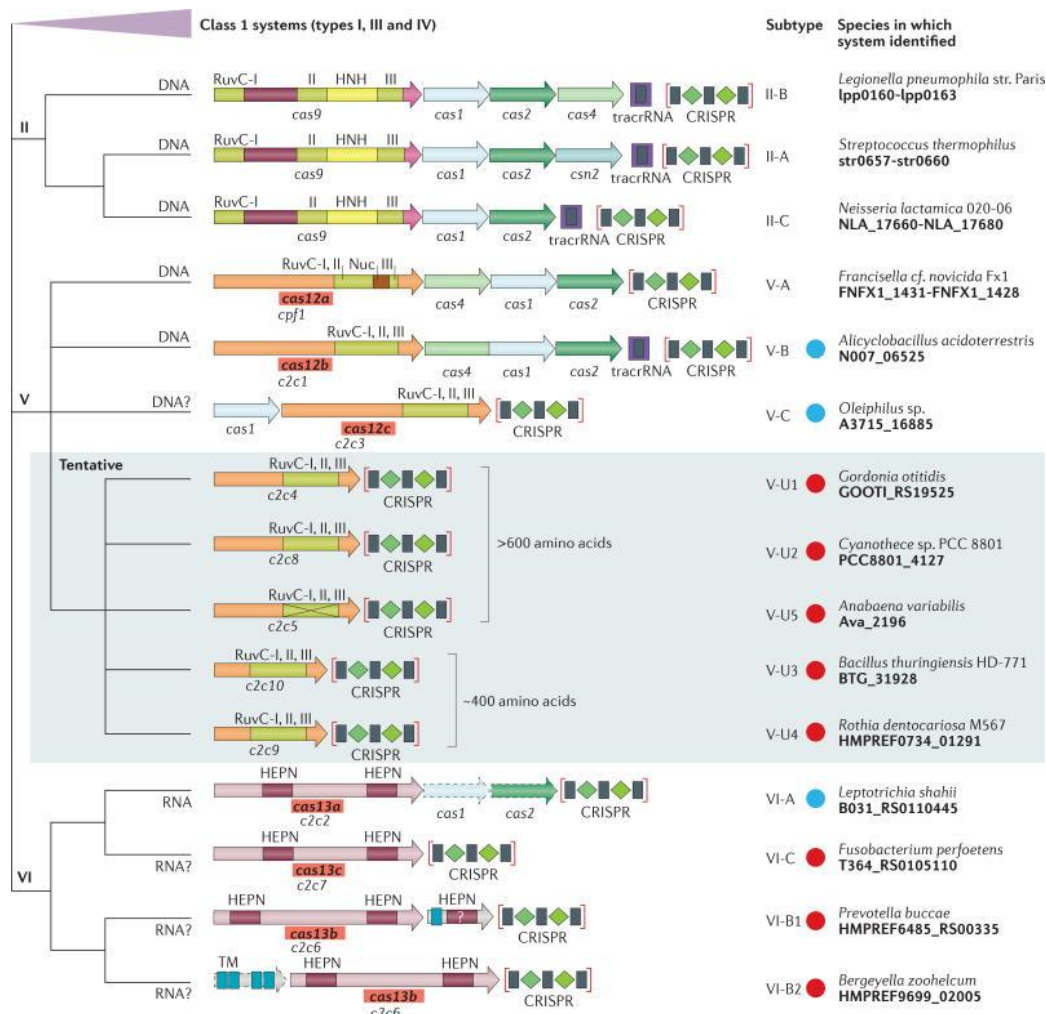


Figure 10: An update of class 2 CRISPR-Cas systems including type VI which consists of Cas 13a, b and c. The locus organization and the domain architecture of the effector and accessory proteins are schematically shown. RuvC contributes to the nuclease catalytic centre and the motif of each RuvC is represented by numerals. The specific strain of bacteria in which these systems were isolated and the locus tags for the respective genes are indicated. TM indicates a predicted transmembrane helix; Systems in blue circles have been described previous and those in red circles are new [139]

Expression involves transcription of the CRISPR array into precursor transcripts (pre-crRNA) which are then processed and matured through cleavage action of RNA endonuclease (usually Cas6) in type I and type III systems [146]. Type II involves an interaction between RNase III and a transactivating CRISPR RNA (tracrRNA) to form mature CRISPR RNA (crRNA) as seen in Cas9 [127]. The process of mature crRNA binding to effector complex (type I and III) or Cas9 (type II) and subsequent cleavage of target DNA region fall within the interference module. In type I, cleavage of target DNA region is catalysed by the HD family nuclease (Cas3'' or a Cas3 domain) [147, 148]. In type III, the combined action of Cas7 and Cas10 proteins are responsible, while Cas9 is responsible in type II [149-152]. It is reemphasized here that Cas9

protein has all the functional components required for CRISPR-based immunity (adaptation, expression and interference).

1.3.1.3 Applications of CRISPR

The driving force behind CRISPR technologies is the potential it holds in disease treatment, crop improvement and genetic engineering among others and the ease to design CRISPR systems for these purposes. For example genome editing using CRISPR was shown to remove the defective gene in mice with Duchenne muscular dystrophy which is among the early successful treatments of genetic disease in animals using CRISPR [153]. CRISPR-Cas9 was used to eliminate the highly conserved 5' and 3' long terminal repeat (LTR) region from HIV-1 DNA in CD4+ T-cells and prevent new HIV-1 infection [154, 155]. CRISPR-Cas9 as a genome editing tool was demonstrated in the *Plasmodium* that causes malaria infection [156]. CRISPR was also used to develop gene drive systems that target gene conversion at high efficiencies in transgene heterozygotes of the malaria carrier, *Anopheles stephensi*. The offspring of the mosquitoes carry dual antiparasite effector genes which target the human malaria parasite *Plasmodium falciparum* thus reducing the ability of the mosquito population to carry the parasite and spread disease [157]. In cancer research, CRISPR applications include but are not limited to modelling cancer mutations [158], screening of cancer related genes [159], genome-editing based cancer therapy [160], and immunotherapy [161]. In crop development, CRISPR was used to alter the size and plant architecture of tomato [162], in livestock, sheep coat colour pattern was altered [163] and in embryos, pathogenic gene mutations were corrected [164]. CRISPR has been applied in other organisms such as fruit fly [165], zebrafish [166] and worms [167]. Most relevant to this study, CRISPR has been applied in archaea and bacteria [168-180].

1.3.1.4 Factors increasing efficiency of CRISPR-Cas gene editing

CRISPR-Cas genome editing results from repair either by NHEJ or HDR. NHEJ mechanism for DSB repair although efficient, is absent in most bacteria. Hence gene editing in bacteria is usually carried out by introducing a repair template for HDR. However, HDR is highly inefficient due to the inefficiency of homologous recombination across living cells. A number of studies have focused on how to increase the efficiency of HR. As stated earlier, the efficiency of HR can be increased

about 1000 fold by introducing a DSB at the HR target site [115]. This can be achieved by RNA-guided Cas9 nuclease activity [181]. Even with RNA-guided Cas9 DSBs, the efficiency is not optimal. Hence several approaches to further increase efficiency have been investigated. One of them includes the use of single stranded oligodeoxyribonucleotides (ssODNs) as DSB repair templates instead of double stranded homology arms. Chemical modifications such as phosphorothioate-modified oligonucleotides even enhanced the efficiency of ssODNs as shown in cultured cells [182]. Another factor that affects the efficiency of HDR is NHEJ. As such, methods used to suppress NHEJ have been also shown to increase the efficiency of HDR. An approach that is becoming popular is the use of a DNA ligase IV inhibitor. DNA ligase is a key enzyme of NHEJ pathway, therefore chemicals like the pyrazine Scr7 (2,3-dihydro-6,7-diphenyl-2-thioxo-4(1H)-pteridinone, 6,7-Diphenyl-2-thio-lumazine (8Cl)) that inhibit DNA ligase and interfere with progression of NHEJ events successfully increased efficiency of HDR [183, 184]. The NHEJ DNA repair mechanism is not common in bacteria. A genome wide study revealed that NHEJ and Class II CRISPR-Cas system co-occur once in 5563 analysed sequences. The mechanism of NHEJ inhibition in bacteria may thus vary slightly. Nonetheless, evidence has been provided for NHEJ inhibition by Csn2 protein in bacteria [178]. The efficiency of HDR was shown to be influenced by DNA repair template design and mode of delivery in human induced pluripotent cells [185]. An important aspect of having a successful CRISPR-Cas DSB is guide RNA design. Although a lot of effort has been put into improving the efficiency of HDR, ways of increasing the efficiency of NHEJ have also been investigated. For example guide RNA orientation has been shown to influence NHEJ efficiency. In a CRISPR study, it was concluded that gRNAs are more effective if they anneal to the template DNA strand of transcribed genes. This enables Cas9 to be dislodged from DNA cleavage site which allows DNA repair mechanisms like DNA ligase IV in mammalian cells better access to the cut site, leading to more efficient NHEJ [186]. There are many factors that influence the successful application of CRISPR-Cas for genome editing – guide RNA design, DNA repair template length and design, toxicity of Cas9, efficient host homologous recombination system and host factors among others. More importantly, for a gene to be edited, it has to be non-essential due to the lethal consequences of editing an essential gene. Hence it is important to

determine gene essentiality before selecting it as a target for genome editing. This is where important forward genetic tools such as TraDIS play a key role in determining experimental designs related to genome editing because of the insight it provides regarding the significance of various genes in an organism.

1.3.2 Transposons

Transposons also known as transposable elements (TEs) or jumping genes are genetic elements that can randomly move from one region of the genome to another by either replicative or 'cut-and-paste' mechanisms. Transposons are abundantly found in both prokaryotes and eukaryotes and in some cases such as the maize plant comprise more than 50% of the genome [187]. They were first discovered by Barbara McClintock in the corn plant where it was shown that the genome was not stationary, but subject to alterations and rearrangements [188]. More importantly it was observed that the movement of genes was sometimes autonomous and other times not [189]. The consequence of genes changing position can manifest in genomic alterations which lead to insertions, duplications, excisions or translocations in the site of integration [190-194]. They can also lead to reorganisation of the genome and loss of genetic DNA by internal deletion. As a result they have been linked to evolution of speciation [195], antibiotic resistance [196] and disease development [197]. Transposons are used for numerous biological and genetic applications. For example the eukaryotic transposon Sleeping Beauty is used as a tool for forward genetic screens for cancer genes *in vivo* [198]. Mariner [199] and P-elements [200] are used as tools for genetic transformation of organisms such as flies. Ac/Ds has been used to identify promoters in tomato [201]. Tn5 has been used as a useful tool to study gene essentiality using TraDIS in *Salmonella* species [35, 202] and *E. coli* [203].

1.3.2.1 Types of Transposons

There are two types of TEs: Class I and Class II. Class I transposons (retrotransposons) first transcribe DNA into RNA and then use reverse transcriptase to make a DNA copy of the RNA to insert in a new location [204]. Depending on the presence of long terminal repeats (LTRs) associated with the retroelement body, Class I is further subdivided into two main groups (Table 4) [205, 206]. DNA transposons move either through a replicative or a non-replicative mechanism and they are made of a transposase and the core transposon flanked by two terminal inverted repeats (TIRs) [207]. When the transposase gene is expressed, the enzyme recognises the TIRs, excises the transposon body and inserts it in a new genomic location. After insertion of the transposon, duplication occurs at the origin of the transposon known as target

site duplication and this is unique to different DNA transposons. For the purpose of applying transposons as tools for forward and reverse genetics this review will be limited to Tc/mariner and Tn5 transposons.

Table 4: Types of transposons, structural features and mechanisms of movements [208]

Type	Structural Features	Mechanism of Movement	Examples
DNA-mediated transposition			
Bacterial replicative transposons	Terminal inverted repeats that flank antibiotic-resistance and transposase genes	Copying of element DNA accompanying each round of insertion into a new target site	Tn3, $\gamma\delta$, phage Mu
Bacterial cut-and-paste transposons	Terminal inverted repeats that flank antibiotic-resistance and transposase genes	Excision of DNA from old target site and insertion into new site	Tn5, Tn10, Tn7, IS911, Tn917
Eukaryotic transposons	Inverted repeats that flank coding region with introns	Excision of DNA from old target site and insertion into new site	P elements (<i>Drosophila</i>), hAT family elements, Tc1/Mariner elements
RNA-mediated transposition			
Virus-like retrotransposons	~250- to 600-bp direct terminal repeats (LTRs) flanking genes for reverse transcriptase, integrase, and retrovirus-like Gag protein	Transcription into RNA from promoter in left LTR by RNA polymerase II followed by reverse transcription and insertion at target site	Ty elements (yeast), Copia elements (<i>Drosophila</i>)
Poly-A retrotransposons	3' A-T-rich sequence and 5' UTR flank genes encoding an RNA-binding protein and reverse transcriptase	Transcription into RNA from internal promoter; target-primed reverse transcription initiated by endonuclease cleavage	F and G elements (<i>Drosophila</i>), LINE and SINE elements (mammals), Alu sequences (humans)

1.3.2.2 Tc1/mariner

The mariner family is often regarded as the most widely distributed family of transposons in nature. It is found in such diverse taxa as insects, plants, nematodes, fungi, fish, ciliates, rotifers and mammals [209-211]. The majority of them harbour inactivating mutations, some of which have been reconstructed to become active such as the Sleeping Beauty from salmonid like fish [212] and Himar 1 from the horn fly [213]. Tc1/mariner elements are between 1300 – 2400 bp long and they contain a single gene encoding a transposase enzyme 282 to 345 amino acids long flanked by two terminal inverted repeats which can vary from 17 to 1100 bp [211]. Two domains are unique to the transposase protein from different Tc1/mariner elements: an amino-terminal region containing the helix-turn-helix (HTH) motif used for recognition and binding of TIRs, and a carboxy-terminal domain harboring the catalytic motif constituted by three amino acids, DDD in the case of mariner-like elements, or DDE in the case of Tc1-like elements. The Tc1/mariner element is mobilised through non-replication process using a cut and paste mechanism and this process does not require ATP because the energy for insertion comes from the

exergonic cleaving of target DNA [214]. The preference of TA dinucleotide targets in genomes make mariner transposon a desired candidate for random genetic mutagenesis applications in organisms [215], especially those with low GC and high TA content such as *Clostridia perfringens* [216] and *Clostridia difficile* [217].

1.3.2.3 Tn5 Transposon

Although eukaryotic transposons have been adapted for use in prokaryotes, several prokaryotic transposons were subsequently discovered, a lot of which are found in *E. coli*. Based on the mechanism of transposition, transposons in *E. coli* fall into three general classes: (I) Those that transpose through a two-step replicative process such as Tn3 and Tn1000; (II) Those that transpose through a conservative cut-and-paste mechanism for example Tn5, Tn10 and Tn903 (Figure 11); (III) Bacteriophage Mu and related viruses. In the last category, transposition can occur through either of the first two mechanisms, depending on the proteins involved and the precise nature of the DNA strand cutting after an intermediate is formed between the transposable element and the target DNA sequence. In both conservative and replicative transposition, two critical elements are required: a unique sequence at the end of the transposon and the transposase enzyme [218]. Tn5 was among the first prokaryotic transposons discovered [219]. Its early discovery and simple macromolecular requirement for transposition made it a choice candidate for study. Tn5 exemplifies the symbiotic relationship viewed between transposons and their bacterial hosts where the former provides means of coping with environmental stress for example antibiotic resistance, and the latter provides the environment necessary for replication [220]. It is a 5820 bp composite transposon that contains genes conferring resistance to the antibiotics kanamycin, bleomycin and streptomycin flanked by a pair of IS50 elements (IS50L and IS50R)[221].

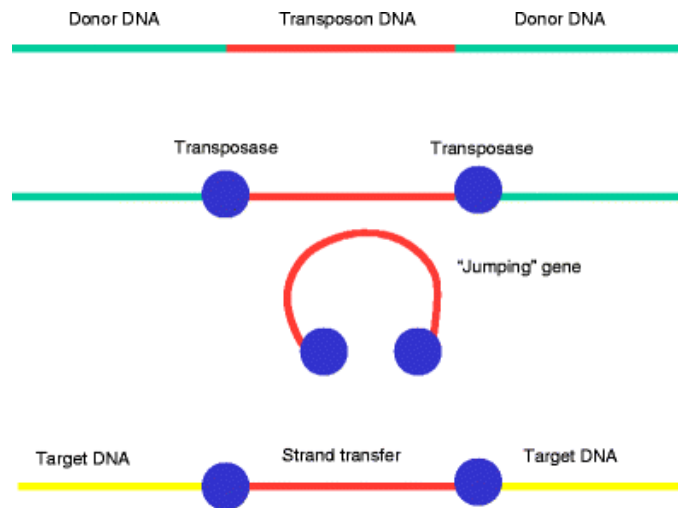


Figure 11: Mechanism of cut and paste transposition. It involves the transposase (blue spheres) binding to specific sites at the ends of the transposon DNA in red. The two ends of the transposon are brought together in a complex, after which the transposase cuts together the transposon DNA from the flanking donor DNA (green). After cleavage, the transposon randomly moves about until it comes across and binds to the target DNA (yellow) [204].

The peripheral 19 bp insertion sequences [222] and the 476 amino acid transposase (Tnp) [223] encoded in the IS50R are essential for transposition. *Inh* is a second IS50R protein expressed in the same reading frame as *tnp* although it has a different promoter, transcriptional and translational start site which results in a 421 bp amino protein that lacks the first 55 residues of Tnp (Figure 12) [224].

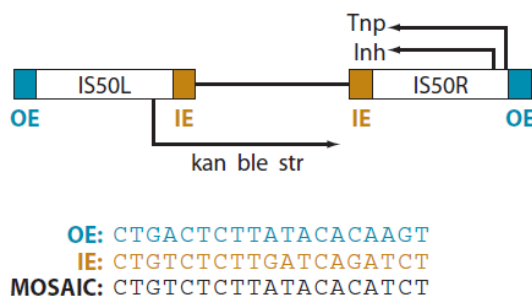


Figure 12: The Tn5 transposon. Tn5 is made of two insertion sequences (IS50L and IS50R) flanking three antibiotic resistant genes. Only the IS50R is active and encodes the *tnp* and *inh* genes. Both IS elements are flanked by two End Sequences (ES) - IE and OE [225].

Tn5 transposition is tightly regulated and has a strong *in vivo* bias for occurring in *cis* [220]. This seems rational as continued transposon and host survival will likely depend on a balance between enough transpositions to allow for maintenance and spread of transposon vs transposition's lethal consequences such as host DNA damage from transposon excision and formation of deleterious mutations. Therefore low transposition frequency decreases the probability of genetic damage to the host

while the *cis* bias leads to transposition coinciding with passage of replication fork during DNA replication, thus resulting in donor DNA loss not being a lethal event [225]. As a result, the tight regulation of Tn5 transposition is elaborate and is influenced by how the *tnp* gene is expressed, Tnp protein interactions and the host carrying the transposon. With regards to regulation of *tnp* gene expression, there is a case of *tnp* gene having a weak promoter [224]. Additionally, *tnp* gene expression is not influenced by read-through activity of adjacent promoters because the transcripts from these promoters interact with the translational start site (TSS) of *tnp* to form an RNA secondary structure. This abolishes the TSS and prevents *tnp* gene expression [226]. Dam methylation has also been shown to influence *tnp* gene expression [227]. After expression and translation into transposase, regulation is carried out primarily by Inh [228]. The activity of Inh is likely as a result of interaction of both proteins that leads to a transposase-inhibitor oligomer. Findings from the same study also suggest an inhibitory action caused by Tnp-Tnp oligomerisation. This can further explain why overexpression of the *tnp* gene can lead to lower transposition efficiencies. The tight regulation of Tn5 can be alleviated by replacing glutamate with alanine at position 110 and 345 of the Tnp. This was shown to increase transposition efficiency by 6 – 16 fold [220].

The information gathered from studying the structure and mechanism of Tn5 action has facilitated its application in many areas such as its use as a surrogate for studying HIV-1 integrase because of the rare protein-DNA co-structural information it provides making it an attractive target for inhibitor development [229]. It has also been used to identify highly expressed genes in methanotrophs [33]. Other genetic applications of Tn5 have been reviewed by Weznikoff [225]. The use of Tn5 as a tool to identify genes responsible for a particular phenotype in an organism (forward genetics) is however becoming more popular due to increasing sequencing methods and capabilities. Tn5 stands out for this application because during transposition, its insertion is random across the genome. This is not the case with some other transposons. For example Tn7 has a bias towards high frequency insertion in specific chromosomal sites in *E. coli* called *attTn7* [230].

1.3.2.4 *TraDIS*

The use of Tn5 for forward genetics is more prevalent in insertional mutagenesis in which a library of bacterial strains, each containing a single randomly located transposon, is assembled. With the advent of massively parallel sequencing (MPS), also known as second generation sequencing, it is now possible to sequence this library of transposon mutants using different techniques referred to as transposon sequencing. Some of these techniques include transposon sequencing (Tn-Seq) [231, 232], high-throughput insertion tracking sequencing (HITS) [233], insertion sequencing (INSeq) [234] and transposon directed insertion-site sequencing (TraDIS) [35]. The principle behind them involves making a library of mutants, extracting their genomic DNA, digesting the extract into short DNA fragments, attaching adaptors to the fragments to enable PCR amplification and finally sequencing the amplified fragments to find out where the transposon has inserted and how many mutants contain insertion in the same site. If a lot of mutants contain insertion in one location or gene, it suggests the gene is not essential otherwise the insertion will be lethal as it will disrupt the gene function. If there are no insertions at certain locations or genes then it is likely that such genes are essential and the mutants did not survive those insertions, hence their genomes were not available for sequencing as they all died [235]. Gene essentiality data are important in metabolic engineering for determining candidate genes for knock-outs. The information it provides can also be used to target virulence genes of bacterial pathogens [236].

The difference between four popular insertion sequencing technologies are illustrated in Figure 13. TraDIS will be more suitable for this study as Tn5 widely used for TraDIS has been shown to work in methanotrophs [33]. Since its introduction in 2009, a few changes have been made to the TraDIS toolkit to improve its efficiency and accuracy. These changes are detailed by Barquist *et al.* [237] and are briefly described as: Redesign of adapters and primers to increase enrichment of genuine transposon-chromosome junctions. This is done by preventing hybridization of the reverse primer until the transposon-specific forward primer has generated a complementary strand; Taq polymerase DNA was used to substitute Kapa HIFI DNA polymerase and magnetic bead-based fragment size selection was substituted for gel-based size selection to

increase yield and allow for easier automation. Other changes of importance that have been made include developing new HiSeq and MiSeq protocols to rectify the challenges encountered by the base-calling algorithms due to dark cycles caused by transposon tags. To support these changes a portable processing and analysis TraDIS pipeline was developed [237].

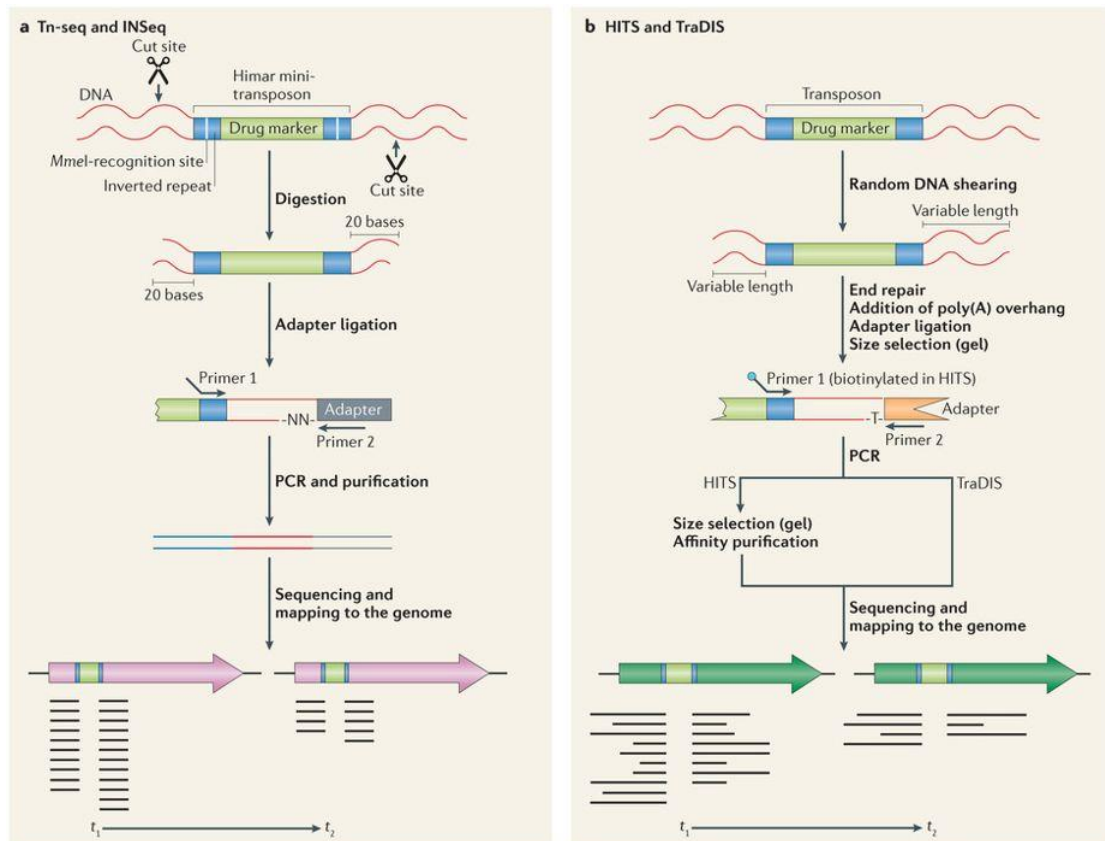


Figure 13: Methods of massively parallel genome sequencing: All four technologies start with a pooled genomic DNA of a mutant library and end with sequencing the right and left transposon junctions. Tn-seq and INSeq are similar except Tn-Seq includes agarose gel purification while INSeq includes a PAGE gel purification after adaptor ligation and PCR to amplify insertion regions. HITS and TraDIS are more similar and differ after the PCR to amplify the insertion regions. In HITS the PCR product undergoes size exclusion and affinity chromatography while in TraDIS the PCR products are sequenced directly [235].

In order to carry out TraDIS, random transposon mutagenesis will have to be established on methanotrophs and a library of mutant has to be generated. Chapter 5 will focus on the preliminary work required to carry out TraDIS.

1.4 Aims and Objectives of study

The aim of this study was to isolate methanotrophs from different environments, characterise them and develop forward and genetic tools for harnessing their potential in bio-based chemical production.

The objectives include:

- 1- Sample collection from environment and enrichment of these samples in different culture media using different protocols for isolation of methane oxidising bacteria.
- 2- Characterisation of isolated strains based on morphology, physiology, metabolism and genetics.
- 3- Establishing tools for random transposon mutagenesis involving suicide plasmids with the ultimate aim of carrying out transposon directed insertion site sequencing (TraDIS) in order to determine gene essentiality in isolated strain(s) of methanotrophs.
- 4- Establishing tools for gene knockout and knock-in using CRISPR genome editing.

2 Chapter 2: Materials and Methods

This chapter focuses on the materials and methods used throughout this study and therefore covers some aspects of all four result chapters of this work. However, it does not cover the materials and methods unique to isolation of methanotrophs; characterisation of methanotrophs; transposon mutagenesis and CRISPR-Cas genome editing. Hence each chapter reporting experiments was accompanied by a specific Material and Methods section. This approach was taken to simplify linking methods used for experiments with the results. Additionally, this approach enabled the avoidance of very long tables such as primer and plasmid tables.

This chapter mainly involves cloning techniques which was carried out in *E. coli* XL-1 Blue and NEB highly competent DH5 α cells. These two strains were used for cloning because better DNA yields were obtained when plasmid miniprep was carried out. Additionally they were both obtained commercially and were found to be reliable. DH5 α in particular was highly competent and more efficient in accepting foreign DNA during cloning which was crucial when transformation of HiFi ligation products was carried out. *E. coli* S17 λ pir on the other hand was used as the conjugative strain because it was capable of transferring plasmid DNA into methanotrophs due to the DNA transfer genes integrated in its chromosome [34, 238].

This chapter also detailed the process of growing methanotrophs on liquid and agar plates; long term storage at -80°C; as well as revival of freeze dried samples of methanotrophs.

2.1 *E. coli* strains and culture conditions

LB-media (Luria-Bertani) was used for culturing *E. coli*: It was made of the following constituents per litre: tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g; agar (for plates only), 10 g. *E. coli* cultures were grown at 37°C and liquid cultures were shaken at 200 rpm. Antibiotics were used at the following concentrations: tetracycline (10 μ g/mL), erythromycin (200 μ g/mL), kanamycin (50 μ g/mL) and chloramphenicol (20 μ g/mL). Long term storage of *E. coli* cultures was carried out at -80°C on microbeads

(Microbank™ Bacterial and Fungal Preservation System, Pro-Lab Diagnostics, UK) according to supplier's instructions.

Table 5: *E. coli* strains used in the study

Strain	Description	Reference
DH5α	<i>F' endA1 hsdR17(r_k⁻,m_k⁻) supE44 thi-1 recA1 gyrA (Nal^r) relA1Δ(lacIZYA-argF) U169 deoR (ϕ 80dLac Δ(lacZ)M15)</i>	[239]
DH5α (High Efficiency from NEB)	<i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	[240]
S17-1	<i>Thi, pro hsdR⁻, hsdμ⁺, recA; integrated plasmid RP4-Tc::Mu-Kn::Tn7</i>	[241]
S17-1 λ pir	<i>E. coli</i> S17-1 λpir strain contains the pir gene (λpir). This strain has conjugal transfer functions (RP4 transfer functions) integrated into the chromosome. The genotype of the strain is <i>TpR SmR recA, thi, pro, hsdR-M+RP4: 2-Tc:Mu: Km Tn7 λpir</i> .	[242]
XL-1 Blue	XL-1 cells are tetracycline resistant. XL1-Blue cells are endonuclease (endA) deficient, which greatly improves the quality of miniprep DNA, and are recombination (recA) deficient, improving insert stability. The hsdR mutation prevents the cleavage of cloned DNA by the <i>EcoK</i> endonuclease system. The <i>lacIqZΔM15</i> gene on the F' episome allows blue-white colour screening. The genotype is: <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tetr)]</i> . Genes listed signify mutant alleles. Genes on the F' episome, however, are wild-type unless indicated otherwise.	[243]

2.2 Preparation of chemically competent *E. coli* cells

Chemically competent *E. coli* cells were prepared using a protocol from NEB [244]. In brief, 0.5 mL bacterial overnight culture was subcultured in 50 mL of LB media and grown at 37°C and 200 rpm till OD₆₀₀ of 0.5 – 0.7 was attained. 25 mL each was transferred to two 50 mL Falcon tubes (25 mL) and spun at 8000 rpm for 5 minutes. The pellet was resuspended in 5 mL ice-cold 30 mM CaCl₂. 1.5 mL each was transferred to six different 2 mL Eppendorf tubes and spun in a micro centrifuge for 30 seconds. Each pellet was resuspended in 0.5 mL of 30 mM ice-cold CaCl₂ by flicking. 50 μL was then aliquoted into a 1 mL Eppendorf tube and stored at -80°C. Cells such as *E. coli* XL-1 Blue and DH5α (High Efficiency from NEB) were in their competent form when purchased from Agilent and NEB respectively.

2.3 Transforming chemically competent *E. coli* cells

Plasmid DNA was diluted to approximately 100 ng/μL before using for transformation. Ligation mixture was used without dilution. 2 μL of DNA was pipetted into a tube of chemically competent *E. coli* cells and incubated on ice. After 30 minutes of incubation on ice, the tube was transferred into a 42°C water bath for 30 seconds (XL-1 Blue, DH5α and DH5α High Efficiency from NEB) or 45 seconds (S17-1, S17-1 λ pir). Afterwards, cells were placed on ice for two minutes before adding 500 μL of super optimal broth (SOC) media. Finally, cells were allowed to recover for 30 – 45 minutes at 37 °C and 200 rpm. 100 μL of recovered cells were spread on antibiotic plates or alternatively, the recovered cells were spun at 8000 rpm for 3 minutes and resuspended in 100 μL which was spread on LB plates containing the desired antibiotics.

2.4 DNA Extraction and purification

2.4.1 Plasmid miniprep

Plasmid miniprep was carried out for plasmid DNA extraction using various kits and was performed according to the manufacturer's instructions. The following kits were used:

I- QIAprep® Miniprep Kit (Qiagen, UK)

II- GenElute™ Plasmid Miniprep Kit (Sigma Aldrich, UK)

III- Monarch® Plasmid Miniprep Kit (NEB, UK)

The choice of kit used was mostly to do with ordering and availability rather than quality. Volume of overnight culture used for miniprep varied from 2 – 10 mL depending on expected DNA yield (high DNA yield was obtained with small volumes when miniprep of high copy number plasmid was carried out). Although DNA elution buffers were included in the kits, elution was also carried out using Nuclease Free Water (NFW). Elution was done using 50 μL of NFW or elution buffer when all kits were used. In some cases where low DNA yield was expected (when dealing with low copy number plasmids), elution with as much as 20 μL of NFW was carried out.

2.4.2 DNA recovery from gel

Gel extraction was carried out using the following kits:

- I- QIAquick® Gel Extraction Kit (Qiagen, UK)
- II- Monarch® DNA Gel Extraction Kit (NEB, UK)
- III- GenElute™ Gel Extraction Kit (Merck, UK)
- IV- Zymoclean™ Gel DNA Recovery (Zymo Research, UK)

The gel content of the DNA was always agarose based. This was weighed prior to using the kits to determine how much of gel dissolution buffer to add. Zymoclean had the lowest elution volume of $\geq 6 \mu\text{L}$ of elution solution.

2.4.3 DNA purification

This was carried out using the following kits:

- I- DNA Clean & Concentrator™ (Zymo Research, UK)
- II- Monarch® PCR & DNA Cleanup (NEB, UK)

Samples used for this application were PCR products and plasmid DNA samples that needed concentrating.

2.4.4 Genomic DNA extraction

2.4.4.1 Genomic DNA extraction using kits:

The following kits were used for genomic DNA extraction:

- I- GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, UK)
- II- Quick DNA Fungal/Bacterial Miniprep Kit (Zymo Research, UK)

Genomic DNA was performed according to kit's manufacturer instructions. Volume of methanotrophs used for DNA extraction was approximately 10 mL at $\text{OD}_{600}1.0$. Quick DNA Fungal/Bacterial Miniprep Kit includes a bead beater step which is especially effective in breaking cell membranes of type II methanotrophs thereby producing high gDNA yields.

2.4.4.2 Phenol-Chloroform Extraction

For health and safety reasons, the use of phenol-chloroform is closely monitored and gDNA extraction using this technique was carried out according to the guidelines of the SBRC Standard Operating Procedure (SOP). This method was used to minimize gDNA fragmentation which is undesirable if the intended use is Next Generation Sequencing (NGS). The following materials and reagents were used:

Lysis buffer (Lysosyme 10 mg/ml in PBS); proteinase K; RNase A; phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma: P3803 – 400 ml); 3M sodium acetate; ethanol; 10% SDS; Phase lock tubes (Phase lock gel heavy – 2.0 ml); and disposable glass pipettes.

The procedure was carried out as follows:

5 – 10 mL of culture approximately OD₆₀₀ 1 was pelleted by centrifugation at 8000 rpm for 5 – 15 minutes. This varies as observation showed *M. parvus* (OBBP) to be particularly difficult to pellet, hence requiring longer centrifugation time (10 – 15 minutes). The pellet was resuspended in 180 µL of lysis buffer and incubated at 37°C for 30 minutes. After addition of 4 µL of RNase A, the mixture was incubated at room temperature for 15 – 30 minutes. 25 µL proteinase K, 85 µL deionised water and 110 µL 10% SDS was then added and mixed by inversion of the tube. This was incubated at 65°C for 30 minutes. An equal volume of phenol:chloroform:isoamyl alcohol was added and the sample was thoroughly mixed, transferred to a heavy phase-lock tube and centrifuged at 13, 300 rpm for 3 minutes. The top phase was transferred into a fresh heavy phase-lock tube containing 400 µL of phenol:chloroform:isoamyl alcohol. This was mixed thoroughly and centrifuged at 13, 300 rpm for three minutes. The top layer was transferred into a fresh heavy phase-lock tube. 10 µL of RNase A was added and incubated for 15 – 30 minutes after which 400 µL of phenol:chloroform:isoamyl alcohol was added, mixed thoroughly and centrifuged at 13, 300 rpm for three minutes. The top layer was transferred into a 1.5 mL Eppendorf tube containing 40 µL of 3M sodium acetate and 800 µL of 100% ethanol. This was mixed gently and placed on ice immediately after which the DNA can be seen falling out of suspension. A glass pipette was used to transfer the DNA pellet to a fresh 1.5 mL Eppendorf tube. 1 ml of

70% ethanol was added to wash the DNA. A glass pipette was then used to transfer the DNA to another Eppendorf tube and allowed time for ethanol to evaporate at room temperature. 50 μ L of NFW was used to dissolve the DNA pellet and stored at -20°C.

2.5 Analysis of DNA

Genomic and plasmid DNA analysis was carried out using SimpliNano spectrophotometer (General Electric USA) to measure nucleic acid concentration at 260 nm and purity was determined using 260/280 nm ratio. Thermo Scientific Qubit 4 Fluorometer was used to analyse genomic DNA extracted for NGS. Additionally, genomic DNA was run on 1% agarose gel to confirm the absence of RNA contamination.

2.5.1 Polymerase Chain Reaction (PCR)

2.5.1.1 Polymerases

DreamTaq Green PCR Master Mix (Thermo Scientific™, UK) was used for routine PCR screening and analysis. Q5 high fidelity polymerase (NEB) was used to amplify DNA fragments intended for cloning as well as DNA fragments requiring high fidelity. KOD Hot Start Master Mix (Merck-Millipore, UK) was used for inverse PCR to identify location of transposon insertion.

2.5.1.2 Colony PCR

Colonies were picked and resuspended in 24 μ L of DreamTaq Green PCR Master Mix containing the required primers. Thermocycler parameters were set up according to the instructions of DNA polymerase manufacturers.

2.5.1.3 PCR of linear or circular DNA

DNA was diluted to a concentration approximately 1 ng/ μ L and 1 μ L was added to 24 μ L of PCR mix. Thermocycler parameters were set up according to the instructions of DNA polymerase manufacturers.

2.5.1.4 Touchdown PCR

Touchdown PCR was carried out when primers had > 5°C difference in annealing temperatures. The lower annealing temperature was selected first followed by an extension time. The higher annealing temperature was then selected followed by a similar extension. Both cycles were made to run 20 times. All other parameters were carried out according to the instructions of DNA polymerase manufacturers.

2.5.1.5 Inverse PCR

In this study, inverse PCR was carried out to determine the genomic location of transposon insertions. It involved genomic DNA extraction; digestion with an enzyme that cuts at the 5' end of the transposon and several times in the genome of organism studied; ligation of the digested genomic fragments so they self-ligate; and finally using 3' – 5' oriented primers to carry out the PCR.

After gDNA extraction, 300 ng of Isolate 12 which has 1633 EcoRI cut sites in its genome and two mega plasmids was digested with 1 µL of EcoRI (NEB) in a 50 µL reaction and incubated at 37°C for 2 hrs. The restriction digest enzyme was inactivated at 65°C for 20 minutes. 1 µL of DNA ligase, 2 µL of DNA ligase buffer and 35 µL of NFW were added to 2µL of digest (6ng/µL) and incubated at room temperature (22°C) for 1 hr in a 40 µL ligation mix. DNA ligase was inactivated at 65°C for 10 minutes.

Inverse PCR was carried out using the KOD Hot Start Polymerase. The reaction mix constituted 10 µL of KOD Hot Start Polymerase, 10 mM of forward and reverse primer, 2 µL of ligation mix and NFW to make up a 20 µL ligation mix. The PCR programme was 95°C for 2 minutes to activate the polymerase, 95°C for 20 seconds denaturation, 67°C for annealing and 70°C extension time for 3 minutes 25 seconds. This was run for 40 cycles. A final extension at 70 for 10 minutes was carried out before pausing at 15°C. The primers KOD_Inv_For and KOD_Inv_Rev were used as forward and reverse primer respectively.

2.6 Cloning Techniques

2.6.1 Restriction digest of DNA

FastDigest™ (Thermo Scientific™, UK) restriction enzymes and NEB restriction enzymes were used according to manufacturer's instructions although minimum digest time used was 1 hr. In some cases, restriction enzymes of NEB and Thermo Scientific were used together with Cutsmart (NEB) buffer. Digest was either heat inactivated or run directly on agarose gel which inactivated restriction enzymes.

2.6.2 DNA blunting using Klenow fragment

DNA Polymerase I, Large (Klenow) Fragment (NEB, UK) was used to produce blunt ended DNA after restriction digest with restriction enzymes according to supplier's instructions. Briefly 1µg of DNA was dissolved in 1 µL T4 DNA ligase and 33 µM of each dNTP. 1 µL of Klenow per µg of DNA was added and incubated at 15°C for 25 minutes. 10mM final concentration of EDTA was added and the mix was heated at 75°C for 20 minutes to stop the reaction.

2.6.3 DNA dephosphorylation using Antarctic Phosphatase

DNA fragments were dephosphorylated using Antarctic Phosphatase (NEB, UK) according to supplier's instructions. Briefly, 2 µL of Antarctic Phosphatase buffer and 1 µL of antartic phosphatase was added to digestion mix after digestion and incubated at 37°C for 30 minutes. This was inactivated either by heating at 80°C for 2 minutes or running on agarose gel.

2.6.4 Agarose gel electrophoresis

A Biometra Compact (analytikjena, DE) system with a PowerPac™ HC High-Current Power Supply (BioRad, UK) was used for gel electrophoresis. Agarose gel was routinely cast at 1 % agarose with SYBR® Safe DNA Stain (ThermoFisher Scientific, UK) at 10 µL/ 100 mL. Electrophoresis was run for 40 – 60 minutes at 120 V. Agarose gels were visualised in a Gel Doc™ XR+ System (BioRad, UK) and if needed, fragments were visualised and cut from gel on a Syngene UltraBright-LED blue light transilluminator (Syngene, UK).

2.6.5 T4 ligation

Ligations were carried out with quick Ligation™ Kit (NEB, UK) according to supplier's instructions and incubated for at least 15 minutes at room temperature (22°C) in a thermocycler for staggered ends. Ligation for blunt ends was carried out at room temperature for 2 hours. Both staggered and blunt end ligations were alternatively carried out overnight at 16°C or on thawing ice.

2.6.6 NEB HiFi plasmid assembly

NEB Hifi was carried out to join fragments of DNA irrespective of their restriction sites. This was done according to the instructions provided by the kit's manufacturer. The number of fragments that can be assembled varies from 2 – 6. For assembling 2 – 3 fragments, the molar vector: insert was 2:1 and the total concentration of the fragments were 0.03 – 0.2 pmols. For fragments ranging from 4 – 6, molar vector: insert was 1:1 and the total concentration of fragments was from 0.2 – 0.5 pmols. 10 µL of NEBuilder HiFi DNA Assembly Master Mix was added and made up to 20 µL with NFW. Samples were incubated in the thermocycler at 50 °C for 15 minutes (2 – 3 fragments) or 60 minutes (4 – 6) fragments and stored at -20°C afterwards. 2 µL of the mix was used to transform NEB *E. coli* DH5-alpha high efficiency competent cells according to the supplier's instructions.

2.7 DNA sequencing

2.7.1 Sanger sequencing

Sanger sequencing was carried out by Source Bioscience in Nottingham. 5 µL of PCR products at a concentration of 10ng/µl or 5 µL of plasmid at a concentration of 100ng/µl were sent. 5 µL of primer samples at a concentration of 3.2pmol/µl were also sent. Results were obtained overnight and DNA sequences of samples sequenced were aligned with known DNA sequence of respective plasmids or organisms using Benchling. DNA sequences for example 16S rRNA were compared to published gene sequences using NCBI BLAST.

2.7.2 Illumina sequencing

Illumina sequencing was carried out on methanotroph samples using the gDNA extracted with phenol:chloroform:isoamyl alcohol gDNA extraction method. At least 5 µg of gDNA having a concentration > 50ng/µL was sent to MicrobesNG genomic services at the University of Birmingham. 1µL of each Sample was run on 1% agarose gel to confirm the absence of RNA. Sequencing was carried out at £50/sample. Illumina sends back trimmed and assembled genomes.

2.7.3 PacBio sequencing

Samples sequenced with PacBio services required strict adherence to laid down guidelines [245]. Among them were a minimum requirement of 10 µg of gDNA at a concentration > 50ng/µL. The gDNA extraction methods recommended were phenol:chloroform extraction or a list of kits provided. The gDNA elution was in buffered solution such as 10 mM Tris, pH 7.0 – pH 8.0. The use of NFW was discouraged for elution. DNA quantification carried out using the Qubit fluorometer and RNA contamination was to be avoided. All samples were run on agarose gel to ascertain the absence of RNA. Services were carried out by McGill University and Genome Quebec Innovation Centre (Canada) at the cost of \$1048 per sample. All samples that were PacBio sequenced were also sequenced using Illumina. Consensus between PacBio and Illumina data was achieved using CLC Genomics Workbench 9.

2.8 Methanotroph culture conditions

2.8.1 Methanotroph Media

2.8.1.1 Nitrate Mineral Salt (NMS) media

This medium was formulated by Whittenbury *et al.* [2] containing per litre of distilled water: MgSO₄·7H₂O, 1 g; KNO₃, 1 g; CaCl₂·H₂O, 0.2 g; 3.8% (w/v) solution Fe-EDTA (FeSO₄·7H₂O, 0.139 g and Na₂EDTA 0.186 g mix although the ready-mixed sample was bought and used in this study), 0.1 mL and 0.1% (w/v) Na₂MoO₄·4H₂O, 0.5 mL. 1 mL of trace elements was added containing per litre of distilled water FeSO₄·7H₂O, 0.5 g; ZnSO₄·7H₂O, 0.4 g; MnCl₂·7H₂O, 0.020 g; CoCl₂·6H₂O, 0.05 g; NiCl₂·6H₂O, 0.01 g; H₃BO₃

(boric acid), 0.015 g; EDTA, 0.25 g [2]. After media was autoclaved and cooled, 10 mL of autoclaved phosphate buffer containing per litre of distilled water KH_2PO_4 , 26 g and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 62 g was added. In addition, 10 mL ($\times 0.1$ stock) of filter sterilised vitamin stock was also added which contained per litre of distilled water biotin, 0.002 g; folic acid, 0.002 g; thiamine HCl, 0.005 g; Ca pantothenate, 0.005 g; vitamin B12, 0.0001 g; riboflavin, 0.005 g and nicotinamide, 0.005 g. To facilitate the growth of *pmoA*-possessing methanotrophs, 10 μM (10 $\mu\text{L/L}$) of $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ was added to the media. Media similar to NMS were used. These include:

2.8.1.2 Dilute NMS (dNMS) media

This is Whittenbury NMS media and components diluted $\times 5$ ($0.2\times$ NMS media). DNMS was also used with varying vitamin stock concentrations ($\times 0.0001$, $\times 0.001$, $\times 0.01$ and $\times 0.1$)

2.8.1.3 Ammonium Mineral Salt (AMS) media

This medium was prepared by substituting KNO_3 in NMS media with NH_4Cl , 0.5 g. Modified NMS (mNMS) media and M2 media [25] were also used.

2.8.1.4 MNMS

Medium contained per litre of distilled water NaCl, 0.1 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.04g; KCl, 0.05g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.015g; Na_2SO_4 , 0.016g and KNO_3 , 0.005g. Phosphate buffer ($1\times$ Whittenbury), 0.81 mL and trace elements (SL 10), 1 mL were added. SL10 was composed of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 1.5 g; 7.7M HCl, 10 ml; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.006 g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.036 g; ZnCl_2 , 0.024 g; H_3BO_3 , 0.002 g; $\text{Na}_2\text{MnO}_4 \cdot 2\text{H}_2\text{O}$, 0.19 g; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.07 g. Modified NMS media was shown to give significantly higher Most Probable Number (MPN) compared to NMS when used for isolation of samples in littoral sediment of a lake [246].

2.8.1.5 M2

Medium contained per litre of distilled water $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02g; CaCl_2 , 0.004g; KNO_3 , 0.2 g; KH_2PO_4 , 0.04 g and trace elements, 0.02% containing per litre of distilled water EDTA, 5 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.03 g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 g; $\text{CuCl}_2 \cdot 5\text{H}_2\text{O}$, 0.1 g; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.02 g and Na_2MoO_4 , 0.03 g. M2 was used to isolate acidophilic methanotrophs from Sphagnum peat bogs. The pH of buffers used

for dNMS, NMS and AMS were 6.8, while that of mNMS and M2 were 3-6 and 7.2 respectively.

For solid media, Oxoid 3 agar, Gellan gum (GG) and Bacto agar (BA) were used at 1.5% concentration.

2.9 Methanotroph growth

2.9.1 Culturing freeze-dried methanotrophs

Methylococcus capsulatus (Texas) and *Methylocystis parvus* (OBPP) obtained from NCIMB were rehydrated using supplier's instruction to a final volume of 1 mL in PBS. Cells were revived as follows:

dNMS media + CH₄: 100 µl of rehydrated methanotrophs were serially diluted. Dilutions (neat, 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) were inoculated in 20 mL of dNMS in 60 mL serum bottles sealed with rubber stoppers and crimps. Serum bottles contained an atmosphere of CH₄:air 50:50, and were incubated in a shaker set at 200 rpm.

dNMS agar + CH₄: 100 µl of rehydrated *M. capsulatus* (Texas) was serially diluted. Dilutions (neat, 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) were spread on 20 mL of dNMS agar slopes in 125 mL serum bottles sealed with rubber tops and crimps. Serum bottles contained an atmosphere of CH₄:air 50:50 and were incubated at 37°C.

2.9.2 Culturing methanotrophs from freezer stock

Methanotrophs stored at -80°C in microbeads (Microbank™ Bacterial and Fungal Preservation System, Pro-Lab Diagnostics, UK) were streaked on NMS BA plates by transferring a whole bead on the plates and streaking all across. The plates were then flushed with CH₄ gas and incubated at the required temperature. The beads can alternatively be transferred directly into liquid NMS media.

2.9.3 Subculturing and restreaking methanotrophs

Methanotrophs were subcultured by transferring a calculated volume of culture (volume if inoculum) into a known volume of NMS that resulted in a starting culture of OD₆₀₀ 0.02 using the formula below:

$$\text{Volume of inoculum} = (\text{OD}_{\text{final}} \times \text{Volume}_{\text{final}}) / (\text{OD}_{\text{inoculum}} - \text{OD}_{\text{final}})$$

Alternatively, a colony of methanotrophs was picked by a sterile loop and dipped into a serum bottle containing NMS. Re-streaking was carried out by dipping a sterile loop on a colony and streaking across agar plate.

3 Chapter 3: Isolation of methanotrophs

3.1 Introduction

The ubiquitous nature of methanotrophs has provided abundant environments from which they can be isolated [18-22]. This has led to their availability and a consequent expansion in knowledge surrounding this group of organisms. However methanotrophs are difficult to isolate in pure culture form. Additionally, some methanotrophs failed to grow in laboratory culture conditions even though their presence was detected in samples using molecular biology techniques. This is common among bacteria and a review on unculturable bacteria has been written by E. J. Stewart [48]. One reason hindering the growth and division of bacteria under laboratory conditions is the failure to replicate essential aspects of their environment. Thus to achieve success in isolating new strains of bacteria, it is important to vary growth conditions such as temperature, pH and media constituents [48].

Scientists and research groups have continued with efforts to isolate and characterise new and novel methanotrophs. There are several reasons why isolation of methanotrophs is still being carried out. One important reason is the search for a superior strain with regards to growth time, tolerance to stress conditions, good resistance to contamination and amenability to DNA transfer for use in everyday laboratory procedures that require methanotrophs. For example the isolation of *Methylobacterium buryatense* fits this category. It was reported to grow relatively quickly with a 3 hr doubling time on pure methane and natural gas. Further studies demonstrated a procedure to conjugate, electroporate and edit its genome [247]. Finally it was shown to be industrially valuable when it was genetically engineered to produce lactate by heterologous introduction of lactate dehydrogenase genes into its genome [34].

Another reason that makes isolations important is acquiring strains that naturally synthesise commercially valuable products. The importance of this is appreciated more when public perception of genetically modified organisms (GMO) and their products is taken into account. Therefore natural producers of valuable products will

require less stringent legislation for industrial and commercial use. Isolating methanotrophs with the desired commercial products can be more attractive as conversion of methane to these products will have the benefit of climate change mitigation and cost savings from using cheap gas resources like natural gas compared to using sugars which can be more expensive. To date so many methanotrophs have been isolated that synthesize valuable products. *Methylocystis parvus* (OBBP) and other type II methanotrophs such as *Methylocystis hirsuta* accumulate poly-3-hydroxybutyrate (PHB) [248, 249]; *Methylococcus capsulatus* has been used as fish feed source in the form of single cell protein (SCP) [105]; and *Methylomonas* species are known to synthesize carotenoids [33].

Other important reasons why isolation of methanotrophs is still carried out include Intellectual Property (IP) protection that provides freedom and flexibility to carry out confidential research. Isolation of new species is also an important way we can learn more about methanotrophs and their diversity and this will help us answer important fundamental science questions.

Studies reported on isolation of methanotrophs have varied in methods. Whittenbury *et al.* used plate assay methods to isolate about 100 methanotrophs [2]; Svenning *et al.* used Soil Substrate Membrane System (SSMS) and Putzer *et al.* used percoll to separate them into different columns. However, the principle applied to all methods remains similar [54, 55]. Primarily, it is to separate contaminants from the desired methanotroph. Sometimes it involves removing the desired methanotrophs from their original sample environment to a controlled environment like Whittenbury's plate assay; sometimes it involves making the sample environment favourable for the methanotrophs and less suitable for the non-methanotrophic bacteria as seen in the SSMS method. Separation of the mixed bacterial cultures based on characteristics that differentiate them as demonstrated with the Percoll method may be undertaken. The technique that is more widely used is most likely making the culture media less favourable for the contaminating bacteria and more suitable for the methanotrophs. This can be done by eliminating carbon sources that can favour contaminants and continuously enriching methanotrophic growth by addition of CH₄. Additionally it helps to subculture and/or restreak samples through several passages thereby

providing media uncontaminated by methanotrophic co-metabolites which can sometimes be carbon sources for contaminants. Subculturing through several passages can be made more effective by making dilutions and transferring the most dilute sample showing growth to the next passage [250].

3.2 Specific Research Aims

This work aimed to isolate diverse methanotrophs from various environments. Emphasis was placed on isolation of fast growing methanotrophic species which led to the screening of fast growing colonies on agar media plates. Although hard to select for, there was high priority placed on isolation of genetically amenable methanotrophs as this would enable the transfer of genetic tools studied and tested in *E. coli*, *C. necator* and *Clostridia* species which are available to the Nottingham synthetic biology research centre (SBRC). This required the isolation of numerous species to increase the chance of getting one that fits this profile. High priority was also placed on isolation of thermotolerant species as this will suit certain industrial processes associated with the author's research group. The involvement of industry in some parts of this project attracted some sense of confidentiality and the need to isolate strains fully owned by the author's collaborators.

3.3 Hypothesis

Chances of isolating pure methanotrophic species increase with decreasing alternative carbon source in growth media and increasing carbon and nutrient source for methanotrophs.

3.4 Materials and methods

3.4.1 Primers

Table 6: List of primers

Name	Sequence	Description
Universal 16S rRNA (F515f/F515r)	Forward - GTGYCAGCMGCCGCGGTA Reverse - GARCTGRCGRCRRCCATGCA	This primer set targets 16S rRNA and produces a fragment approximately 500 bp when bacterial DNA is amplified.
pMMOI (A189f/A682r)	Forward - GGNGACTGGGACTTCTGG Reverse - GAASGCNGAGAAGAASGC	This primer combinations A189f/A682r amplify a region of <i>pmoA</i> gene approximately 525 bp in size [251].
pMMOII (A189f/A650r)	Forward - GGNGACTGGGACTTCTGG Reverse - ACGTCCTTACCGAAGGT	The <i>pmoA</i> primer combinations A189f/A650r amplify a region of <i>pmoA</i> gene approximately 500 bp in size [251].
pMMOIII (A189f/mb661r)	Forward - GGNGACTGGGACTTCTGG Reverse - CCGGMGCAACGTCYTTACC	This primer combinations A189 and mb661 amplify a region of <i>pmoA</i> approximately 470-bp large [75].
sMMO (A166f/A1401r)	Forward - ACCAAGGARCARTTCAAG Reverse - CCGATCCAGATDCCRCCCCA	The primer combinations amplify an <i>mmoX</i> region 800 bp in size [252].

3.4.2 Enrichment 1

Environmental samples were collected from the University of Nottingham Sutton Bonington campus farm on the 27th of November 2014. Samples collected included fresh cow dung (CMF) excreted the same day; old cow dung (CMO); anaerobic digester samples; food waste samples; slurry from surface of a cow manure slurry tank (SS) and slurry from the bottom of the tank (BS). Samples not from the farm including food waste and anaerobic digester samples were also collected from Helen West at the Nottingham University department of Environmental Biology. Four conditions of dNMS media were set up to isolate methanotrophs from the samples. Media (dNMS) with and without 10 μ M CuSO₄·7H₂O, each with headspace containing CH₄: air 50:50 and 20:80. Headspace pressure was not measured as it was common practice to use CH₄: air volume ratio in headspace rather than measure headspace pressure [34, 252]. CH₄: air volume ratio was used throughout the study.

Samples collected were stored at 4°C for six weeks as enrichment could not commence immediately. 1 mL of each sample was added to two 60 mL serum bottles containing 10 mL dNMS, and two other 60 mL serum bottles containing 10 mL dNMS and 10 µM CuSO₄·7H₂O. Two different amounts of CH₄ were added to each medium to provide CH₄: air of 50:50 and 80:20. Serum bottles were incubated at 30°C and 200 rpm for four days. After four days, 1 mL of all grown samples were sub-cultured into dNMS media containing 10 µM CuSO₄·7H₂O, and CH₄: air 50:50 was added. Serum bottles were incubated at 30°C and 200 rpm for four days. Serial dilutions (neat – 10⁻³) of the subcultures were then made in PBS and streaked on 20 mL of dNMS Number 3 Oxoid agar slopes in 125 mL serum bottles containing 10 µM CuSO₄·7H₂O. CH₄:air 50:50 were added and bottles were incubated at 30°C. After growth of colonies on agar slopes, 16S rRNA gene and methanotroph-specific genes were targeted for colony PCR using primers listed in Table 6. Electrophoresis was used to run PCR products on agarose gel. Visible bands were cut, DNA was extracted and sequenced by Source Bioscience. Colonies were restreaked multiple times and PCR-screened with 16S rRNA primers to identify the species growing on plates.

3.4.3 Enrichment 2

Environmental samples were collected from two areas near the University of Nottingham lake at the University Park campus on 16th of March, 2015. One site was selected for enrichment as it contained more excretory wastes from birds, thus suggesting a richer organic environment more habitable for methanotrophic growth.

Enrichment started within 24 hours. The sample near the ramp was vortexed for a few seconds and centrifuged at 1000 rpm for 1 minute. 10 µL of the supernatant was added to 60 mL serum bottles containing 10 mL of dNMS with various vitamin stock concentrations – 0×, 0.1×, 0.01×, 0.001× and 0.0001×. 10 µL of supernatant was also added to 60 mL serum bottles, each containing 10 mL of either dNMS, NMS, AMS, mNMS or M2 media. Three sets of control cultures were grown using dNMS media. The first and second controls had 10 µL of environmental samples and were spiked with *M. capsulatus* (Texas). Addition of *M. capsulatus* (Texas) was to see if methanotrophs could grow under the media conditions that were used. Only the first

control had air:CH₄:CO₂ of 76:20:4. The third control had contaminating bacteria previously shown to grow under CH₄ conditions. All media contained 10 µM concentration of CuSO₄·7H₂O. Serum bottles were sealed and the headspace gas ratio fixed – air:CH₄:CO₂ of 76:20:4 except for the second control. Serum bottles were incubated at 30°C and 150 rpm for 25 days. All samples were made in duplicates. After turbidity was observed in some serum bottles, DNA extraction was carried out using Sigma-Aldrich GenElute™ Bacterial Genomic DNA Kit. To determine the presence of methanotrophs in the samples, PCR was carried out on gDNA samples using methanotroph-specific primers pMMOI (A189f/A682r) and sMMO (*mmoXa/mmoXd*).

Only samples which grew in the first round of culturing were sub-cultured. However, all control samples were sub-cultured. 10 µL of culture was transferred to 60 mL serum bottles containing 10 mL of the same set of media used in the first round of culturing. The same gas and incubation conditions were also applied. After five days, serial dilution (neat – 10⁻⁵) was carried out. 100 µL was spread on dNMS media plates with Bacto Agar (BA) used as solidifying agent. Plates were incubated in Oxoid jars for five days at 30°C and air:CH₄:CO₂ of 76:20:4.

After five days, plates were observed for growth. Two distinct colonies were picked from plates using sterile loops dipped into 15 µL NFW and immediately streaked from the NFW to new dNMS BA plates using the same loop. The plates were incubated in conditions similar to the first round of plating and left until colonies formed. PCR using Universal 16S rRNA (F515f/F515r) and methanotroph-specific primers (pMMOI) was carried out using the inoculated NFW. Gel electrophoresis followed using the PCR products. DNA bands were visualised, extracted from gels and sequenced. After 21 days, colonies were picked again and re-streaked. PCR was carried out using primers mentioned earlier. In addition, two other sets of pMMO primers were used – pMMOII (A189f/A650r) and pMMOIII (A189f/mb661r) in order to increase the likelihood of detecting methanotrophs as not all *pmoA* genes are amplified by A189f/A682r set of primers. Colonies positive for *pmoA* and *mmoX* were grown in dNMS media, diluted and streaked on plate repeatedly to obtain pure methanotroph colonies.

3.4.4 Enrichment 3

Samples used for the third round of enrichment were obtained from the lake in Wollaton Park, Nottingham on the 23rd of March 2015. In total, six different samples were obtained: Lake Stream Sediment (LSS); Shallow Lake Sediment (SLS); Oily Lake Stream Sediments (LSSO); Reed Bed (RB); Moss (Moss) and Lake Tree Sediment (LTS). Enrichment 2 protocol was modified and used for growth, identification and isolation. The modifications include removal of centrifugation step at the beginning. Instead, samples were allowed to stand in falcon tubes until particles settled. Also inoculum of environmental samples was increased at the beginning from 10 µL in 10 mL of media to 100 µL in 10 mL of media. Additionally, the first round of culturing lasted for 16 days before sub-culturing was carried out instead of 25 days in Enrichment 2. The incubation temperature was kept at 30°C and were applicable, shaking was done at 200 rpm. Only LTS was selected for culturing under the various media conditions used in Enrichment 2 (dNMS with 0×, 0.1×, 0.01×, 0.001× and 0.0001× vitamin stock, as well as dNMS, NMS, AMS, mNMS and M2 media). The remaining samples (LSS, SLS, LSSO, RB and Moss) were grown only in dNMS media. After Subculturing, samples that grew were streaked on dNMS BA agar plates. Colonies that grew were screened for *pmoA* and *mmoX* genes. Colonies positive for either gene were enriched to purity.

3.4.5 Enrichment 4

Samples were obtained from a Bog in Mosely on the 31st of July 2015, and a landfill site in Nottingham on the 3rd of August 2015, and stored at room temperature overnight. A 3 mL liquid of landfill sample from lake sediment and 3 g solid of bog sample were homogenized using a vortex in 27 mL of NMS media with 10 µM of CuSO₄·7H₂O concentration. Sediments in the samples were allowed to settle after which 11 mL was transferred to a 60 mL serum bottle which was subsequently diluted to 10⁻⁷ in dNMS media. Serum bottle head space was adjusted to 20:80 CH₄:air ratio and samples were incubated for five weeks at 30°C.

After five weeks, the highest dilutions with growth were transferred from 60 mL serum bottles to dNMS BA plates and dNMS Gellan Gum (GG) plates. Colonies that

grew on plates were picked with sterile loops and re-streaked through two additional passages with the same growth conditions. Colonies from the third passage were screened for sMMO and pMMO genes. Colonies positive for either gene were transferred into 200 μ L of dNMS without vitamins, diluted to 10^{-7} in 96-well plates and incubated at 30°C and 200 RPM in gas-tight box for two weeks. 5 μ L of the highest dilutions (most dilute) were streaked on dNMS BA plates for 10 days. Colonies that grew on plates were grown and diluted in dNMS media to 10^{-7} and the highest dilutions streaked again on dNMS BA plates. This process was repeated until pure isolates were obtained.

In subsequent experiments carried out to obtain purity of the methanotrophs grown in Enrichments 1 – 3, vitamin supplements were removed from all growth media. Additionally, colonies from agar plates were transferred to liquid media. From liquid media, cultures were diluted and transferred back to agar plates. This alternation from liquid to solid media was different from earlier experiments where colonies or liquid cultures were diluted and streaked continuously on agar plates. Alternating from liquid to solid growth media was repeated until purity was obtained. As for the sample from cow dung, in addition to alternating liquid and solid media, its growth temperature conditions were also alternated from 37°C to 42°C in liquid to enhance the purification process. Shaking was carried out at 200 rpm.

3.4.6 Enrichment 5

Samples were obtained from the Roman baths in Bath on the 31st of July 2015 and kept for four months at 4°C. The long duration taken to enrich this sample was due to many other available samples which were already in the process of enrichment. Isolation was carried out by transferring 2 mL of moss sample from the King Spring bath into 35 mL of PBS and vortexing. 200 μ L of the vortexed and settled PBS sample was transferred to 10 mL of dNMS media without vitamins and copper. This was incubated at 45°C for 12 days after which 10 μ L was inoculated in 96-well plates and diluted to 10^{-7} . After 13 days, 5 μ L of all the dilutions were streaked on dNMS BA plates. Colonies grew on these plates up to 10^{-4} dilutions which were screened for pMMO and sMMO and re-streaked to purity.

3.4.7 Enrichment 6

Environmental sample obtained from dry lake sediment (DLS) at a covered landfill in Abuja (Nigeria) on the 17th of January 2017 was kept at room temperature for 48 hours during transportation to England. Quadruplicate of 1 g of the sample was measured out and each resuspended in 25 mL of dNMS. Two of the four samples were grown with 10 μ M concentration of $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ while two were only grown with dNMS. CH_4 :air 50:50 was added and samples were incubated at 45°C. After three days, 1 mL of each sample was taken, spun at 7500 RPM for 5 minutes and supernatant discarded. Pellet was resuspended in 1 mL and transferred to 50 mL falcon tube. 35 mL of deionised water was added to the falcon tube to dilute any carbon source available. The sample was spun and resuspended in fresh 1 mL of deionised water which was transferred to 25 mL of dNMS and incubated with CH_4 :air 50:50 at 45°C. After 5 days, dilutions were made (neat – 10^{-3}) and each dilution was subcultured in 2 mL of dNMS. After two additional days, growth was observed and a further dilution (neat – 10^{-3}) was carried out and 10 μ L of the dilution was spread on dNMS BA incubated at 45°C. After nine days, colonies appeared and were screened with pMMO, sMMO and 16S rRNA primers using colony PCR. Samples were restreaked on dNMS BA plates till purity was obtained.

In all enrichments carried out, test for purity involved streaking 10 μ L of growing culture on agar plates containing either LB, TSA, 10% LB or 10% TSA. Absence of growth on all the rich media plates was regarded as purity. Additionally, NCBI BLAST search using 16S rRNA analyses data of colony PCR products were used to identify the species isolated.

3.5 Results of methanotroph isolation experiments

3.5.1 Enrichment 1

The six environmental samples obtained from Sutton Bonington – Surface Slurry (SS), Bottom Slurry (BS), Cow Manure Fresh (CMF), Cow Manure Old (OCM), Food Sample (FS) and Anaerobic Digester Sample (ADS) – showed growth after four days in 10 mL dNMS liquid media. 1 mL culture of the samples in 10 mL dNMS also showed turbidity after four days for most of the samples. Significant growth of colonies was observed on dNMS agar slopes streaked with neat, 10^{-1} , 10^{-2} and 10^{-3} dilutions. For most samples, colonies grew confluent on dNMS agar plates in neat dilution. However, 10^{-2} and 10^{-3} dilutions resulted in single colonies. Less growth were seen in ADS and FS samples while no growth was seen in control samples which contained deionised water in dNMS agar with and without methane. The morphology of cells ranged from small (0.5 mm) and medium (2 mm), to large (4 mm) colonies which were mostly round and irregular in shape. A lot of colonies were cream in colour with some being transparent and others showing opaque appearance. Colourless transparent colonies were also common. Samples having distinct single colonies were selected for 16S rRNA identification using PCR, electrophoresis and DNA sequencing. Electrophoresis of PCR products showed bands approximately 500 bp in size (Figure 14). Except in Enrichment 5 where NEB 2 log DNA ladder was used, NEB 1kb ladder was used in all gel images shown in this chapter.



Figure 14: Electrophoresis gel image of PCR products using 16S rRNA primers (1) 2-CMF.1A, 10^{-3} (2) 2-CMF.1C, 10^{-3} (3) 2-CMF.2A, 10^{-3} (4) 2-CMF.2B, 10^{-2} (5) 2CMF.3C, 10^{-3} (6) 2-CMF.3D, 10^{-3} (7) CMO.1C, 10^{-3} (8) CMO.1C, 10^{-2} (9) 2-SS.1C, 10^{-2} (10) 2-SS.1D, 10^{-2} (11) BS.1C, 10^{-3} (12) BS.1A, 10^{-2} (13) FS.1B, 10^{-2} (14) FS.1D, 10^{-2} (15) ADS, OTC25.B,neat (16) ADS, OTC25.D,neat (17) ADS, OTC37.B, 10^{-2} (18) ADS, OTC37.D, 10^{-3} (19) ADS, TYL25.D,neat (20) ADS, TYL25.A,neat

After DNA was extracted from bands and sequenced with F515f/r primers, the species of the colonies was identified using NCBI BLAST (Table 7).

Table 7: Organisms identified on BLAST using 16S rRNA sequence data from colonies growing on dNMS agar plates

Sample ID	Sample Number	Organism	Max score	Total score	Query cover	E value	Ident
2-CMF1.a (10^{-3})	1	<i>Mucilaginibacter paludis</i> DSM 18603 chromosome, whole genome shotgun sequence	710	2824	93%	0	93%
2-CMF1.c (10^{-3})	2	<i>Mucilaginibacter paludis</i> DSM 18603 chromosome, whole genome shotgun sequence	682	2707	93%	0	92%
2-CMF2.a (10^{-3})	3	<i>Sphingobium japonicum</i> UT26S chromosome 2, complete sequence	774	1549	93%	0	95%
2-CMF2.b (10^{-2})	4	<i>Acinetobacter calcoaceticus</i> PHEA-2 chromosome, complete genome	848	1693	73%	0	98%
2-CMF3.c (10^{-3})	5	<i>Mucilaginibacter paludis</i> DSM 18603 chromosome, whole genome shotgun sequence	693	2768	95%	0	92%
2-CMF3.d (10^{-3})	6	<i>Pedobacter saltans</i> DSM 12145 chromosome, complete genome	676	2707	90%	0	92%
CMO.c (10^{-3})	7	<i>Mucilaginibacter paludis</i> DSM 18603 chromosome, whole genome shotgun sequence	675	2683	96%	0	91%
CMO.c (10^{-2})	8	<i>Mucilaginibacter paludis</i> DSM 18603 chromosome, whole genome shotgun sequence	691	2761	93%	0	92%
2-SS.c (10^{-2})	9	<i>Pedobacter saltans</i> DSM 12145 chromosome, complete genome	678	2715	92%	0	92%
2-SS.b (10^{-2})	10	<i>Mucilaginibacter paludis</i> DSM 18603 chromosome, whole genome shotgun sequence	693	2768	96%	0	92%
BS.c (10^{-3})	11	<i>Acinetobacter baumannii</i> ATCC 17978 chromosome, complete genome	850	4252	94%	0	97%
BS.a (10^{-2})	12	<i>Mucilaginibacter paludis</i> DSM 18603 chromosome, whole genome shotgun sequence	676	2702	91%	0	92%
FS.b (10^{-2})	13	<i>Brevundimonas subvibrioides</i> ATCC 15264 chromosome, complete genome	850	1701	93%	0	98%
ADS.b (OTC 25) N	15	<i>Bacillus sp.</i> 1NLA3E, complete genome	852	10157	93%	0	98%
ADS.d (OTC 25) N	16	<i>Streptomyces albus</i> J1074, complete genome	909	6367	95%	0	99%
ADS.b (OTC 37) 10^{-2})	17	<i>Brevibacillus brevis</i> NBRC 100599, complete genome	885	13240	92%	0	99%
ADS.d (OTC 37) (10^{-3})	18	<i>Sphingobium sp.</i> SYK-6 chromosome, complete genome	739	1479	95%	0	93%
ADS.d (TYL 25) N	19	<i>Brevibacillus brevis</i> NBRC 100599, complete genome	904	13501	93%	0	99%
ADS.a (TYL 25) N	20	<i>Brevibacillus brevis</i> NBRC 100599, complete genome	907	13556	93%	0	99%

No methanotroph was identified in the first round of detection. A second batch of samples were selected for colony PCR using 16S rRNA, and in addition, methanotroph-specific primers – *pmoA* (A189f/A682r) and *mmoX* (166f/1401r). Electrophoresis carried out with PCR products showed bands approximately 525 bp and 800 bp for *pmoA* and *mmoX* respectively (Figure 15).

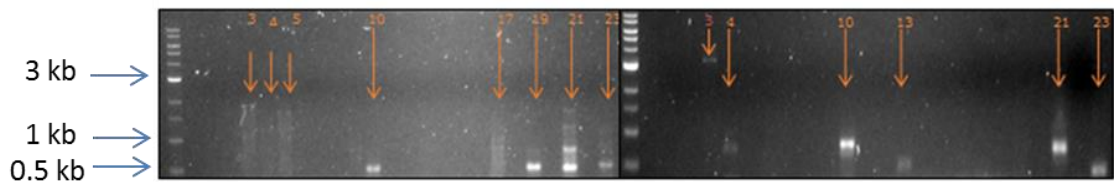


Figure 15: Bands from PCR products for *pmoA* (left) and *mmoX* (right): (3) ADS.D (TYL 25), 10⁻¹ (4) CMO.c10⁻³ (5) CMO.A, 10⁻³ (10) BS.D, neat (13) 2-CMF.A, 10⁻³ (17) CMO.B, 10⁻² (19) BS.C, neat (21) 2-CMF.1B, 10⁻³ (23) 2-SS.C, 10⁻³

All samples positive for *pmoA* and/or *mmoX* restreaked on dNMS Oxoid 3 agar grew after five days. Colony PCR using *pmoA*, *mmoX* and Universal 16S rRNA primers gave positive results. Details of DNA extracted from cut out bands of 16S rRNA PCR and sequenced are shown in Table 8.

Table 8: Organisms identified on NCBI BLAST using 16S rRNA sequence data obtained from samples growing on dNMS agar plates

Sample ID	Dilution	No.	Organism	<i>pmoA</i>	<i>mmoX</i>	Max score	Total score	Query cover	E value	Ident
ADS.d (TYL 25) (10^{-1})	1	U1a	<i>Brevibacillus brevis</i> NBRC 100599 DNA, complete genome			907	13556	98%	0	99%
CMO.c (10^{-3})	3	U2a	<i>Pedobacter saltans</i> DSM 12145, complete genome	✓.		536	2146	98%	7E-150	88%
CMO.a (10^{-3})	3	U3a	<i>Pedobacter saltans</i> DSM 12145, complete genome			580	2323	97%	3E-163	87%
BS.d N	N	U4a	<i>Niastella koreensis</i> GR20-10, complete genome		✓	773	2319	96%	0	95%
CMO.b (10^{-2})	2	U5a	<i>Riemerella anatipestifer</i> DSM 15868, complete genome	✓.	✓	568	1704	88%	3E-159	88%
BS.c N	N	U6a	<i>Niastella koreensis</i> GR20-10, complete genome	✓.		726	2180	97%	0	94%
2-CMF1.b N	"	U7b	<i>Mucilaginibacter paludis</i> DSM 18603 chromosome	✓.		675	2678	99%	0	91%
2-SS.c (10^{-3})	3	U8a	<i>Pedobacter saltans</i> DSM 12145, complete genome	✓.		682	2730	96%	0	92%
2-SS.b (10^{-3})	"	U9b	<i>Niastella koreensis</i> GR20-10, complete genome	✓.	✓	725	2175	99%	0	95%
2-SS.d (10^{-2})	2	J10a	<i>Niastella koreensis</i> GR20-10, complete genome			712	2136	97%	0	93%
BS.a (10^{-2})	2	J11a	<i>Stenotrophomonas maltophilia</i> K279a chromosome	✓.		909	3627	96%	0	99%
2-CMF1.c (10^{-3})	3	J12a	<i>Mucilaginibacter paludis</i> DSM 18603 chromosome			664	2634	97%	0	92%
2-CMF1.c (10^{-3})	"	J12b	<i>Chitinophaga pinensis</i> DSM 2588 chromosome	✓.		821	4915	96%	0	97%
2-SS.a (10^{-3})	3	J13a	<i>Niastella koreensis</i> GR20-10, complete genome		✓	721	2163	98%	0	93%
CMF U14a N	N	J14a	<i>Xanthomonas hortorum</i> pv. <i>carotae</i> str. M081 chromosome			771	771	99%	0	94%
CMF N	N	J15a	<i>Niastella koreensis</i> GR20-10, complete genome	✓.		743	2230	94%	0	94%
SSU16a (10^{-1})	1	J16a	<i>Niastella koreensis</i> GR20-10, complete genome		✓	725	2175	97%	0	93%

No methanotrophs were identified in NCBI BLAST using 16S rRNA Universal primers. A third round of identification carried out to determine the species of colonies growing on dNMS agar slopes found colonies positive for *pmoA* and *mmoX* gene. The positive samples were restreaked on Bacto agar (BA) instead of Oxoid 3 agar which led to the growth of brown coloured colonies on plates of CMF environmental sample (Figure 16). These colonies when sequenced showed 16S rRNA sequence similarity to *Methylocaldum* species. However, colony appearance from these plates was heterogeneous (brown and cream) which suggested cohabitation possibly due to cross-feeding between methanotrophic species and non-methanotrophs. Further streaking on LB media for purity check showed the cream colours to be more contaminated than the brown colours. Several re-streaking of the brown coloured colonies on dNMS plates reduced the appearance of cream colonies (Figure 17). After alternating growth in liquid and agar media, and at 42°C and 37°C, brown colonies that did not grow on LB media plates were obtained. Further tests for purity on 0.1% LB, 0.1% TSA, LB and TSA media showed no growth.

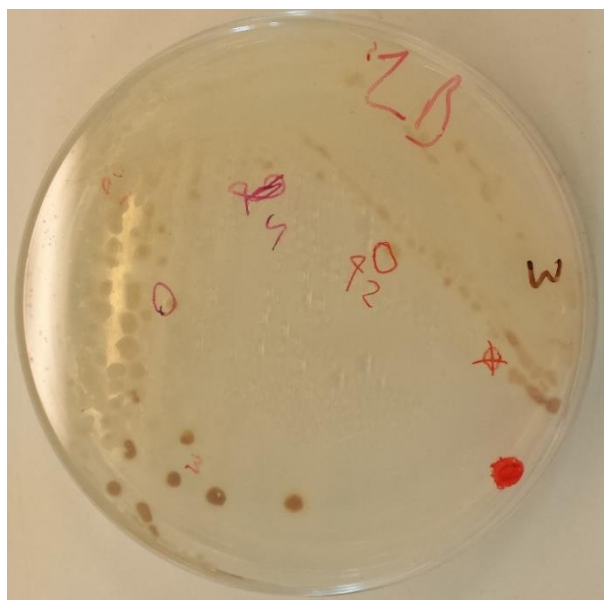


Figure 16: Fresh cow manure restreak consisting of cream and brown colonies. Brown colonies were less contaminated with non-methanotrophs



Figure 17: Brown colonies of CMF sample after restreaking



Figure 18: Appearance of pure Isolate 10 colonies

Sequenced 16S rRNA PCR products of these colonies with brown appearance showed they were species of the *Methylocaldum*. The pure bacteria isolated from fresh cow manure (CMF) was designated Isolate 10 (Figure 18).

Table 9: 16S rRNA Blast search result for Isolate 10

Description	Total score	Query cover	Ident	Accession
<i>Methylocaldum gracile</i> strain KAR5Ro7 16S ribosomal RNA gene, partial sequence	2756	97%	99%	KY621548.1
<i>Methylocaldum</i> sp. 05J-I-7 16S ribosomal RNA gene, partial sequence	2750	97%	99%	EU275146.1
<i>Methylocaldum</i> sp. 0510-P-2 16S ribosomal RNA gene, partial sequence	2737	97%	99%	EU275144.1
Uncultured delta proteobacterium clone MAB5 16S ribosomal RNA gene, partial sequence	2736	97%	99%	DQ069211.1
<i>Methylocaldum marinum</i> DNA, complete genome, strain: S8	5468	100%	99%	AP017928.1

3.5.2 Enrichment 2

Lake Sediment (LS) from University of Nottingham was grown in different liquid media – dNMS, NMS, AMS, M2, mNMS, dNMS (0× vit), dNMS (0.1× vit), dNMS (0.01× vit), dNMS (0.001× vit) and dNMS (0.0001× vit). Turbidity was observed after 25 days for LS samples (Table 6) grown in dNMS, AMS, dNMS (0× vit), dNMS (0.1× vit), dNMS (0.01× vit) and dNMS (0.001× vit). Only one replicate of these samples showed growth. Controls were also tested. The first and second controls had 10 µL of environmental samples and were spiked with *M. capsulatus* (Texas). Addition of *M. capsulatus* (Texas) was to see if methanotrophs could grow under the media conditions that were used. Only the first control had CH₄. The third control had contaminating bacteria previously shown to grow under methane conditions. However, none of the control samples grew.

Table 10: Growth of LS samples after 25 days incubation

Media	Replicates	
	A	B
dNMS	+	-
NMS	-	-
AMS	-	+
M2	-	-
mNMS	-	-

Controls	Replicates	
	A	B
1	-	-
2	-	-
3	-	-
0.01×	-	+
0.1×	+	-

Controls	Replicates	
	A	B
1	-	-
2	-	-
3	-	-

Electrophoresis carried out on PCR products of genomic DNA extracted from grown samples showed bands for *pmoA*, *mmoX* and Universal 16s rRNA primers. For *pmoA*, 4 out of 6 samples showed bands approximately 525 bp in size which suggested the presence of methanotrophs in the culture. For *mmoX*, 3 out of 6 samples showed bands approximately 800 bp which also suggested methanotroph presence (Figure 19).

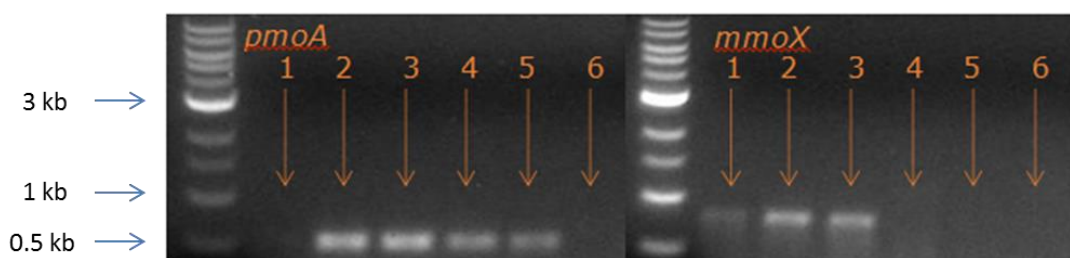


Figure 19: PCR of methanotroph-specific genes in gDNA extracted for growth culture of methanotrophs (1) LT in dNMS (2) LS in AMS (3) LS in dNMS – 0× vit (4) LS in dNMS – 0.001× dNMS (5) LS in dNMS – 0.01× vit (6) LS in dNMS – 0.1× vit.

Some samples which grew in the first 25 days turned turbid after subculturing for five days (Table 11).

Table 11: Conditions under which growth was seen in the 5 days LS samples were subcultured

Replicates		
Media	A	B
dNMS	+	-
NMS	-	-
ANMS	-	+
M2	-	-
MNMS	-	-

Replicates		
Vitamin	A	B
0×	+	-
0.0001×	-	-
0.001×	-	+
0.01×	-	-
0.1×	+	+

Replicates		
Controls	A	B
1	-	-
2	-	-
3	-	-

All dilutions (neat – 10^{-5}) of five-day subculture showed growth after spreading on plates and incubating for five days in CH_4 at 30°C . Colony PCR of selected colonies with *pmoA* and *mmoX* primers, and subsequent electrophoresis was carried out. No bands appeared which suggests that colonies growing in the first five days were contaminating microorganisms with fast growth rate and not methanotrophs. After further 16 days of incubation, more growth was observed on plates. New colonies were picked and analysed for presence of *pmoA* and *mmoX*. Only *M. capsulatus* (Texas) control had colonies positive for *mmoX*. Table 12 shows samples that had colonies positive for *pmoA*. These were restreaked on fresh solid media to obtain pure colonies.

Table 12: Colonies of LS samples positive for *pmoA* and *mmoX* gene

Sample number	Sample description	Samples positive for <i>pmoA</i>	Samples positive for <i>mmoX</i>
1	LS in dNMS	+	-
2	LS in AMS (a)	+	-
3	LS in AMS (b)	-	-
4	LS in 0× vit (a)	-	-
5	LS in 0× vit (b)	-	-
6	LS in 0.1× vit (a)	+	-
7	LS in 0.1× vit (b)	+	-
8	LS in 0.001× vit (a)	-	-
9	LS in 0.001× vit (b)	-	-
10	Control 3B (a)	-	-
11	Control 3B (a)	-	-
12	<i>M. capsulatus</i> (Texas) control	+	+

When the positive samples shown in Table 12 were restreaked, colonies of different morphologies grew, some of which were positive for *pmoA* gene. Dilutions of the positive colonies were restreaked on NMS agar plates and incubated at 30°C. Alternated streaking on plates, culturing in liquid, making dilutions of the culture and streaking on plate resulted in obtaining pure cultures which did not grow on any of the rich media plates (Figure 20). This was designated Isolate 6.

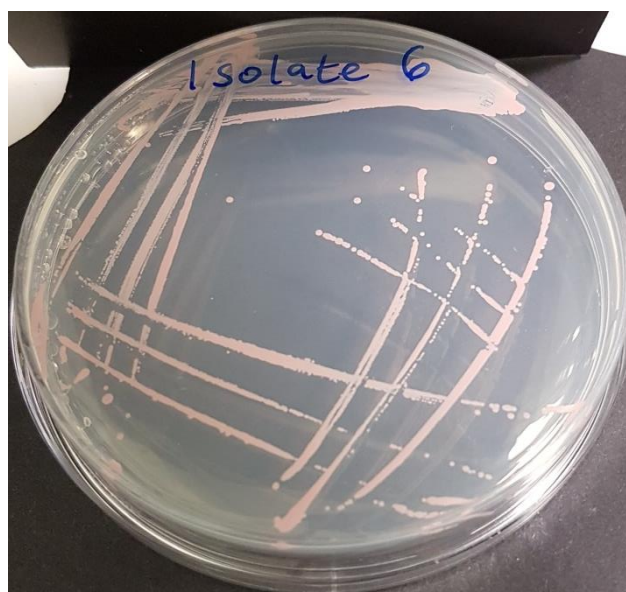


Figure 20: Appearance of pure Isolate 6 colonies on BA plates - Nottingham lake sample

16S rRNA PCR of the pure colonies was carried out and the PCR product sequenced. The sequence results were analysed on NCBI BLAST and *Methylocystis* species was obtained (Table 13).

Table 13: 16S rRNA BLAST search result of Isolate6

Description	Total score	Query cover	Ident	Accession
<i>Methylocystis</i> sp. SC2 complete genome	2700	100%	99%	HE956757.1
<i>Methylocystis</i> sp. m231 16S ribosomal RNA gene, partial sequence	2658	99%	99%	DQ852350.1
<i>Methylocystis rosea</i> strain SV97 16S ribosomal RNA, partial sequence	2656	96%	100%	NR_042108.1
Type II methanotroph AML-A6 16S ribosomal RNA gene, complete sequence	2652	98%	99%	AF177299.1
Uncultured bacterium HOCi42 16S ribosomal RNA gene, partial sequence	2643	99%	99%	AY328591.1

The 16s rRNA of Isolate 3* and Isolate 6 were identical which led to comparison of 23S rRNA. The 23S rRNA of Isolate 3* from enrichment 4 and Isolate 6 differed in six nucleotide positions.

Table 14: 23S rRNA BLAST search result of Isolate 6

Description	Total score	Query cover	Ident	Accession
<i>Methylocystis</i> sp. SC2 strain SC2 23S ribosomal RNA, complete sequence	5158	100%	99%	NR_077052.1
<i>Methylocystis</i> sp. SC2 complete genome	5158	100%	99%	HE956757.1
<i>Methylocystis bryophila</i> strain S285 chromosome, complete genome	8818	100%	95%	CP019948.1
<i>Methylosinus trichosporium</i> OB3b chromosome, complete genome	12911	100%	94%	CP023737.1
<i>Beijerinckia indica</i> subsp. indica ATCC 9039, complete genome	12012	99%	93%	CP001016.1

3.5.3 Enrichment 3

Environmental samples were collected from Wollaton lake. Only Lake Tree Sediment (LTS) was grown in different liquid media – dNMS, NMS, AMS, M2, mNMS, dNMS (0× vit), dNMS (0.1× vit), dNMS (0.01× vit), dNMS (0.001× vit) and dNMS (0.0001× vit). Turbidity was observed for some LTS samples growing for 16 days in liquid media (Table 15). All the other lake samples – Lake Stream Sediment (LSS); Shallow Lake Sediment (SLS); Oily Lake Stream Sediments (LSSO); Reed Bed (RB); and Moss (Moss) – cultured in dNMS media showed growth Table 16.

Table 15: Growth of LTS samples controls after 16 days

	Replicates			Replicates			Replicates	
	A	B		A	B		A	B
Media			Vitamin			Controls		
dNMS	+	-	0×	-	-	1	+	-
NMS	-	-	0.0001×	-	-	2	-	-
AMS	-	-	0.001×	+	-	3	-	-
M2	-	-	0.01×	-	-			
mNMS	-	-	0.1×	-	-			

Table 16: Growth of Wollaton lake samples in dNMS media after 16 days

	Replicates	
	A	B
Env samples		
RB	+	-
LSS	+	-
LSSO	+	-
Moss	-	+
SLS	+	-

Electrophoresis carried out on PCR products of genomic DNA extracted from grown samples showed bands for *pmoA*, *mmoX* and Universal 16S rRNA primers suggesting there were methanotrophs in the culture. For *pmoA*, all eight samples showed bands approximately 525 bp in size which further supports the presence of methanotrophs. For *mmoX*, 3 out of 8 samples showed bands approximately 800 bp which also suggests methanotroph presence (Figure 21).

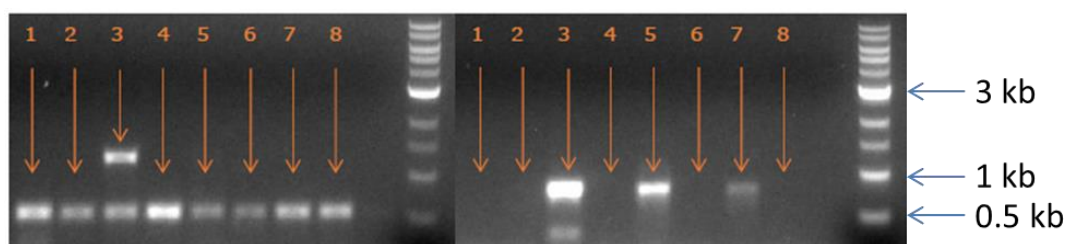


Figure 21: *pmoA* (left) and *mmoX* (right) bands for samples obtained from Wollaton lake – (1) LTS in dNMS (2) LTS in dNMS – 0.001× dNMS (3) LTS in dNMS – *M. capsulatus* control 0.01× vit (4) RB in dNMS (5) LSS in dNMS (6) LSSO in dNMS (7) Moss in dNMS (8) SLS in dNMS.

Most samples which grew in the first 16 days turned turbid after sub-culturing for five days (Table 17).

Table 17: Growth of samples obtained from Wollaton lake five days after subculturing

Replicates			Replicates			Replicates			Replicates		
Media	A	B	Vitamin	A	B	Control	A	B	Env. samples	A	B
dNMS	-	-	0×	-	-	1	+	-	RB	+	+
NMS	-	-	0.0001×	-	-	2	-	-	LSS	+	-
ANMS	-	-	0.001×	+	+	3	-	-	LSSO	-	+
M2	-	-	0.01×	-	-				Moss	+	-
MINM	-	-	0.1×	-	-				SLS	+	+
S	-	-									

All dilutions (neat – 10^{-5}) of five-day subculture showed growth after spreading on BA plates and incubating in CH_4 atmosphere at 30°C . Colony PCR with *pmoA* and *mmoX* primers, and subsequent electrophoresis was carried out. No bands appeared when DNA was run on agarose gel which suggested colonies growing in the first five days were contaminating microorganisms with fast growth rate and were not methanotrophs. After a further 16 days of incubation, more growth was observed on plates. New colonies were picked and analysed for presence of *pmoA* and *mmoX*. Only *M. capsulatus* (Texas) control had colonies positive for *mmoX*. Table 18 shows samples that had colonies positive for *pmoA*. These were restreaked on fresh solid media to obtain pure colonies.

Table 18: Colonies of Wollaton lake samples positive for *pmoA* and *mmoX*

Sample number	Sample description	Samples positive for <i>pmoA</i>	Samples positive for <i>mmoX</i>
1	LTS in dNMS (0.001× vit) (a)	-	-
2	LTS in dNMS (0.001× vit) (b)	-	-
3	BR in dNMS (a)	+	-
4	BR in dNMS (b)	+	-
5	LSS in dNMS (a)	-	-
6	LSS in dNMS (b)	-	-
7	LSSO in dNMS (a)	-	-
8	LSSO in dNMS (b)	+	-
9	Moss in dNMS (a)	+	-
10	Moss in dNMS (b)	-	-
11	SLS in dNMS (a)	+	-
12	SLS in dNMS (b)	+	-
13	Control 1a	+	-
14	Control 1b	-	-
15	Control 2a	-	-
16	Control 2b	-	-
17	Control 3a	-	-
18	Control 3b	-	-
19	<i>M. capsulatus</i> control	+	+

In addition to showing bands for *pmoA*, colonies of SLS in dNMS acquired a pink colouration. Restreaks acquired the same colouration. To get pure isolates, colonies were picked, grown in liquid dNMS, diluted and grown on plates. After 10 days, purple and light cream coloured colonies grew. However, the pink colonies suspected to be *M. extorquens* were negative for *pmoA* when PCR and electrophoresis were carried out. However, sequencing of 16S rRNA confirmed purple colonies were *M. extorquens*. Light cream colonies positive for *pmoA* were diluted (10^{-1} – 10^{-7}) and streaked on dNMS BA plates. Growth was observed and the colonies obtained were restreaked to purity (Figure 22).

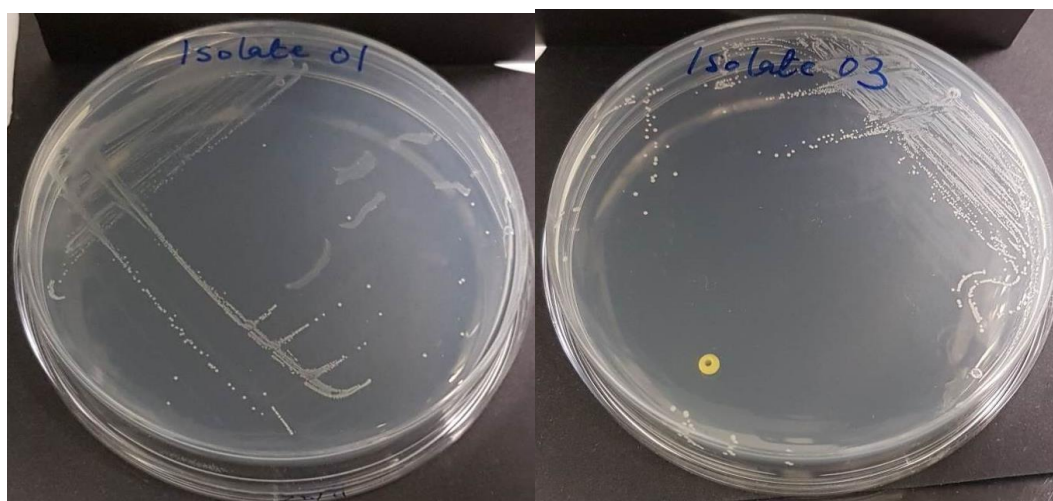


Figure 22: Appearance of Isolate 01 and Isolate 03 colonies

Using 16S rRNA PCR and Sanger sequencing, two *Methylocystis* species were isolated from SLS samples (a and b) designated as Isolate 01 and Isolate 03 (Table 19). The 16S rRNA of these isolates was identical.

Table 19: 16S rRNA BLAST search result for Isolate 01 and Isolate 03

Description	Total score	Query cover	Ident	Accession
Uncultured <i>Methylocystaceae</i> bacterium clone Pad-181 16S ribosomal RNA gene, partial sequence	2145	100%	100%	JX505428.1
<i>Methylocystis</i> sp. H9a partial 16S rRNA gene, strain H9a	2145	100%	100%	AJ458490.1
Uncultured <i>Methylocystaceae</i> bacterium clone Pad-170 16S ribosomal RNA gene, partial sequence	2139	100%	99%	JX505417.1
Uncultured <i>Methylocystaceae</i> bacterium clone Pad-133 16S ribosomal RNA gene, partial sequence	2139	100%	99%	JX505380.1
Uncultured <i>Methylocystis</i> sp. clone Pad-32 16S ribosomal RNA gene, partial sequence	2139	100%	99%	JX505279.1

3.5.4 Enrichment 4

After five weeks, growth was observed for Moseley bog and landfill samples grown in duplicates. The highest dilutions streaked on dNMS (Gellan Gum) GG and dNMS (Bacto Agar) BA plates all grew. GG plates were runny and acquired contaminations easily although they supported growth of methanotrophs. After three passages of restreaking, colonies obtained were still contaminated as they had different cell morphologies on the same plates. Some of the colonies streaked were positive for both *pmoA* and *mmoX* genes. Dilutions of these colonies remained contaminated after growing on dNMS BA. After removal of vitamin from media; growth and dilution in liquid media; and alternating samples in liquid and solid dNMS media, purity was obtained. Pink colonies were obtained from shallow lake landfill sediment (Figure 23).

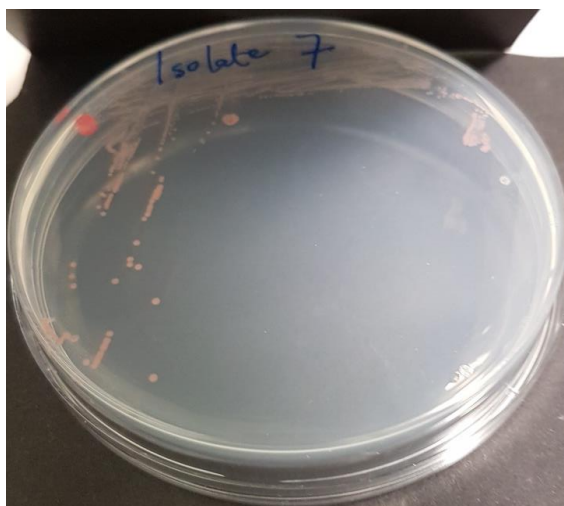


Figure 23: Appearance of pure Isolate 7 colonies isolated from landfill

16S rRNA DNA sequencing indicated this was *Methylobacter* species and was designated Isolate 7 (Table 20).

Table 20: 16S rRNA BLAST search result for Isolate 7

Description	Total score	Query cover	Ident	Accession
<i>Methylobacter methanica</i> MC09, complete genome	5371	100%	97%	CP002738.1
<i>Methylobacter rubra</i> 16S ribosomal RNA gene, partial sequence	1812	100%	97%	AF150807.1
Uncultured bacterium clone A26 16S ribosomal RNA gene, partial sequence	1807	100%	97%	KC442837.1
<i>Methylobacter lenta</i> strain R-45377 16S ribosomal RNA, partial sequence	1797	100%	97%	NR_133783.1
<i>Methylobacter lenta</i> partial 16S rRNA gene, isolate R-45370	1796	100%	97%	FR798956.1

Pink and cream colonies were obtained from samples obtained from Moseley bog gravel and Moseley bog sediment which were designated as Isolate 3* and Isolate 12 respectively (Figure 24). 16S rRNA sequencing of these isolates showed they were both *Methylocystis* species (Table 21).



Figure 24: Isolate 3* was Moseley bog gravel sediment first grown on dNMS gellan gum plates and transferred to dNMS BA plates. Isolate 12 was Moseley bog sediment grown in BA from the start.

Table 21: 16S rRNA BLAST search result of Isolate 3*

Description	Total score	Query cover	Ident	Accession
<i>Methylocystis</i> sp. SC2 complete genome	2700	100%	99%	HE956757.1
<i>Methylocystis</i> sp. m231 16S ribosomal RNA gene, partial sequence	2658	99%	99%	DQ852350.1
<i>Methylocystis rosea</i> strain SV97 16S ribosomal RNA, partial sequence	2656	96%	100%	NR_042108.1
Type II methanotroph AML-A6 16S ribosomal RNA gene, complete sequence	2652	98%	99%	AF177299.1
Uncultured bacterium HOCiCi42 16S ribosomal RNA gene, partial sequence	2643	99%	99%	AY328591.1

Table 22: 16S rRNA BLAST result of Isolate 12

Description	Total score	Query cover	Ident	Accession
Uncultured bacterium clone E44 16S ribosomal RNA gene, partial sequence	2662	99%	99%	KC683261.1
<i>Methylocystis parvus</i> 16S ribosomal RNA gene, partial sequence	2662	97%	99%	AF150805.1
<i>Methylosinus</i> sp. ok-BEg7 gene for 16S ribosomal RNA, partial sequence	2628	97%	99%	AB845163.1
Uncultured alpha proteobacterium clone KWK23F.18 16S ribosomal RNA gene, partial sequence	2606	97%	99%	JN656883.1
<i>Methylocystis parvus</i> strain OBBP 16S ribosomal RNA, partial sequence	2601	94%	100%	NR_044946.1

3.5.5 Enrichment 5

Turbidity in dNMS media was observed after 10-day growth of moss samples obtained from King Spring bath. Samples diluted and streaked grew into colonies. These colonies were screened for *pmoA* and *mmoX* genes. Colonies positive for both *pmoA* and *mmoX* genes were restreaked to purity. Figure 25 shows colony appearance while Figure 26 show *mmoX* and *pmoA* bands of Isolate 14. NEB 2 log 1kb ladder was used in the gel image below.



Figure 25: Appearance of pure colonies of Isolate 14

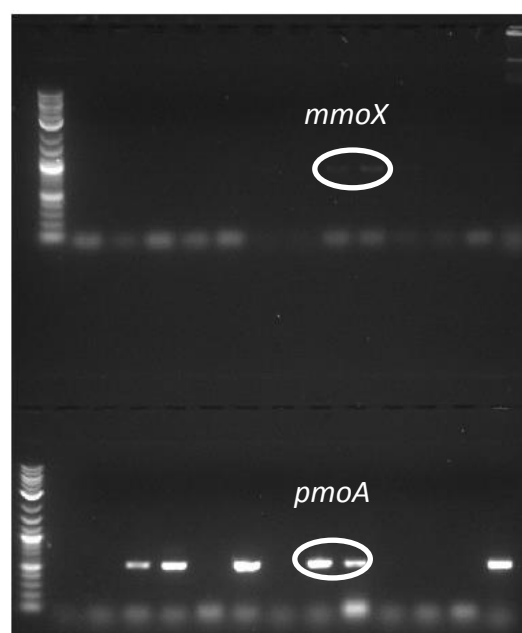


Figure 26: *mmoX* and *pmoA* PCR screen of colonies from KS Moss colonies growing on dNMS BA no copper no vitamins plates

PCR of pure colonies and 16S rRNA sequence suggested the isolate to be *Methylococcus capsulatus* (Table 23).

Table 23: 16S rRNA BLAST search result of Isolate 14

Description	Total score	Query cover	Ident	Accession
<i>Methylococcus capsulatus</i> str. Bath, complete genome	5660	100%	100%	AE017282.2
<i>Methylococcus capsulatus</i> strain Texas 16S ribosomal RNA gene, partial sequence	2728	96%	99%	NR_042183.1
Uncultured bacterium clone BG14 16S ribosomal RNA gene, partial sequence	2680	95%	99%	HM362450.1
Uncultured bacterium clone BG068 16S ribosomal RNA gene, partial sequence	2665	95%	99%	HM362489.1
<i>Methylococcus capsulatus</i> strain Cla 16S ribosomal RNA (rrs) gene, partial sequence	2651	93%	99%	JN166980.1

3.5.6 Enrichment 6

Samples obtained from dry lake sediment in a Nigerian landfill enriched in dNMS BA with and without copper grew in the first five days which were subcultured. The subculture grew after three days for dNMS BA without copper. Neat and 10^{-1} dilutions of these cultures were spread on dNMS BA plates and colonies from these plates were screen with *pmoA* and *mmoX* primers. These were restreaked till they appeared pure. After several restreaks, growth on rich media was carried out to check purity. Colonies that appeared to be pure were screened with *pmoA* and *mmoX* media.

Table 24: 16S rRNA BLAST search result of Isolate 9

Description	Total score	Query cover	Ident	Accession
<i>Methylococcus capsulatus</i> strain OnlyC-B1 16S ribosomal RNA gene, partial sequence	837	100%	99%	KF952440.1
<i>Methylococcus capsulatus</i> strain Cla 16S ribosomal RNA (rrs) gene, partial sequence	837	100%	99%	JN166980.1
Uncultured bacterium clone BG068 16S ribosomal RNA gene, partial sequence	837	100%	99%	HM362489.1
<i>Methylococcus capsulatus</i> str. Bath, complete genome	1675	100%	99%	AE017282.2
<i>Methylococcus capsulatus</i> strain Texas 16S ribosomal RNA gene, partial sequence	837	100%	99%	NR_042183.1

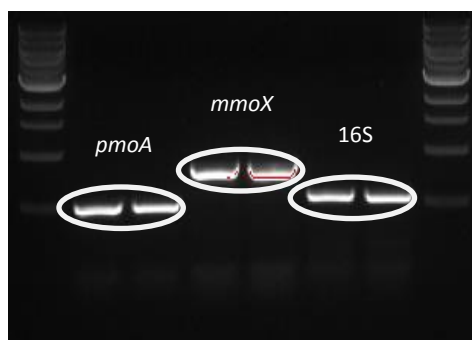


Figure 27: PCR confirmation of Isolate 9 showing it possess *pmoA* and *mmoX* genes



Figure 28: Appearance of pure Isolate 9 colonies

Sequencing 16S rRNA PCR products of these colonies showed the isolated species was *Methylococcus capsulatus* and was designated Isolate 9.

Table 25: Summary of Isolations carried out in this study

Isolate	Species	Type	Enrichment	Sample Environment and location	Isolation technique	Growth temperature (°C)
1	<i>Methylocystis</i>	II	3	Wollaton lake sediment	Diluted and restreaked to purity	30°C
3	<i>Methylocystis</i>	II	3	Wollaton lake sediment	Diluted and restreaked to purity	30°C
3*	<i>Methylocystis</i>	II	4	Moseley bog gravel	Dilution, media alternation and removal of vitamin	30°C
6	<i>Methylocystis</i>	II	2	University Of Nottingham lake sediment	Dilution, media alternation and removal of vitamin from media	30°C
7	<i>Methylomonas</i>	I	4	Nottingham landfill lake	Dilution, media alternation and removal of vitamin from media	30°C
9	<i>M. capsulatus</i>	X	6	Abuja (Nigeria) Landfill	Diluted and restreaked to purity at 45°C	37 - 45°C
10	<i>Methylocaldum</i>	I	1	Fresh cow manure Sutton Bonington farm	alternating incubation temperature between 37 - 42°C	30 - 42°C
12	<i>Methylocystis</i>	II	4	Moseley bog sediment	Dilution, media alternation and removal of vitamin from media	30°C
14	<i>M. capsulatus</i>	X	5	Kings Spring bath Moss (Bath)	Diluted and restreaked to purity at 45°C	37 - 45°C

3.6 Discussion

Samples collected from different environments (cow manure, lakes, bogs, landfills and warm springs) led to the successful isolation of pure methanotrophs which support findings from previous studies suggesting methanotrophs are ubiquitous [15-19]. Environmental samples were stored for various durations before enrichment which suggest methanotrophs remain viable even after long term isolation from their natural environments. For example, landfill sediment from Nottingham landfill and King Spring moss sample from Bath from which isolate 7 and Isolate 14 were isolated respectively, were stored at 4°C for over four months before they were enriched with media and CH₄ gas. This is important to know as it reduces the need to transfer samples immediately into NMS media and methane/air atmosphere when they are removed from their natural environments. It also shows that environmental samples can be obtained from anywhere in the world, considering the Nagoya protocol status of the country, transported at room temperature without CH₄ and still be used for isolation of methanotrophs. For the King Spring moss Bath sample, it must be emphasized that the sample obtained was not only starved of CH₄ but also stored at temperatures significantly lower than its natural environmental temperature (42°C).

The amount of starting sample used for enrichment can have a significant effect on the onset of methanotrophic growth. For example when 10 µL of starting sample was inoculated in 10 mL dNMS media in Enrichment 2, it took about 25 days before growth was observed in most liquid cultures. This was significantly slower compared to all other enrichments which had at least 100 µL as starting sample. The reason for low volumes of starting materials was to reduce the growth advantage of undesired microbes by minimizing their population and carbon source present in the starting sample. Observations from all the enrichments carried out showed using relatively lower sample volumes did not have a significant effect on reduction of available carbon source and presence of non-methanotrophs. This is because contamination was also observed on dNMS agar media plates streaked with growth cultures with the lowest starting sample volume (10 µL). Observations during this study point to a better solution for minimizing contaminating microbes and carbon source. This can be done

by growing the 100 μL – 1 mL of starting sample in 10 mL liquid media and making dilutions (at least neat – 10^{-4}) of the grown culture sample and spreading 10 – 50 μL of the dilutions on media plates.

Throughout this study, dNMS media which is one fifth concentration of NMS media was used for isolation of methanotrophs. Although this medium resulted in the successful isolation of pure methanotroph cultures, its dilute nature may result in early depletion of important nutrients by contaminating bacteria. This is likely to starve and hinder the growth of methanotrophs which are slow growers thereby shifting the balance of dominance towards non-methanotrophs. Hence future experiments can investigate the effect of using NMS for isolations in order to verify the validity of this assumption. Previous studies already showed successful isolation of methanotrophs with NMS [2, 253, 254].

One important skill acquired from this study was the ability to visually distinguish likely methanotrophic colonies from non-methanotrophic colonies on media agar plates. A key observation made about the appearance of methanotrophs on plate was that all transparent colonies screened turned out not to be methanotrophs. This is crucial because during the course of isolation experiments, a lot of transparent colonies appeared on agar media and a considerable amount of time and resources were spent trying to identify them. Hence methanotrophs are likely to be cream, pink and brown such as Isolates 12, 7 and 10 respectively, but highly unlikely to be transparent. The most common mistake repeated in the first three enrichments was expecting fast growing bacterial colonies to be methanotrophs. The 16S rRNA sequence of all the colonies that fully grew in the first week on media plates showed they were not methanotrophs. Throughout the study, methanotrophs isolated were always from colonies that were significantly smaller than surrounding colonies in the first few days of growth. This was supported by growth observation of pure methanotrophs which are almost invisible in the first three days on agar plates, very small in the first week and fully grown after two weeks. For methanotrophs such as Isolate 01 and 03, full growth was observed after one month. However, screening of fast growing bacteria in the first three enrichments was connected to one of the aims of this project which was isolation of fast growing methanotrophs.

Contrary to growth on agar media, growth in liquid media was much faster as OD₆₀₀ of 0.5 is typically attained in two days for pure isolates. This suggests that methanotrophs in environmental samples grown in liquid media can dominate the culture in 5 – 10 days thereby eliminating the need for prolonged enrichment of environmental samples. The difference in methanotroph growth time between media agar and liquid media is likely as a result of mass exchange of CH₄ and air between the headspace of growth environment and the media/methanotrophs. Methanotrophs grown in liquid media are constantly shaking at 200 rpm which enhances gas exchange, whereas methanotrophs grown on media agar are static under atmospheric pressure.

In addition to visual identification of methanotrophs, a more objective and evidence based identification of methanotrophs was colony PCR screening using the conserved regions of *pmoA* and *mmoX* genes as targets [255-257]. It is from these genes that the particulate and soluble methane monooxygenase (pMMO and sMMO) are transcribed, hence they are most likely essential and at least one of them is present in most methanotrophs. Taking into consideration the number of environmental samples screened, colony PCR of these target regions determined which colonies were enriched further. Colonies positive for either *pmoA* or *mmoX* genes were further enriched, while colonies negative for either gene were discontinued. Although this screening technique was instrumental in isolating pure methanotrophs, this method of screening can be counterproductive as poor PCR setup or results can lead to the discontinuity in enrichment of methanotroph colonies. Additionally, false positives can lead to waste of time and resources trying to enrich colonies that are not methanotrophs. More importantly, methanotrophs have been reported with a novel type of *pmoA* gene sequence [258]. These methanotrophs or others with diverging *pmoA* or *mmoX* sequence may fail detection using colony PCR screen with the set of primers used in this study. Nonetheless, colony PCR was the key molecular biology tool that aided isolation of pure methanotrophs. In addition to *pmoA* and *mmoX* gene targets, 16S rRNA colony PCR was used to identify the species of bacterial colonies that were screened. Except during the first three enrichment experiments, this screen was usually towards the end of the enrichment process when purity of isolates was suspected or confirmed.

This study lasted for approximately 18 months. The first nine months of this study did not result in isolation of methanotrophs. Certain adjustments were made to the experimental set-up that resulted in isolation of pure methanotrophs. The first change that yielded results and significantly reduced non-methanotrophs was the removal of vitamins. It has always been suspected that vitamins supported the growth of both methanotrophs and non-methanotrophs. Therefore it was not unexpected when removal of vitamins generally lowered the growth of both suspected methanotrophs and non-methanotrophs. This led to the loss of methanotrophs as a lot of colonies positive for methanotroph-specific genes failed to grow when transferred to vitamin-free dNMS media. Those that survived grew slower. However the absence of vitamins significantly lowered the population of contaminants. The isolation of methanotrophs that grow in vitamin-free media is advantageous as it can reduce cost of growth media if any of the isolates is to be used for industrial applications. The elimination of vitamins should however be done with caution as removal at the early stages of enrichment can prevent the isolation of novel methanotrophs that require vitamins. It is emphasized here that although the isolates in this enrichment 1, 2, 4 and 5 were isolated in vitamin-free media, subsequent routine experiments were carried out in the presence of vitamins as tolerance to lack of vitamins may not be uniform amongst the species used in this study.

Another important adjustment made was the alternation of enriched environmental samples from agar media to liquid media. Samples enriched in liquid media were also diluted before transferring the dilutions to solid media. This in addition to vitamin removal accelerated the isolation of pure methanotrophs. The media used for isolation was devoid of carbon sources. However the agar used for growing colonies was made of 0.3 g sugars per 100 g which was likely enough to support the growth of heterotrophic contaminants. Additionally, methanotrophs produce metabolites such as acetate, formate and lactate which can support the growth of contaminants [259]. Hence transferring colonies from agar media to liquid media likely reduced the presence of carbon sources and favoured the growth of methanotrophs. The dilution of the cultures that grew in liquid significantly reduced the contaminating bacteria and carbon sources carried along and produced by methanotrophs. It is worth noting that

no colony of methanotroph has been found consisting of two different methanotrophs which suggests that methanotrophs are unlikely to contaminate other methanotroph colonies. This could be due to the absence of mutual benefits from association unlike heterotrophs that can consume metabolites produced by methanotrophs which can be toxic to methanotrophs when they accumulate.

In addition to the general adjustments made, changes peculiar to isolates were carried out. For example the growth temperature of Isolate 10, *Methylocaldum* species, was alternated from 42°C to 37°C and back to eliminate the contaminating bacteria associated with it during the isolation process. Isolate 10 took the longest time (more than six months) to purify. Isolates 9 and 14 were restreaked to purity after the first round of dilutions as they appeared to be free from contaminants at early stages of their enrichments. The samples from which Isolate 9 and Isolate 14 were isolated came from different environments (landfill and warm spring respectively). They were both isolated at different times. However it took less than two months to isolate them, the quickest isolation throughout the study. This was likely due to their growth temperature of 45°C which did not favour the growth of mesophilic non-methanotrophs which are likely more prevalent in environmental samples. It should be noted that the isolate names/numbers are random and unrelated to the sequence of their isolation.

Determination of methanotroph purity involved their appearance on plate, under the microscope and growth in rich media. It is paramount that they are grown in all the rich media listed in the methods section. This is because suspected pure isolates in the past have failed to grow in as many as three out of four of the media used for testing purity. It is also important to grow the suspected pure methanotrophs for at least a week as sometimes growth has occurred after three days.

3.7 Conclusion

Environmental samples from lakes, landfills, bogs, warm springs and cow manure were enriched using dNMS media to isolate nine methanotrophs in their pure form over 18 months. The methanotrophs isolated include type I (*Methylomonas* and *Methylocaldum* species), type II (*Methylocystis* species) and type X (*Methylococcus capsulatus*). PCR screening of *pmoA* and *mmoX* genes of bacterial colonies is a reliable way to determine methanotrophic presence on agar media plates and has proved instrumental in the isolation of pure methanotrophs. The removal of vitamins from dNMS media and alternating growth of enrichment from liquid to solid media significantly impacted the outcome of isolation. *Methylococcus capsulatus* species (Isolate 9 and 14) were the easiest and quickest methanotrophs to isolate, possibly due to their ability to grow at 45°C, a temperature likely unsuitable for most contaminating heterotrophs.

4 Chapter 4: Characterisation of Methanotrophs

4.1 Introduction

In studies involving isolation of methanotrophs, characterisation experiments were usually designed to identify the novel properties of isolated strains in addition to other properties that will determine the taxonomy of isolates. Hence characteristics such as cell morphology, biochemistry and physiology were often the focus. In this study, characterisation was involved with but not limited to the listed criteria used in past studies. Additional characterisation parameters were investigated in order to determine the properties of methanotrophs relevant to the applications the isolates from this study were intended for. As such, characterisation was carried out with emphasis on methanotroph genetics which will provide insight into genetic modification of methanotrophs for biofuel and bio-based chemical production. Most importantly, attention was on those characteristics that make methanotrophs suitable for development of forward and reverse genetics which will provide the relevant information and needed tools for successful genome engineering of methanotrophs.

Analyses of 16S rRNA sequence carried out in Chapter 3 showed that all three types of methanotrophs were isolated in this study – type I (*Methylobomonas* and *Methylocaldum* species); type II (*Methylocystis* species) and type X (*Methylococcus capsulatus*). Further observation of the 16S rRNA data suggested Isolate 01 was identical to Isolate 03. Isolate 3* was also identical to Isolate 6. Characterisation of isolates will likely provide evidence to back the similarity or dissimilarity of these strains. For example, the difference in cell shape can be a decisive determinant of species dissimilarity. Furthermore, morphology of isolates is likely to provide more information such as the nature of internal structure and cell membranes of these isolates.

A basic prerequisite for carrying out genetic studies is knowledge regarding antibiotic susceptibility as it will provide information required for selecting antibiotic markers that enable counter selection of desired strains. Another important characteristic that was investigated was amenability to DNA transfer via conjugation and its efficiency in isolated strains. Strains with high DNA transfer efficiency make good candidates for

use in transposon library generation, an area investigated by this study. The ability to transform methanotrophs using electroporation is an important tool in genetic engineering which makes a good substitute for conjugation especially in cases involving the introduction of linear DNA into cells.

Many methanotrophs are obligate and use CH₄ as sole carbon and energy source. However, facultative species have been identified [31]. The ability of methanotrophs to use various carbon sources has various ramifications. For example, the use of carbon sources in solid and liquid form can be good substitutes in assays such as growth monitoring using the BioLector which requires retrofitting to use CH₄ gas. On the other hand, industrial applications that require consumption of CH₄ can be compromised if the facultative methanotrophs end up selecting alternative carbon sources for growth. Hence it was important to determine if isolated species could grow on alternative carbon sources.

The Nottingham SBRC intends to produce chemicals from methanotrophs in the future. However it is unlikely that methanotrophs can produce these chemicals in amounts beyond their tolerance threshold. Therefore the ability of isolates to tolerate different chemical compounds was investigated. The principal chemicals this the Nottingham SBRC will aim to produce using methanotrophs include poly-3-hydroxybutyrate (PHB), 3-hydroxypropionic acid (3HP), methanol (CH₃OH) and Isobutanol ((CH₃)₂CHCH₂OH). PHB toxicity seemed unlikely as it is produced in large quantities by type II methanotrophs. CH₃OH is also produced by methanotrophs but it is rapidly converted to formaldehyde which is converted to formate. 3HP is an important platform chemical for bioplastic production and isobutanol a biofuel source. Therefore it was important to see the effect they had on methanotrophs as toxicity to these chemicals will suggest inability to produce them in relatively large quantities even when heterologous genes are introduced. Reports of both 3HP and isobutanol synthesis by methanotrophs have not been found by the author. However, a methylotrophic bacterium *Methylobacterium extorquens* AM1 was able to grow on methanol and produce 3HP after expressing native genes of *Chloroflexus aurantiacus*. *Methylobacterium extorquens* AM1 is not a methanotroph as it does not grow on CH₄ [260].

Under laboratory conditions, methanotrophs are usually grown with NMS media. In addition to mineral salt, components including trace elements, Fe-EDTA, vitamins, phosphate buffer and $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ are added. The addition of these components at an industrial scale will increase cost of production. Furthermore, introducing these components into NMS media at the wrong concentrations can result in sub-optimal growth of methanotrophs. Finally, elimination of components such as vitamins can reduce the risk of contamination, a problem commonly encountered during laboratory growth of methanotrophs [261]. Hence it was important to determine the effect of these components on isolated methanotrophs. The use of nitrate as a nitrogen source in NMS media may also not be the cheapest and most suitable. The investigation of an alternative nitrogen source was carried out.

The growth of methanotrophs in pure CH_4 is suitable under laboratory conditions as CH_4 is cheap when purchased in small quantities. At industrial scales, the use of pure CH_4 may not be viable for the production of chemical commodities due to competition for other purposes such as electricity generation, heating and lighting. One way around this limitation is the use of low grade waste CH_4 gas which is flared or released into the atmosphere. Low grade CH_4 produced as biogas or emitted as landfill gas usually has a low percentage of CH_4 that can lead to knocking of engines if used for electricity generation [262]. Additionally, low grade biogas and landfill gases contain trace gases that make them unsuitable for electricity generation [263]. This study therefore investigated the use of biogas for growth of methanotrophs and PHB production.

The genome sequence of methanotrophs will likely provide genetic information backing the phenotype of characterised isolates. In addition, phenotypes can be predicted using information from genome sequences. Whereas Illumina provides sufficient genomic data, complementing these data with PacBio genome sequence data results in more accurate and reliable genome information. The advancement of PacBio enabled by Single Molecular Real-Time (SMRT) sequencing provides more long read DNA sequences which can be advantageous when determining genome sequencing. PacBio also provide Restriction Modification Systems (RM system) data that shows bacterial DNA methylation pattern which can be exploited to improve

transformation efficiencies of subject strains. Hence genome sequences of isolates was acquired and studied.

The expression of genes introduced into methanotrophs cannot be predicted as promoters that work in other organisms may not necessarily work in methanotrophs. The importance of having promoters of different strength cannot be overemphasized as some genes need to be expressed at the right level for optimal translation of messenger RNA into protein [4, 264]. A promoter probe assay was therefore carried out to test the activity of selected promoters.

4.2 Specific Research Aims

The aim of this chapter was to investigate the properties and behaviour of methanotrophs isolated in this study in order to determine their novelty. This chapter also aimed to determine the suitability of isolated methanotrophs for applications such as metabolic engineering, development of forward and reverse genetic tools for methanotrophs, and production of bio-based chemicals. Finally, this chapter involved gathering as much information as possible on isolated strains in order to better understand the nature of methanotrophs.

4.3 Material and methods

4.3.1 Primers

Table 26: Primers

Name	Sequence	Description
Universal 16S rRNA (F515f/f515r)	Forward- GTGYCAGCMGCCGCGGTA Reverse- GARCTGRCGRCRRCCATGCA	This primer set targets 16S rRNA and produces a fragment approximately 500 bp when bacterial DNA is amplified.
pMMOI (A189f/A682r)	Forward- GGNGACTGGGACTTCTGG Reverse- GAASGCNGAGAAGAASGC	This primer combinations A189f/A682r amplify a region of <i>pmoA</i> gene approximately 525 bp in size [251].
pMMOII (A189f/A650r)	Forward- GGNGACTGGGACTTCTGG Reverse- ACGTCCTACCGAAGGT	The <i>pmoA</i> primer combinations A189f/A650r amplify a region of <i>pmoA</i> gene approximately 500 bp in size [251].
pMMOIII (A189f/mb661r)	Forward- GGNGACTGGGACTTCTGG Reverse- CCGGMGCAACGTCYTTACC	This primer combinations A189 and mb661 amplify a region of <i>pmoA</i> approximately 470-bp large [75].
sMMO (A166f/A1401r)	Forward- ACCAAGGARCARTTCAAG Reverse- CCGATCCAGATDCCRCCCCA	The primer combinations amplify an <i>mmoX</i> region 800 bp in size [252].
ColEI_for	ACGGTTCCTGGCCTTTTGCT	Primers for sequencing promoters
EYFP_rev	CTTGTCAGCTCGTCCATGC	Primer for sequencing yellow fluorescent protein and promoters

4.3.2 Strains

Table 27: Strains

Strain	Description	Reference
<i>Methylocystis parvus</i> (OBBP)	Wild-type strain	Purchased from NCIMB
<i>Methylococcus capsulatus</i> (Bath)	Wild-type strain	Andrew Crombie, University of East Anglia

4.3.3 Plasmids

The plasmid pMTL90882 was used in many studies involving bacterial transformation in the work carried out in this thesis. A lot of other plasmids used were also derived from pMTL90882. To make this plasmid, pMHA199 reported to work for methanotrophs, was first assembled using genes from three plasmids purchased from BIOMATIK – pBSK1, pBSK2 and pJET1.2 [265]. These plasmids were electroporated into *E. coli* DH5 α and plated on LB ampicillin (100 μ g/mL). Colonies from all three plates

were grown and miniprep was used to extract plasmids. Plasmids of pBSK1, pBSK2 and pJET1.2 were digested with NotI/BamHI, NotI/XhoI and BamHI/XhoI respectively.

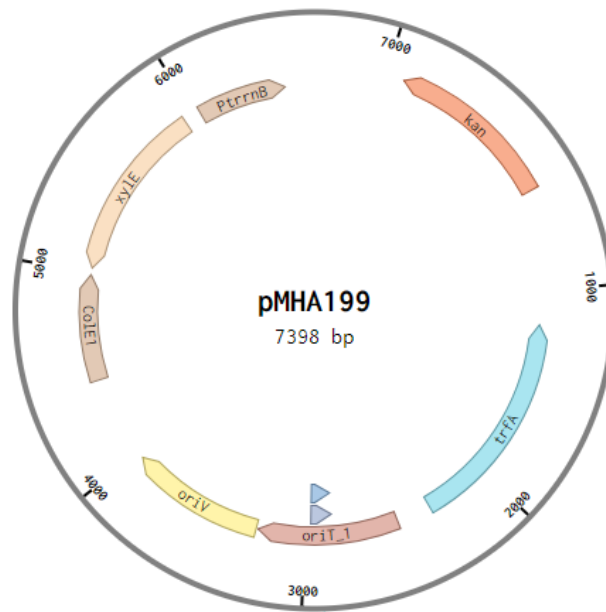


Figure 29: pMHA199 plasmid assembled by digesting components of pBSK1, pBSK2 pJET 1.2 plasmids.

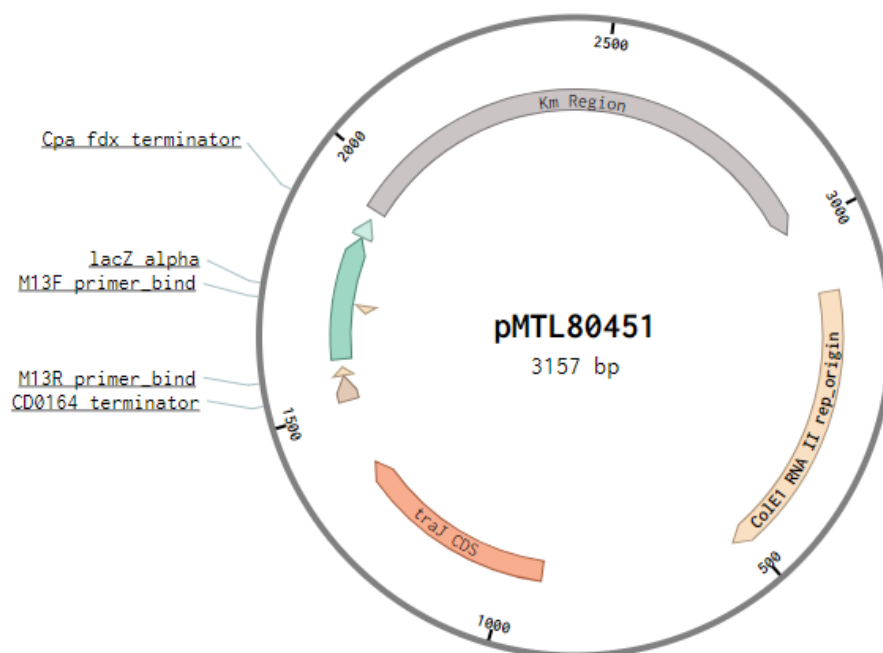


Figure 30: pMTL80451 plasmid with kanamycin resistant cassette and modular restriction sites available in SBRC Nottingham culture collection.

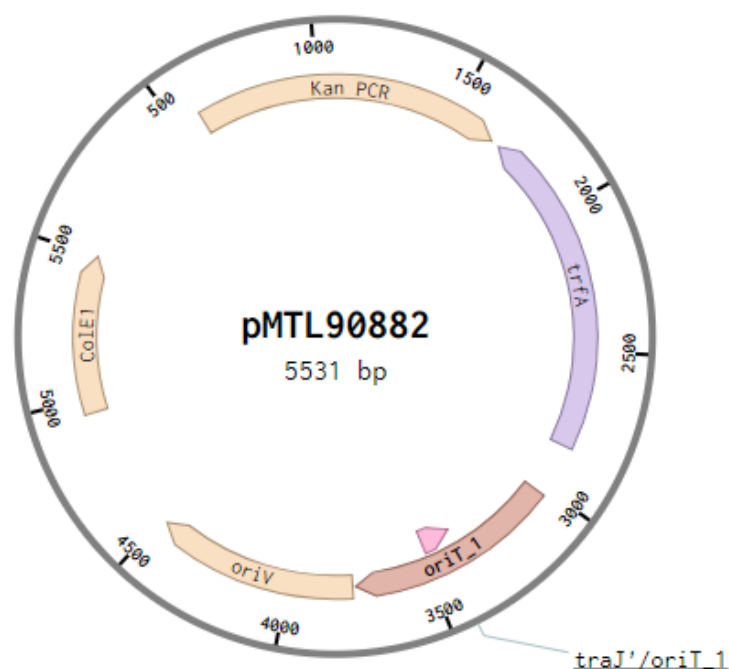


Figure 31: pMTL90882 assembled from pMHA199 and pMTL80451. This plasmid is used as the backbone for new plasmids of methanotrophs. It is a modular plasmid which has a ColE1 origin of replication, an oriV for vegetative replication and trfA which binds to oriV to initiate replication, a kanamycin resistant cassette and a multiple cloning site.

The digests were ligated to form pMHA199. PCR of pMHA199 region between ColE1 and trfA was carried out using trfA_pme-F and ColE1_SbfI-R. The fragment was ligated into a modular plasmid pMTL80451 which has a kanamycin resistant cassette and a multiple cloning site.

4.3.4 Conjugation of methanotrophs

Conjugation was carried out based on modifications of the method used by Martin and Murrell [238]. 10 mL of *E. coli* S17-1 λ pir harbouring the plasmid intended for conjugation was grown overnight in LB media containing kanamycin (50 μ g/mL). The absorbance (OD₆₀₀) of the grown culture was measured and the *E. coli* was washed three times with NMS to remove the antibiotics by centrifuging at 8000 rpm for 5 minutes. After the third wash, *E. coli* was resuspended in 10 mL NMS and the absorbance (OD₆₀₀) re-measured. A calculated volume of the *E. coli* suspension and a calculated volume of methanotroph culture was mixed to have 1:1 donor:recipient. The calculated values are important to provide a standard ratio due to OD₆₀₀ variation

of *E. coli* overnight cultures as well as that of methanotrophs grown for 2 – 3 days. Therefore *E. coli* resuspension having an OD₆₀₀ of 2.5 was calculated as follows:

Volume of *E. coli* that will give OD₆₀₀ of 1 at 1 mL = 1 mL/2.5 = 0.4 mL

If methanotroph OD₆₀₀ was measured as 0.7,

Volume that will give OD₆₀₀ of 1 at 1 mL = 1 mL/0.7 = 1.25 mL

Hence in the example above, 0.4 mL of *E. coli* will be mixed with 1.25 mL of the methanotroph to have a 1:1 ratio at OD₆₀₀ 1 and 1 mL.

The mixture of *E. coli* and methanotroph was spun at 8000 rpm for three minutes and resuspended in 50 µL of NMS which was spotted on a dry plate of NMS BA containing 0.5% yeast extract. The spot was allowed to dry and then incubated at the required temperature in CH₄ typically for 48 hrs or as required in the particular experiment. After mating, the spot was scraped with a sterile loop and resuspended in 1 mL of NMS. Depending on the species, 10 µL was diluted in 90 µL of NMS (for *Methylocystis* species as they have high efficiencies of conjugations) and spread on NMS plates with the required antibiotics. For *M. capsulatus* (any strain) or *Methylocaldum* species, the 1 mL mating suspension was resuspended in 100 µL and spread on NMS with the required antibiotic. Colonies appeared between 10 – 15 days.

4.3.5 Determination of conjugation efficiency in methanotrophs

Conjugation efficiency was calculated as number of transconjugants per donor cell. In other words, number of methanotrophs growing on antibiotic plates per number of live *E. coli* present during mating. This can be derived from the conjugation mix spotted on mating plates. After re-suspending the spot in NMS media (1 mL), dilution series of the suspension were made from 10⁻¹ – 10⁻⁷. 10 µL of each dilution was spotted on LB kanamycin (50 µg/mL) for calculating the number of donors. To calculate the number of recipients, 10 µL of each dilution was spotted on NMS kanamycin (15 µg/mL) for type I and NMS kanamycin (50 µg/mL) for type II methanotrophs. Kanamycin plates for type I and type II contained nalidixic acid (25 µg/mL) to prevent *E. coli* from growing. The spots were made in triplicates for *E. coli* and in quadruplicate

for methanotrophs. The average number of colonies from the highest dilution spotted was multiplied by a factor of that dilution to determine how many cells grew in 10 μ L of the neat sample. For example if 10 colonies grew on each of the three 10^{-7} spots, 10 the average is multiplied by 10^7 (10×10^7) to get number of colonies per 10 μ L of neat. This was further multiplied by 100 to determine how many colonies were in 1 ml of the conjugation mix spotted. The efficiency thus is:

$$\text{Conjugation efficiency} = \text{Number of transconjugants in 1 mL} / \text{Number of donors in 1 mL}$$

Therefore the number transconjugants and number of donors were calculated by counting the number of colonies per mL of neat mating suspension that grew on NMS and LB antibiotic plates respectively [266].

4.3.6 Electroporation of methanotrophs

The ability to carry out electroporation on methanotrophs was determined. Type II methanotrophs were tested (Isolate 3*, Isolate 6 and Isolate 12). Modifications of protocol used by Crombie and Murrell, as well as Baani and Liesack was adopted for this purpose [85, 267]. Methanotrophs were grown in NMS media to low OD₆₀₀ (0.3 – 0.4) and high OD₆₀₀ (0.7 – 0.8). After OD₆₀₀ measurement, samples were harvested by spinning at 6000 $\times$ g, for 15 min at 4°C; washing twice and resuspending in phosphate buffer containing 1mM MgCl₂ and 270 mM sucrose [268]. After OD₆₀₀ measurement of suspension, 100 μ L was electroporated with Biorad Genepulser Xcell (2.5kV, 400 Ω and 25 μ F). After electroporation, samples were re-suspended in 1 mL of NMS and incubated in electroporation cuvette or 60 mL serum bottles and spun at 200 rpm for 6 hrs to recover at 30°C. After recovery, samples were re-suspended in 100 μ L of NMS and plated on NMS kanamycin 50 μ g/mL media for 2 – 3 weeks. 100 ng of pMTL90882 or NPR3 was used as electroporation plasmids which both carry a kanamycin antibiotic marker. NPR3 also had an Enhanced Yellow Fluorescent Protein (EYFP) expressed with P3 promoter. PCR was carried out on colonies to determine the presence of plasmids in colonies obtained.

4.3.7 Phylogeny of Isolates

16S rRNA sequence was obtained from the genomes of selected Isolates that were sequenced using Illumina and/or PacBio (Isolate 3*, Isolate 6, Isolate 10, Isolate 12 and Isolate 14). The 16S rRNA sequence of other isolates was obtained from PCR amplification using primers F515f and F515r (Isolate 01, Isolate 03 and Isolate 7). FASTA sequences of 16S were uploaded to Molecular Evolutionary Genetics Analysis software (MEGA version 10) and aligned using Clustal Omega alignment tool. Phylogenetic tree of the aligned sequences was obtained using Maximum Likelihood Tree as method of statistical analysis. Tamura-Nei model was used and Nearest Neighbour Interchange was the Tree Interference option that was selected. Everything else was left as default under the MLT statistical method of generating phylogenetic trees in MEGA. Methanotrophs isolated in this study were computed first. Another phylogenetic tree was constructed which consisted of additional species to gain insights into the relationship between methanotrophs and other species.

4.3.8 Microscopy

Phase contrast microscopy

Nikon Eclipse Ci phase contrast microscope with digital camera was used to observe the morphology of cells. Colonies were picked from solid agar slopes using a sterile loop and dipped into 10 μ L of PBS. 1 μ L of the solution was pipetted on a microscope slide and covered with a cover slip. Immersion oil was added to the coverslip and the slide was viewed using $\times 400$ magnification. Images were taken using Nikon microscope camera.

Electron microscopy

Transition Electron Microscope (TEM) was used to view images of isolated methanotrophs. Cultures were centrifuged 5,000 rpm for 5 minutes and suspended in 0.1M cacodylate buffer overnight then re-suspended again in the same solution a further 3 times. They were then post-fixed in 1% osmium tetroxide and dehydrated in ethanol. Dehydration was carried out by washing once for 1 min in distilled water then dehydrating 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 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 uranyl 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.

4.3.9 Antibiotic minimum inhibitory concentration (MIC) test

To determine what antibiotic selection markers to use during molecular microbiology work it was important to measure the minimum inhibitory concentration of selected antibiotics. Antibiotic strips from Lifolichem ranging in concentration from 0.016 – 256 µg/mL were laid on dNMS BA plates spread with 200 µL of growing culture and incubated with flushed CH₄ at 30°C (*Methylocystis* species) and 37°C (*Methylococcus capsulatus* and *Methylocaldum* species) for two weeks. Kanamycin, nalidixic acid, streptomycin, ampicillin, tetracycline and gentamycin were the antibiotics tested.

4.3.10 Growth on alternative carbon sources

The use of CH₄ is not always convenient as a carbon and energy source especially in microbiological applications such as growth measurements where taking time points may alter the headspace gas composition. As such alternative sources of carbon were selected and tested in one methanotroph isolated in this study (Isolate 3*). Cell cultures in 10 mL of dNMS media were grown in triplicates. CH₄, D (-) fructose, sodium acetate, sodium pyruvate, sodium formate, sodium succinate dibasic, sodium fumarate, disodium-DL-malate, D-lactose monophosphate, and sodium-DL-lactate were added as sources of carbon at 0.1% concentrations. OD₆₀₀ of all samples was taken at day 0, 3, 6, 9, 12 and 15. These were grown in 48 well plates. The CH₄ control

plates were also grown in 48 well plates but were incubated in an air-tight chamber flushed with CH₄.

4.3.11 Growth on methanol

Methanotrophs were grown in NMS media with varying concentrations of methanol – 0.01%, 0.1%, 1%, 0.1% vapour and 50:50 CH₄:air as control. Methanol was added to 10 mL of media containing methanotrophs at starting OD₆₀₀ of 0.02 and grown for 15 days. To prevent the escape of methanol from serum bottles, only one time point was taken at the end of the experiment. Samples that grew were spotted on LB plates to ensure that they were not heterotrophic contaminants.

4.3.12 Tolerance test

The use of CH₄ to produce bio-based chemicals such as isobutanol and 3-hydroxypropionic acid (3HP) holds a lot of potential in the biotechnology industry. Therefore it is important to determine the toxicity effect of these chemicals at known concentrations. The tolerance of methanotrophs to extreme environments (acidic, basic and saline) will also give an insight into optimum media conditions. Isobutanol, 3HP and methanol were tested at various concentrations. The tolerance of methanotrophs in acidic, basic and saline environments was also tested. The ability of one methanotroph (Isolate 3*) to grow in the absence of nitrogen and in the presence of CO₂ was also determined.

4.3.13 Growth of methanotrophs in biogas

M. parvus (OBBP), *M. capsulatus* (Bath) and isolated species of methanotrophs were grown in biogas and CH₄. The first biogas sample was collected in Sigma-Aldrich 2 L Teddlar bags from an anaerobic digester plant in Staffordshire fed with plant biomass (shredded maize). The second biogas sample (BG2) was collected in Teddlar bags from an anaerobic digester also located in Staffordshire but fed with food waste including animal blood. Bulk gas composition was measured using Trace GC while trace gas composition was measured by Lucideon in Staffordshire. 9 mL of culture at starting OD₆₀₀ of 0.02 was added to 165 mL serum bottle together with 75% air and 25% (39 mL) of BG1, BG2 and CH₄ as carbon source in a volume ratio. Each sample was grown in

triplicate. Samples were incubated at 30°C for type II and 37°C for type I and *M. capsulatus* (Bath) for three – four days. After growth, 1 mL of headspace was taken for headspace analysis using Trace GC. OD₆₀₀ of the samples were then taken and the rest were pelleted to check for PHB production. The pellet was also used to confirm the presence of *pmoA* in these strains to ensure methanotrophs were the ones growing in these samples.

4.3.14 Headspace analysis

Trace GC was used to carry out headspace analysis of selected gases consumed and produced by methanotrophs grown in CH₄ and biogas. A 2.5 mL airtight Hamilton SampleLock syringe was used to remove 1 mL of headspace from serum bottles before and after growth of methanotrophs. This was injected into a Trace 1310 GC machine at initial temperature of 40°C which rose to 250°C at the end of the run time which lasted 10.7 minutes. Shin carbon ST 100/120 column was used with 2mm/1mmID and flow rate of 5 mL/min. Helium was the carrier gas and a thermal conductivity detector (TCD) was used. Standards (CH₄, O₂, CO₂ and N₂) were injected in triplicates first before other samples were run. After run time, retention time and % amount of gases were recorded. The percentage of each gas in the headspace was calculated from the calibration curve of the standards measured.

4.3.15 PHB analysis

PHB analysis was carried out using the SBRC Nottingham Analytics Suite Standard Operating Procedure. Samples grown on CH₄ and biogas were pelleted in 2 mL Eppendorf tubes and freeze dried overnight using the Thermo Micromodulyo Freeze Dryer. The pellets were transferred to a pre-weighed 2 mL Eppendorf and weighed again. These pellets were then transferred to 16 mm diameter round-bottomed screw cap centrifuge tube. In a fume cupboard, 100µl of 1mg/ml Benzoic acid solution in 1-propanol was added. This was followed by 4ml of 25% concentrated hydrochloric acid in 1-propanol. The glass tubes were then heated at 100°C for two hours. After cooling, 4 mL of deionised water was added and each tube was vortexed for 30 seconds to cause phase separation. The top layer was discarded, 4 mL of deionised was added and each tube was vortexed again for 30 seconds. The top layer was discarded for the

second time and 1 mL of the bottom layer was transferred into a GC snap vial cap which was used for PHB measurement using GC/MS in the SBRC Analytics Suite programme PHB_T1.M.

Another PHB analysis was carried out using a different method for growing methanotrophs but the same sample preparation from freeze-drying to the GC/MS run. During the growth phase, methanotrophs were grown in dilute NMS media (0.1× dilution) except FeEDTA which was kept at NMS levels. 30 mL of samples were grown in 165 mL serum bottles for nine days using CH₄ and biogas as carbon source. Starting OD₆₀₀ of growth averaged 0.02. After three days, the serum bottles were vented and 25% of CH₄/biogas was added to 75% of air in the serum bottles. This was repeated after day six. After day nine, the serum bottles were degassed, 50 µL was spotted on LB to check for purity and the rest were pelleted after checking the OD₆₀₀. The pellets were freeze-dried overnight, weighed and then analysed using the SBRC Analytics Suite SOP.

4.4 Results

4.4.1 Morphology of methanotrophs

Isolate 01

When grown in NMS BA plates, Isolate 01 colonies appeared cream in colour with a concave morphology. The colonies grew to a 2 mm diameter after one month of growth from glycerol stock and incubation at 30°C. Phase contrast morphology showed the cells to be diplococci in shape (Figure 32). Isolate 01 is a *Methylocystis* species.

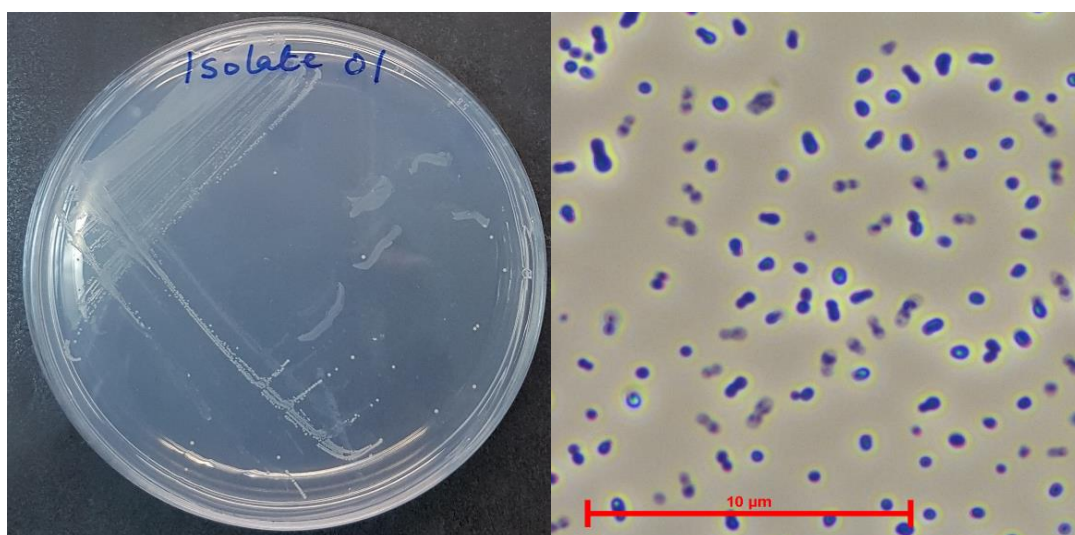


Figure 32: Colony morphology of Isolate 01 (left) and Phase Contrast Microscopy image (right).

Isolate 03

Isolate 03 (*Methylocystis* species) growth on plate was similar to Isolate 01 with regards to shape and colour. It took one month for colonies to reach full growth. Just like Isolate 01, the phase-contrast microscopy images showed the cells were diplococci (Figure 33).

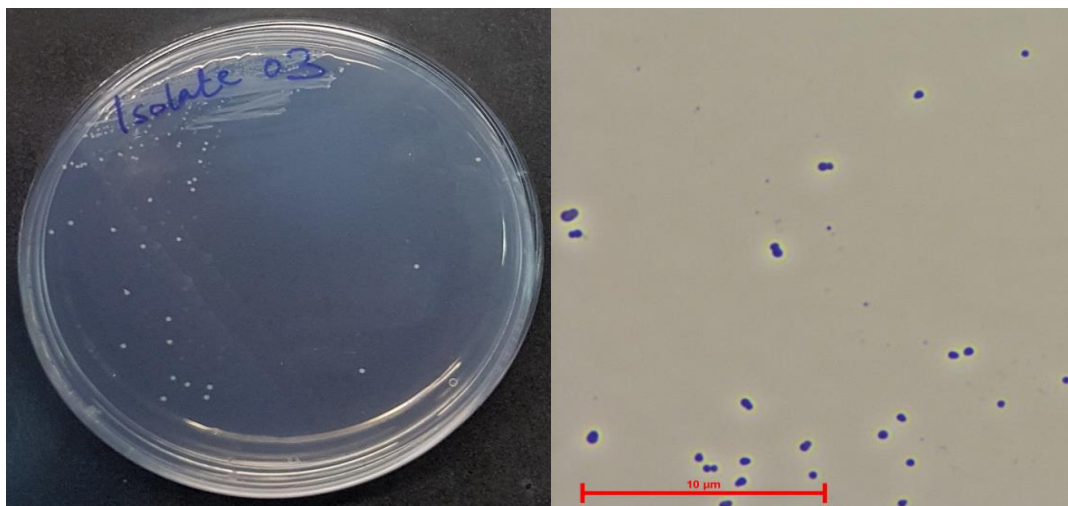


Figure 33: Colony morphology of Isolate 03 (left) and Phase Contrast Microscopy image (right).

Isolate 3*

Colonies of isolate 3* appeared creamy after 10 days of growth on NMS BA plate. After 2 – 4 weeks, the cream colour gradually transformed into and remained pink. The colonies were concave and grew as large as 3 mm in diameter. Microscopy images showed they are diplococci (Figure 34)

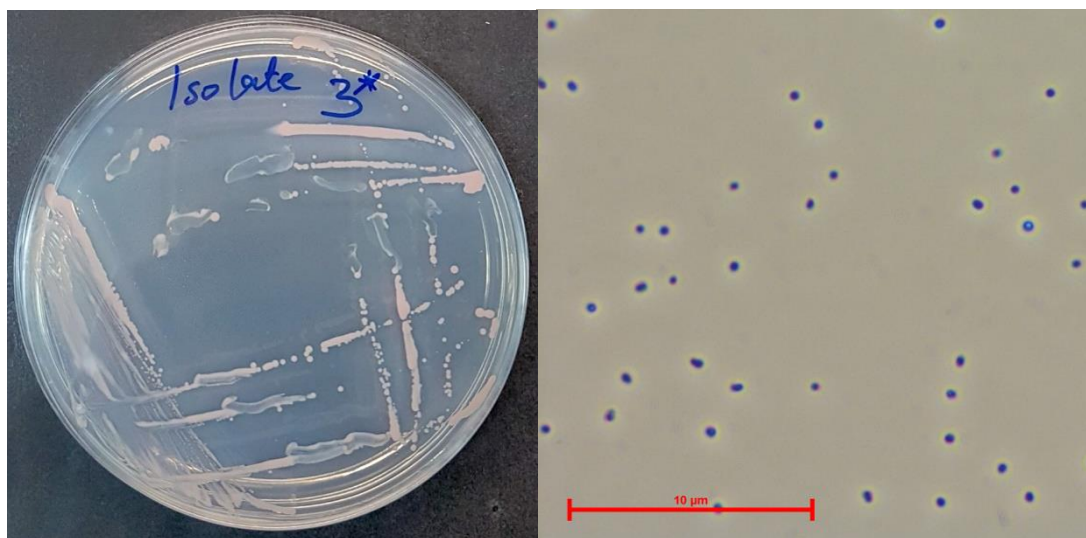


Figure 34: Colony morphology of Isolate 3* (left) and Phase Contrast Microscopy image (right).

Transmission Electron Microscopy (TEM) images of Isolate 3* further suggests they are diplococci (Figure 35). The scale of the single cell image is 200 nm as represented by the white line at the bottom right corner and it suggests the size of the cell to be approximately 500 nm from the view shown. It has a magnification of 87000×. The prominent white storage granule is suspected to be PHB while the striations running

round the cell periphery are the Intra Cytoplasmic Membranes (ICM) where CH_4 is oxidised. Isolate 3* also appears to have fimbriae running round the entire cell surface. The image on the right has a 4200 $\times$ magnification and is at a scale of 5000 nm. It portrays Isolate 3* in different views and stages of development. Isolate 3* is also a *Methylocystis* species.

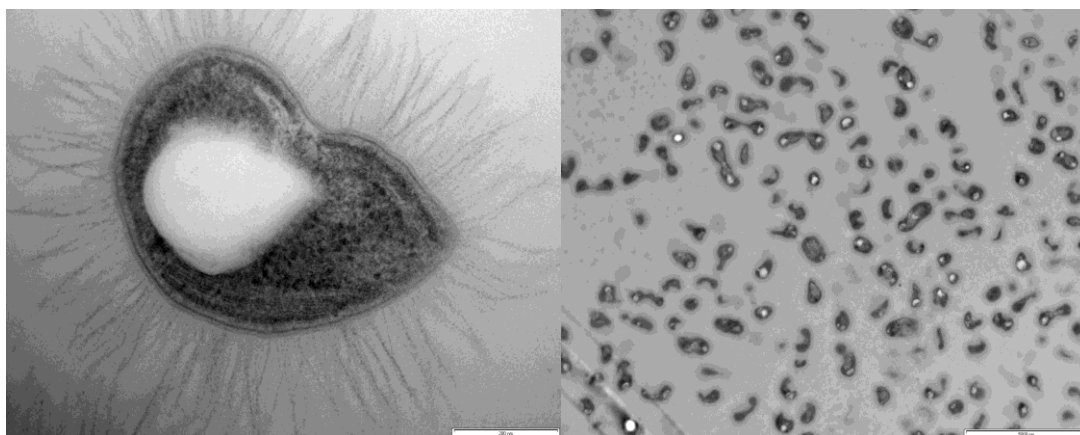


Figure 35: TEM images of Isolate 3*. Single cell image (left) shows storage granule, ICM running round the periphery of the cell membrane. Fimbriae were also visible around the surface of the cell. The image on the right is a lower magnification and shows different views of the cells.

Isolate 6

Colonies of isolate 6 appeared creamy after 10 days of growth on NMS BA plate. After 2 – 4 weeks, the cream colour gradually transformed into and remained pink as described for Isolate 3*. The colonies were concave and grew as large as 3 mm in diameter. Microscopy images showed they are diplococci (Figure 36). Images from TEM suggest that Isolate 6 is also diplococci in shape (Figure 37). The scale of the single cell image is 200 nm as represented by the white line at the bottom right corner. The image is at a magnification of 60000 $\times$. The prominent white storage granule is suspected to be PHB while the striations running round the cell periphery are the ICM.

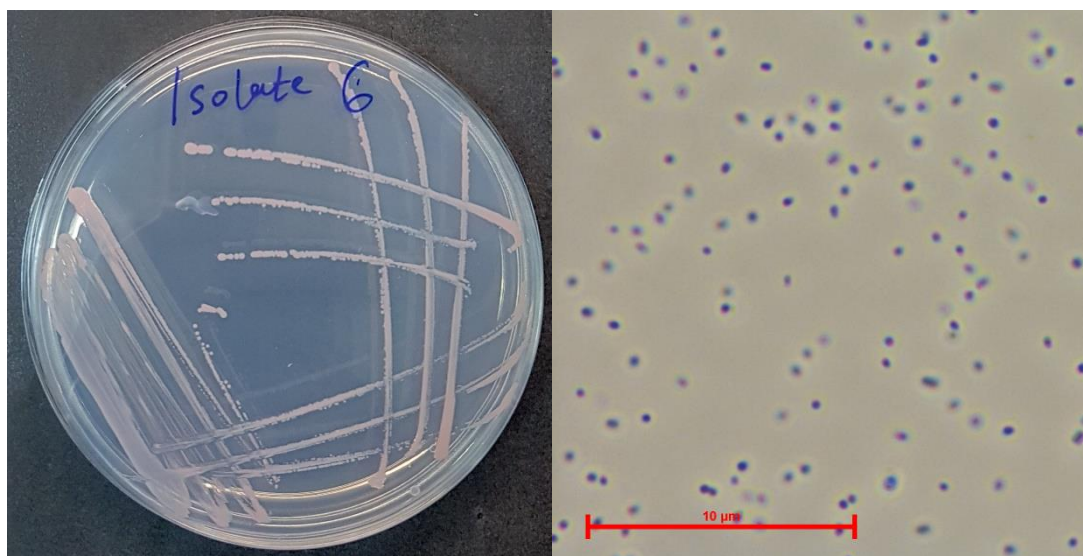


Figure 36: Colony morphology of Isolate 6 (left) and Phase Contrast Microscopy image (right).

The image on the right has a 4200× magnification and is at a scale of 5000 nm. It portrays Isolate 6 in different views and stages of development.

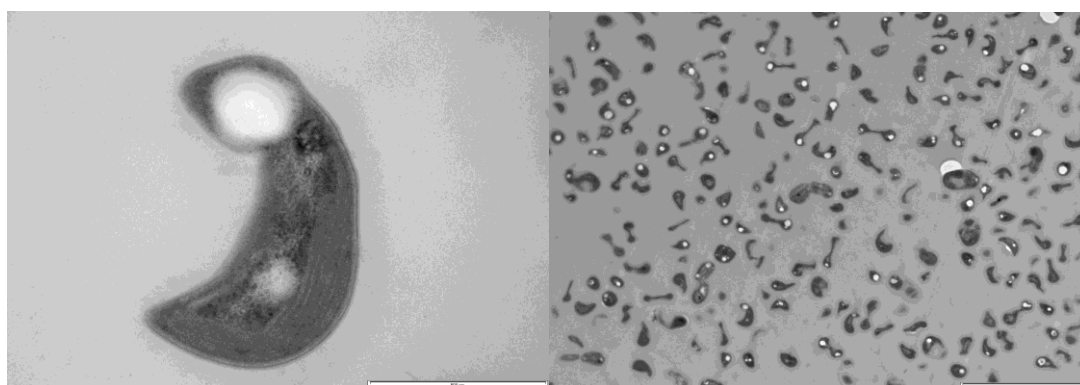


Figure 37: TEM images of Isolate 6. Single cell image (left) shows storage granule and ICM running round the periphery of the cell membrane. The image on the right is a lower magnification and shows different views of the cells.

Isolate 7

Colonies of isolate 7 appeared pink early in their growth stage and remain pink after three weeks when maximum growth was attained (Figure 38). Afterwards, the colonies dug in and fused with the agar making it difficult to pick with loops. At early growth stage, they appear as concave and later flatten out just as they begin to fuse with agar. Like the other isolates, Isolate 7 cells appeared as diplococci when viewed using Phase-contrast microscopy. Isolate 7 is a *Methylomonas* species.

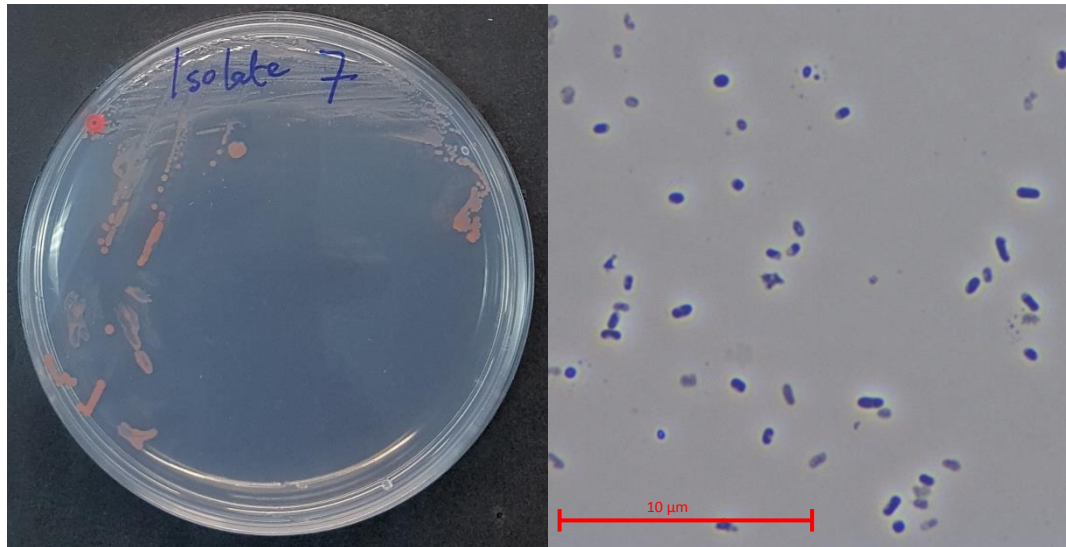


Figure 38: Colony morphology of Isolate 7 (left) and Phase Contrast Microscopy image (right).

Isolate 9

Colonies of Isolate 9 appeared and remained creamy throughout growth stage (Figure 39). The colonies have a concave morphology and appear as diplococci when viewed with phase contrast microscope. Isolate 9 is a *Methylococcus capsulatus* species.

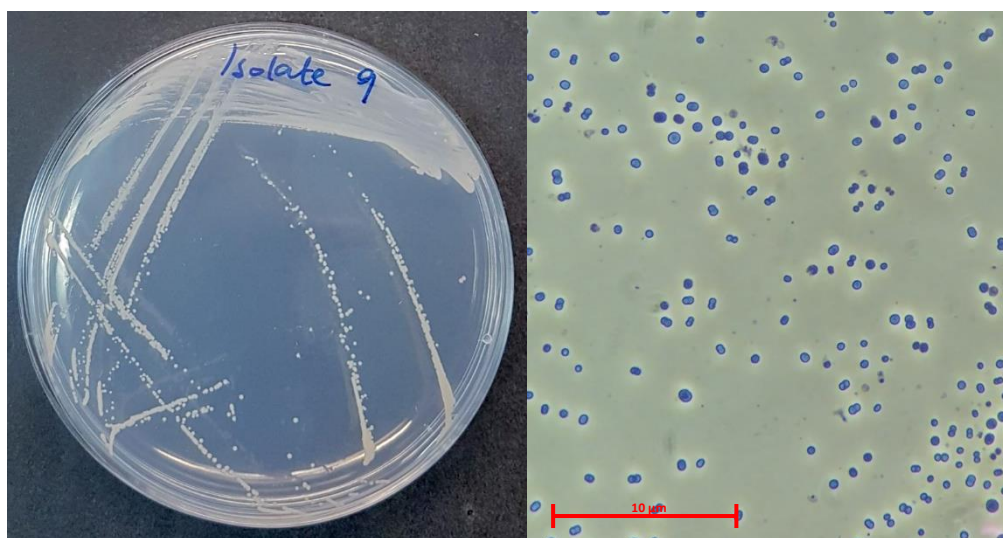


Figure 39: Colony morphology of Isolate 9 (left) and Phase Contrast Microscopy image (right).

Isolate 10

Colonies of Isolate 10 appearances were temperature dependent. At 30 – 37°C, they appeared as cream colonies and gradually turned brown when full colony sizes were attained. At 42°C, the onset of brown colouration was earlier. The colonies were concave morphology in the first two weeks of growth but gradually dried up, flattened out, dug into and fused with agar. Under the microscope, they appeared as cocci and diplococci (Figure 40). Isolate 10 is a *Methylocaldum* species.

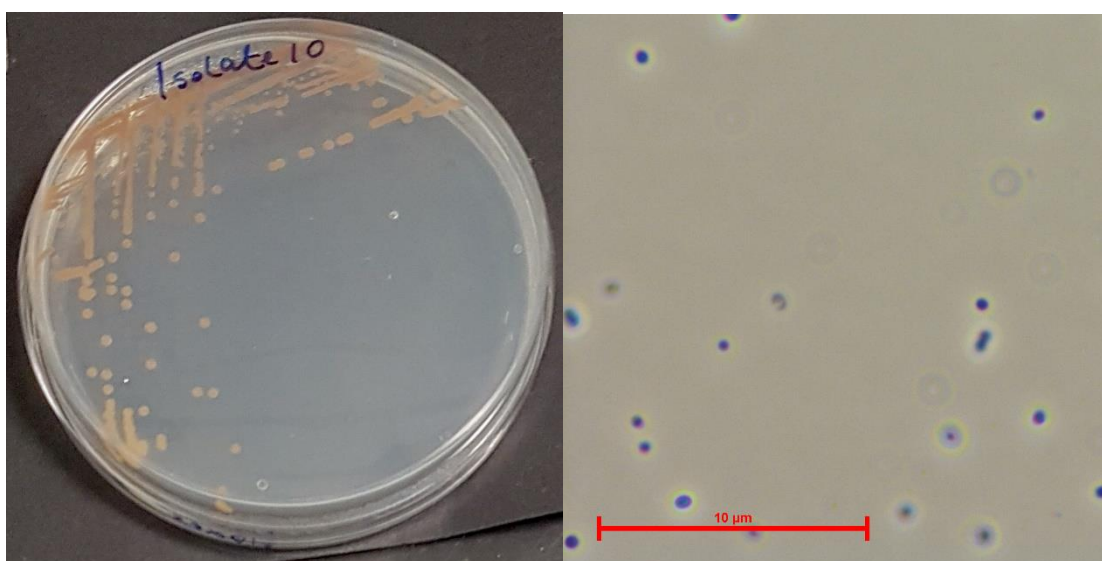


Figure 40: Colony morphology of Isolate 10 (left) and Phase Contrast Microscopy image (right).

TEM images of Isolate 10 suggest they are mostly cocci (Figure 41). The scale of the single cell image (left) is 500 nm. It has a magnification of 43000×. Striations can be

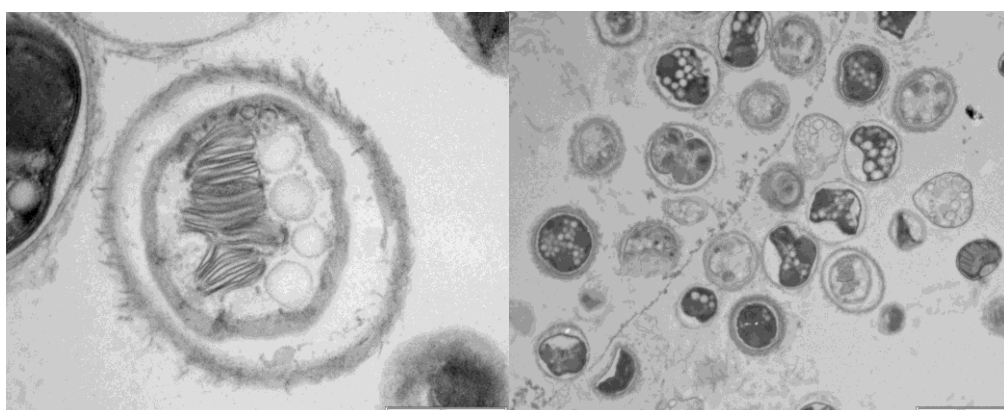


Figure 41: TEM images of Isolate 10. Single cell image (left) shows storage granule and ICM in bundles. A rough hairy surface is seen covering the external membrane as well as regular spherical granules. The image on the right is a lower magnification and shows different views of the cells.

clearly seen in bundles, a property that is characteristic of type I methanotrophs. A rough hairy covering of the external membrane was observed. Round regular granules were also observed although they are likely to be glycogen as type I methanotroph's ability to produce PHB is still speculated [269]. The image on the right has 8200 $\times$ magnification and is at a scale of 5000 nm.

Isolate 12

Colonies of Isolate 12 appeared creamy and grew at 30° (Figure 42). The morphology of the colonies was concave. Although prolonged incubation did not lead to colonies fusing with agar, colonies were observed to dry up and solidify. Under the microscope, they appeared as diplococci with white spots (Figure 42). Isolate 12 is a *Methylocystis* species.

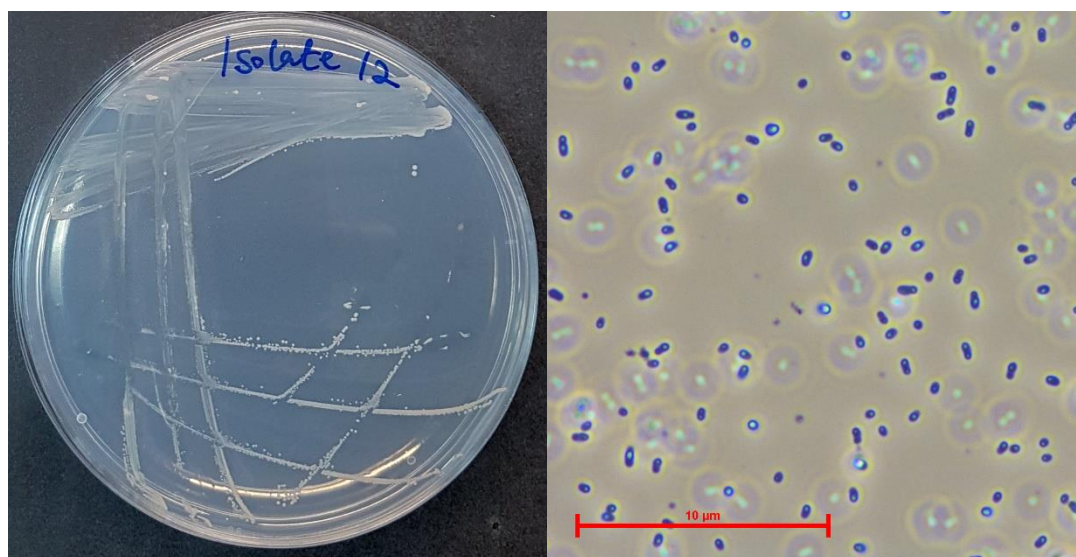


Figure 42: Colony morphology of Isolate 12 (left) and Phase Contrast Microscopy image (right).

TEM images of Isolate 12 also suggested diplococci shape (Figure 43). The scale of the single cell image is 500 nm and has a magnification of 60000 $\times$. The prominent white storage granule is suspected to be PHB and can be seen occupying most of the cell. This is observed for many other Isolate 12 cells viewed with the TEM. In cells that appear diplococci, the PHB granule usually accumulated at one pole of the cell. The image on the right has 8200 $\times$ magnification and is at a scale of 2000 nm. It portrays Isolate 12 in different views and stages of development. Isolate 12 is also a *Methylocystis* species.

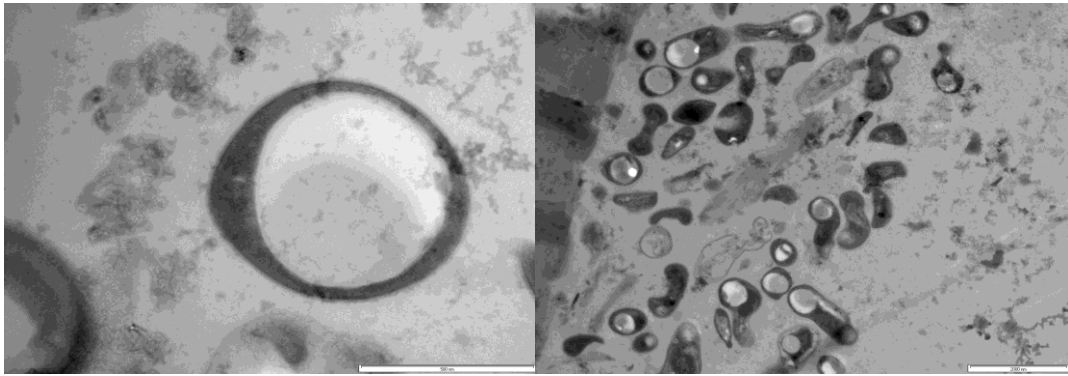


Figure 43: TEM images of Isolate 12. Single cell image (left) shows storage granule occupy most of the cell. The image on the right is a lower magnification showing how surrounding cells also accumulate a lot of PHB.

Isolate 14

The appearance of Isolate 14 colonies on agar plates was similar to Isolate 9. The morphology of the colonies was concave. Colonies of Isolate 14 appeared and remained creamy throughout growth stage (Figure 44). They appeared as cocci and diplococci when viewed with phase contrast microscopy. Isolate 14 is a *Methylococcus capsulatus* species.

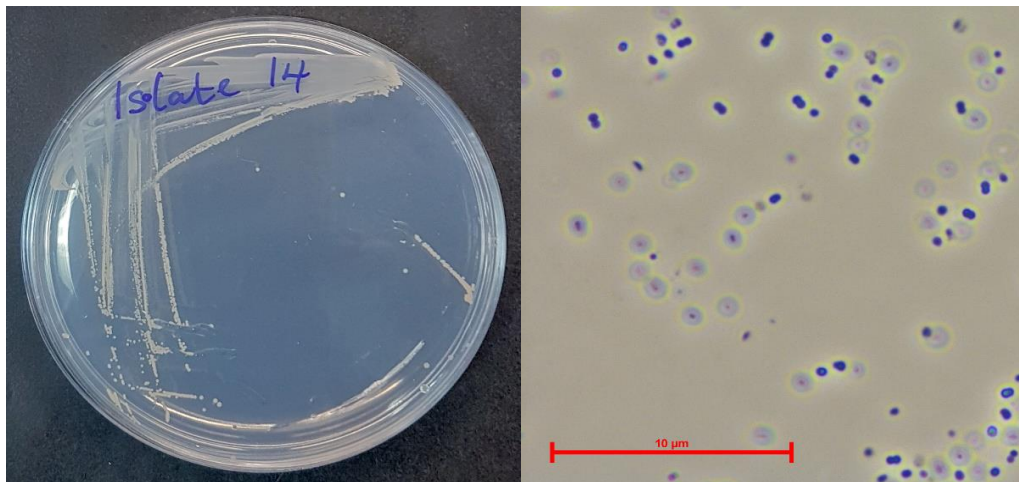


Figure 44: Colony morphology of Isolate 14 (left) and Phase Contrast Microscopy image (right).

Although phase contrast microscopy images suggested Isolate 14 to be mostly diplococci, TEM images of Isolate 14 suggested more cocci shaped cells (Figure 45). The scale of the single cell image is 500 nm and has a magnification of 60000 $\times$. The ICM seen in Isolate 10 appear in bundles, a property that is characteristic of type I methanotrophs but also found in type X. Round regular granules suspected to be glycogen were also observed. The image on the right has 6000 $\times$ magnification and is at a scale of 5000 nm.

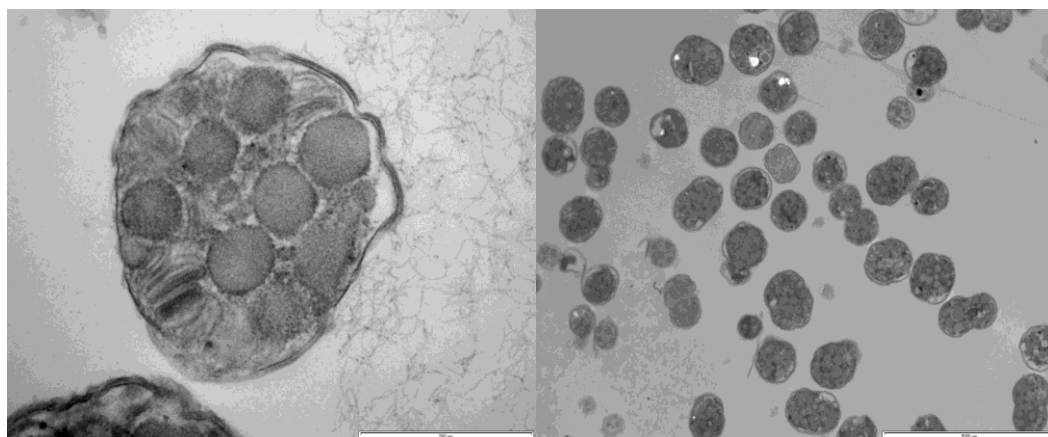


Figure 45: TEM images of Isolate 14. Single cell image (left) shows storage granule and ICM in bundles. The image on the right has a lower magnification showing how surrounding cells appear.

4.4.2 Conjugation Efficiency of Methanotrophs

Comparison of pMTL90882 and pMTL70401 conjugation efficiency

The Conjugation Efficiency (CE) of ColE1 based plasmid and a pBBR1 based plasmid was determined in type I (Isolate 10) and type II (Isolate 3* and Isolate 12) methanotrophs. The design of these plasmids was detailed in section 115.3. CE was measured as number of transconjugants per donor cells. The ColEI-based plasmid was pMTL90882 and CE values were 2.75×10^{-2} , 6.1×10^{-6} , 1.79×10^{-2} and 4.34×10^{-3} for Isolate 3*, Isolate 10, Isolate 12 and *M. parvus* (OBBP) respectively. pMTL70401 the pBBR1-based plasmid had lower CE values of 4.25×10^{-3} , 6.53×10^{-6} , 9×10^{-4} and 2.65×10^{-4} for Isolate 3*, Isolate 10, Isolate 12 and *M. parvus* (OBBP) respectively (Figure 46).

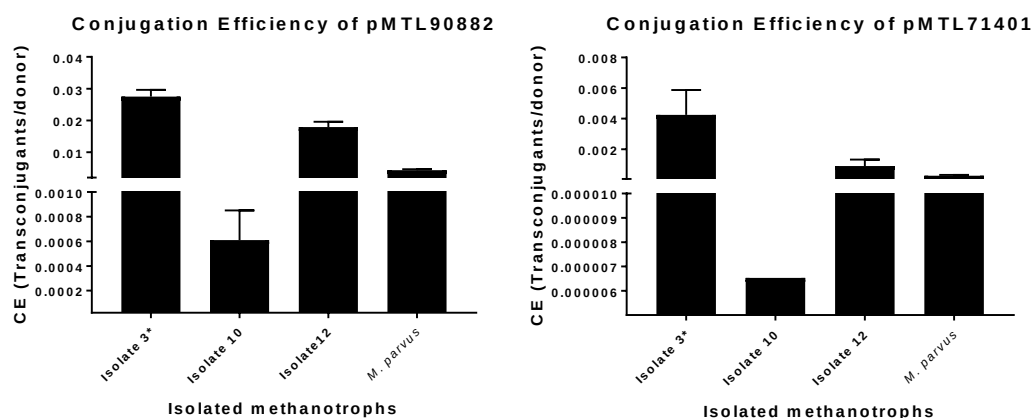


Figure 46: Conjugation efficiency of pMTL90882 (left) and pMTL71401 (right) in Isolate 3*, 10 12 and *M. parvus* (OBBP). Conjugation efficiency measured as number of transconjugants per donor cell.

Effect of mating time on conjugation efficiency

Isolate 12 was conjugated with *E. coli* S17 λ pir carrying pMTL90882. Mating was carried out at 3, 6, 12, 24 and 48 hrs before spreading on antibiotic plates. Results of the experiment showed that 6 hr mating had the highest conjugation efficiency (0.02445) while 12 hr mating was the lowest (0.00855) with $P < 0.002$ (Figure 47).

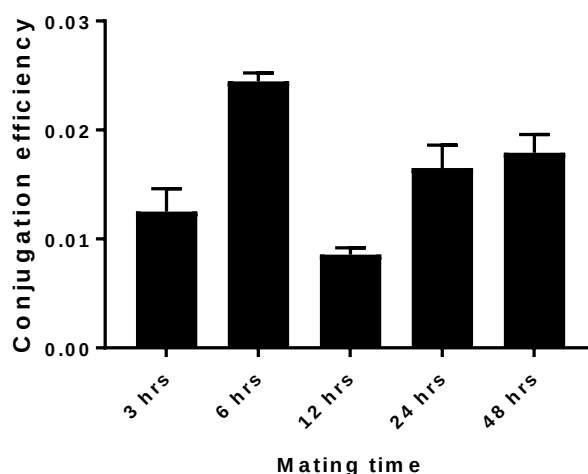


Figure 47: Conjugation efficiency of pMTL90882 in Isolate 12 at different mating times. P value of 0.002 was obtained when 6 hrs and 12 hrs growth was compared.

4.4.3 Electroporation of methanotrophs

All electroporation experiments were conducted with methanotrophs made electrocompetent on the same day. Isolate 3*, 6 and 12 were electroporated. After making cells electrocompetent and resuspending in 50 μ L, the OD₆₀₀ of resuspension ranged between 8.38 and 12.08. After electroporation with 100 ng of DNA, spreading on antibiotic NMS media plates and incubating at 30°C for three weeks, colonies were observed on most plates except Isolate 12 which only had one colony for one of the variables (high OD growth electroporated with pBBR1_NPR3 and recovered in cuvette) (Figure 48). PCR screening using primer sets ColEI_F/Kan_IV_rev and NotI_P3/EYFP_rev respectively to amplify regions of pMTL90882 and pBBR1_NPR3 were all negative and did not show bands. Additionally, pBBR1_NPR3 which had EYFP and a strong promoter failed to fluoresce under blue light further supporting the likelihood of unsuccessful plasmid transfer.

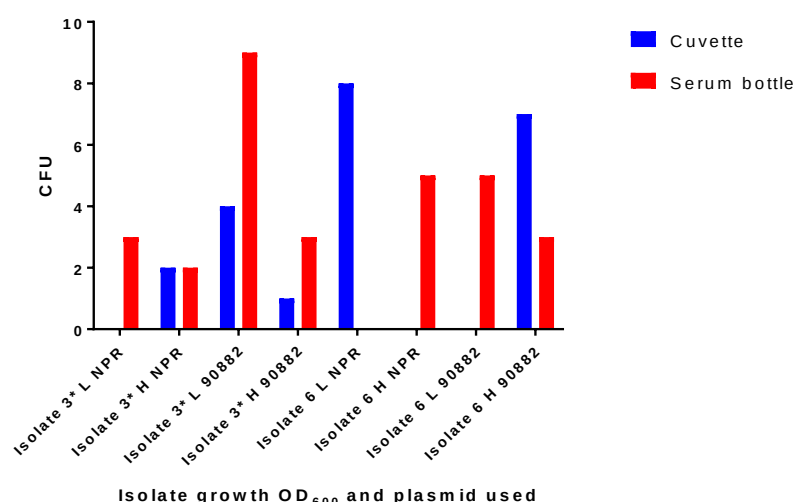


Figure 48: Electroporation efficiency in CFU per mL of recovery culture of isolated methanotrophs - Electroporated samples recovered in cuvettes and serum bottles are represented by blue and red columns respectively.

4.4.4 Phylogeny

Phylogenetic analyses showed Isolate 01 and 03 to be closely related. The same was shown for Isolate 3* and Isolate 6. The close relationship was expected due to the similarity of their 16S rRNA sequence. Both groups were closely related to Isolate 12 which is also *Methylocystis* species. Phylogenetic tree showed that Isolate 10 (*Methylocaldum* species) and Isolate 7 (*Methylomonas* species) were closer in relationship compared to Isolate 14 (*Methylococcus capsulatus*) (Figure 49).

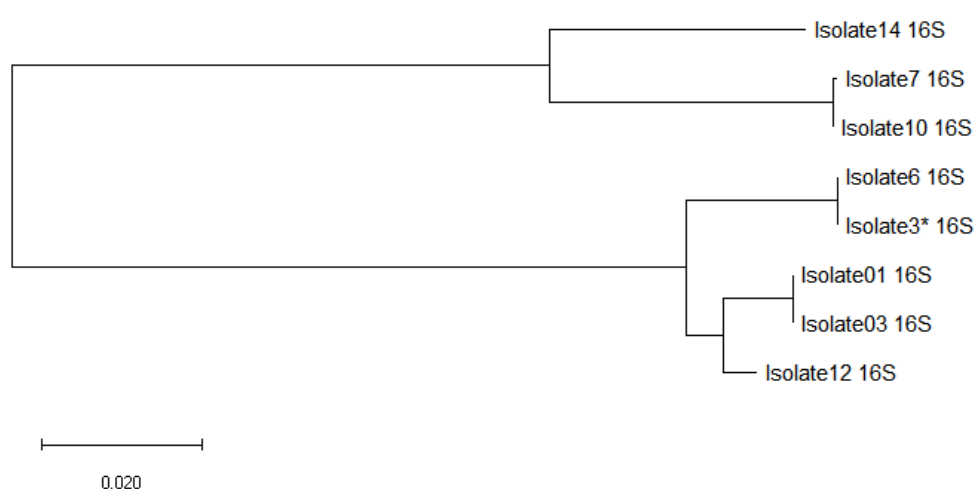
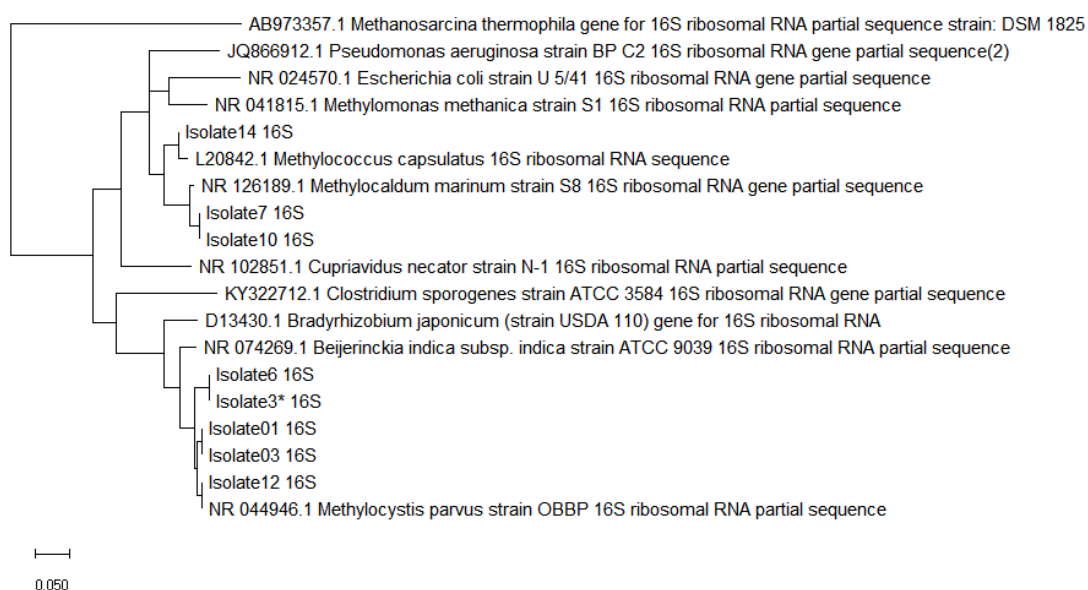


Figure 49: Molecular phylogenetic analysis of isolated methanotrophs inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-3637.23) is shown. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1536 positions in the final dataset.

Comparing all isolates to other bacteria and archaea showed *Methylocystis* species (type II) are closer to other bacteria such as *Bradyrhizobium japonicum* than they are to type I methanotrophs such as *Methylococcus capsulatus*. It also showed that type I methanotrophs are closer to bacteria such as *E. coli* than to type II bacteria. 16S rRNA showed a closer relationship between methanotrophs and all bacteria included compared to a methanogenic archaea (*Methanosarcina thermophilea*) (Figure 50).

Figure 50: Molecular phylogenetic analysis of isolated methanotrophs inferred by using the Maximum



Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-9964.04) is shown. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1577 positions in the final dataset.

4.4.5 Antibiotic Minimum Inhibitory Concentration (MIC) test

After incubation of isolates on agar plates with antibiotic strips, all isolates tested were found to be resistant to nalidixic acid. Isolate 6 and 10 also showed resistant to erythromycin. Type I isolates showed higher degree of sensitivity to kanamycin, gentamicin and streptomycin compared to Type II isolates (Table 28).

Table 28: Minimum inhibitory concentration of kanamycin, tetracycline, nalidixic acid, gentamicin, streptomycin and erythromycin when tested on Isolate 3*, 6, 10, 12 and 14

Antibiotics	Isolate 3 ($\mu\text{g/mL}$)	Isolate 6 ($\mu\text{g/mL}$)	Isolate 10 ($\mu\text{g/mL}$)	Isolate 12 ($\mu\text{g/mL}$)	Isolate 14 ($\mu\text{g/mL}$)
Kanamycin	0.19	1	0.047	0.5	0.064
Tetracycline	2	4	3	0.025	NA
Nalidixic acid	Resistant	Resistant	Resistant	Resistant	Resistant
Gentamicin	12	5	0.19	6	0.25
Streptomycin	5	1	0.19	1	0.19
Erythromycin	1.5	Resistant	Resistant	0.0016	0.5

4.4.6 Growth on alternative carbon sources

Among the carbon sources tested only sodium acetate and methane supported growth. Methane growth rate was faster than acetate. The growth rate on CH_4 was faster than growth on acetate. There was no significant difference between growth on acetate and growth in dNMS without CH_4 for the first five days of growth. Growth significantly increased after days 5. After 15 days growth was OD_{600} 0.085 (Figure 51).

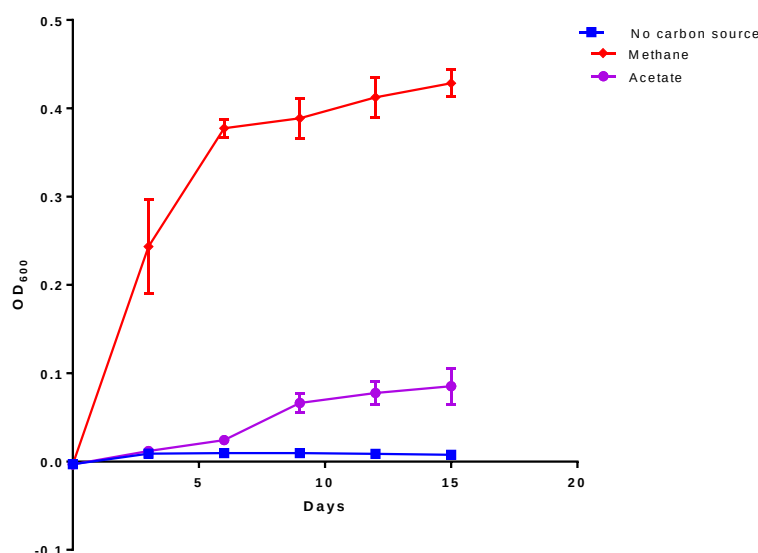


Figure 51: Growth of Isolate 3* with CH_4 , without CH_4 and with acetate only as carbon source.

4.4.6.1 Growth on methanol (CH_3OH)

After 10 days of growth, Isolate 3*, Isolate 12 and *M. parvus* (OBBP) showed growth on CH_3OH , and CH_4 . Isolate 3* only grew at CH_3OH concentration of 0.01% while Isolate 12 and *M. parvus* (OBBP) grew at 0.1% CH_3OH concentration (Figure 52). Grown samples spotted on LB plates did not show any growth which suggests there were no contaminants in the CH_3OH culture.

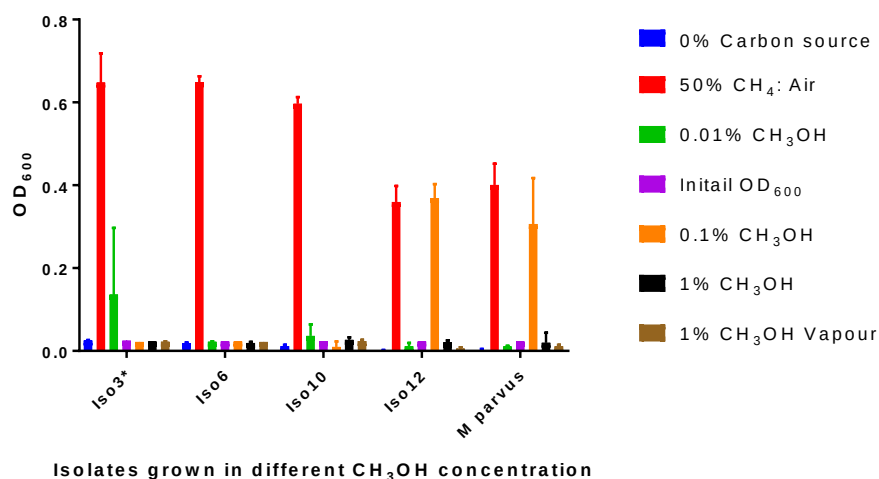


Figure 52: Growth of methanotrophs with different CH₃OH concentrations. Growth in CH₄ was used as control.

4.4.7 Genomic DNA sequencing and analyses

After phenol chloroform genomic DNA extraction, gDNA samples for Isolate 3*, 6, 10, 12 and 14 run on agarose gel did not result in a smear across the gel which suggested the absence of RNA (Figure 53).

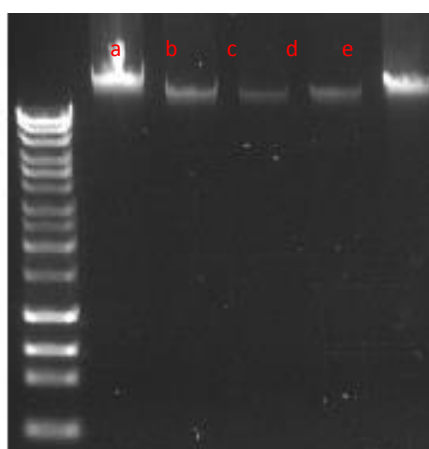


Figure 53: BioRad gel doc image after running gDNA samples on agarose gel - Isolates 3* (a), 6 (b), 10 (c), 12 (d) and 14 (e). Bioline 1 kb ladder was used in the gel image.

Samples sent for Illumina sequencing were received as two files – genome sequence assemblies and trimmed genome sequence paired reads. The genome sequence assemblies were already annotated and available in .gb and FASTA format. The trimmed paired reads were assembled by mapping to reference genomes using CLC Genomics Workbench 9. The PacBio data was sent as assembly files with one contig for Isolate 14, three contigs for Isolate 12 and four contigs for Isolate 6. The contigs were mapped to Illumina sequence using CLC Genomics Workbench 9. Table 29 highlights

some genomic features of the isolates. Isolate 10 had the lowest G+C content and longest genome. Isolate 12 had 2 mega plasmids and Isolate 14 had the highest G+C content.

Table 29: Genomic profile of isolated methanotrophs

Isolate	3*	6	10	12	14
Species	<i>Methylocystis</i>	<i>Methylocystis</i>	<i>Methylocaldum</i>	<i>Methylocystis</i>	<i>Methylococcus capsulatus</i>
Methanotroph Type	II	II	I	II	X
Illumina sequence	✓	✓	✓	✓	✓
PacBio sequence	X	✓	X	✓	✓
ReBase data	X	X	X	✓	✓
Genome size (bp)	3889428	3386331	5869838	4529043	3357575
G+C content	62.61	62.69	58.08	63.35	63.51
Mega plasmid	X	X	X	2 mega plasmids	X
N50	204241	223744	189908	101750	247129
Number of contigs (Illumina)	61	56	111	92	31
Number of contigs (PacBio)	X	4	X	3	1

The Analyses file received from PacBio was sent to Rebase to reveal the Restriction Modification (RM) systems of Isolate 12 and 14. In addition to type I RM systems possessed by Isolate 14, both isolates were shown to have type II and III. Isolate 12 was shown to possess RM systems on its genome and mega plasmid that recognise the sequence GANTC, GATC and CTCGAG (Figure 54 and Table 30). The Rebase predicted sequence recognised by Isolate 14 RM systems was RGATCY (Figure 55 and Table 31).

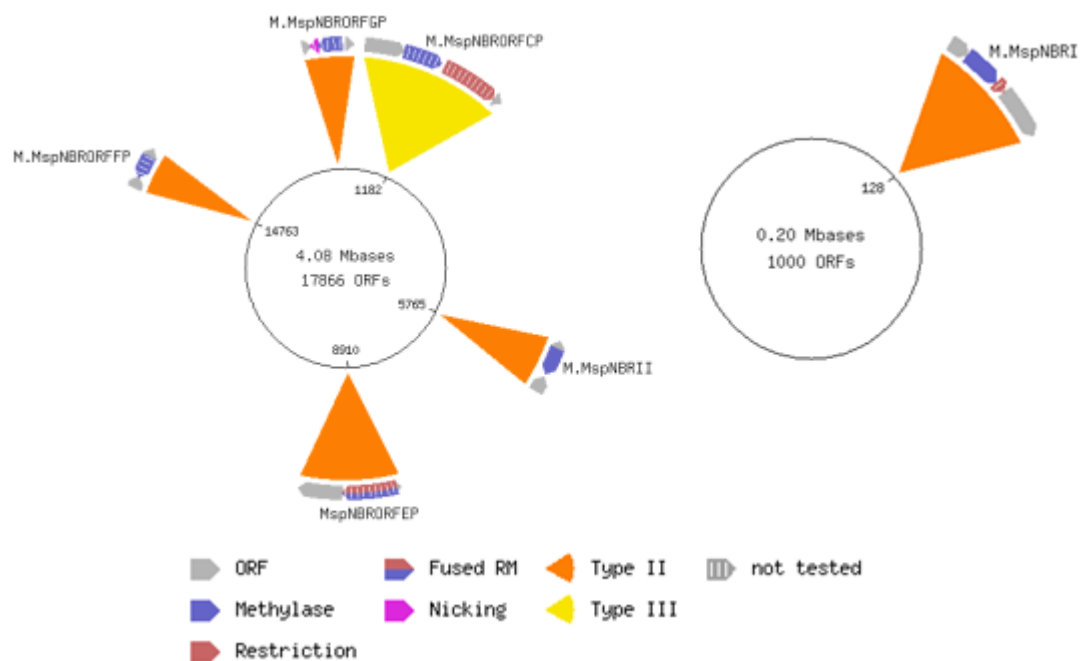


Figure 54: Restriction modification system of Isolate 12 on genome (left) and second mega plasmid (right). Only one type II RM system is present on the mega plasmid while type II and III systems are present in the genome.

Table 30: Putative Isolate 12 RM systems and predicted recognition sequence

Type	Gene	Name	Predicted Recognition Sequence	Location	Coordinates
II	M	M.MspNBRII	GATC	genome	1315067-1316356
II	RM	MspNBRORFEP			2022083-2024881
II	M	M.MspNBRORFPP	GATC		3367098-3368321 c
II	V	V.MspNBRORFGP			4025438-4025851 c
II	M	M.MspNBRORFGP			4025856-4027121 c
III	M	M.MspNBRORFCP			270030-272033
III	R	MspNBRORFCP			272044-275073
II	M	M.MspNBRI	CTCGAG	plasmid	25768-27519
II	R	MspNBRIIP	CTCGAG		27509-28273

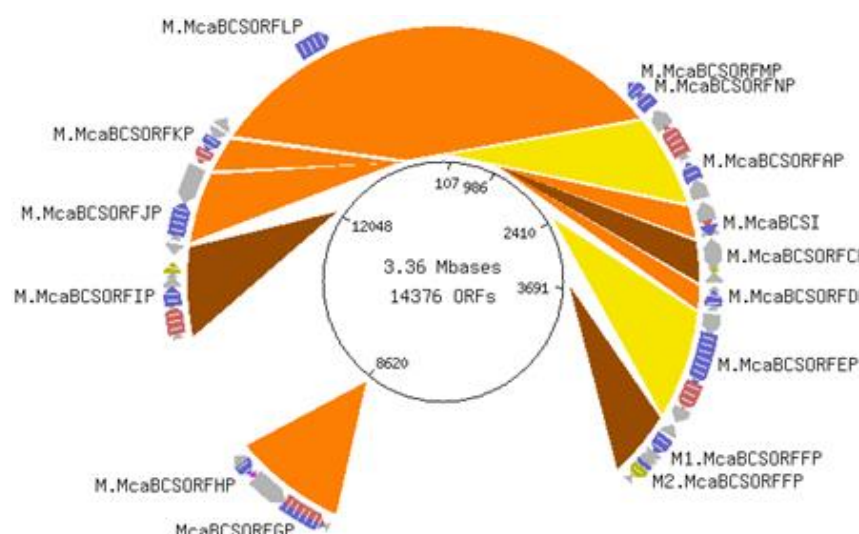


Figure 55: Restriction modification system of Isolate 14. Type I, II and III RM system present

Table 31: Putative Isolate 14 RM systems and predicted recognition sequence

Type	Gene	Name	Predicted Recognition Sequence	Coordinates
II	R	McaBCSIP	RGATCY	232750-233462
II	M	M.McaBCSI	RGATCY	233443-234093

Data from genome analyses in this study was used for many purposes such as designing promoters, identifying location of transposon insertions, identifying genes for knockout, homology arm and CRISPR guide RNA design. Table 29 compares some genes shared by five isolates sequenced in this study.

Table 32: Comparison of genes shared by isolates sequenced in this study

Isolate	3*	6	10	12	14
DNA ligase genes for NHEJ					
<i>ligA</i>	✓	✓	✓	✓	✓
<i>ligD</i>	✓	X	✓	✓	X
<i>ligD</i> Rv0938/MT0965	✓	✓	X	✓	X
<i>ykoU</i>	✓	✓	X	X	X
DNA repair proteins for NHEJ					
Ku	X	X	X	✓	X
<i>ykoV</i>	✓	✓	X	✓	X
Homologous recombination genes					
<i>recBCD</i>	X	X	✓	X	✓
recombinase A	✓	✓	✓	✓	✓
<i>recF</i> (DNA replication and repair protein)	✓	✓	✓	✓	✓
<i>recG</i> ATP dependent DNA helicase	✓	✓	✓	✓	✓
<i>recJ</i> (Single stranded DNA-specific exonuclease)	✓	✓	✓	✓	✓
<i>recN</i> (DNA repair protein)	✓	✓	✓	✓	✓
<i>recO</i> (DNA repair protein)	✓	✓	✓	✓	✓
<i>recQ</i> (ATP dependant DNA helicase)	✓	✓	✓	✓	✓
Recombination protein RecR	✓	✓	✓	✓	✓
<i>radA</i>	✓	✓	✓	✓	✓
Polyhydroxybutyrate metabolism genes					
<i>phbC1</i> - Poly-beta hydroxybutyrate polymerase	✓	✓	✓	✓	X
PHB/PHA accumulation regulator DNA-binding domain	✓	✓	✓	✓	X
<i>phbA</i> Acetyl-coa Acetyltransferase	✓	✓	✓	✓	X
<i>phbB</i> Acetoacetyl-CoA reductase	✓	✓	✓	✓	X
<i>phbC2</i> - Poly-beta hydroxybutyrate polymerase	✓	✓	✓	✓	X
phb depolymerase C terminus (2 copies for iso12)	✓	✓	X	✓	X
Esterase PHB depolymerase	✓	✓	✓	X	X
<i>phaE</i> Poly(R) - hydroxyalkanoic synthase subunit	X	X	✓	X	X
<i>bdhA</i> D beta hydroxybutyrate dehydrogenase	✓	✓	✓	✓	X
Phasin protein	✓	✓	✓	✓	X

4.4.8 Chemical tolerance for methanotrophs

The ability of Isolate 3*, Isolate 12 and *M. parvus* (OBBP) to grow in the presence of 3HP was tested. The results showed that Isolate 3* doubled its growth in the presence of 5 mM 3HP from a starting OD₆₀₀ 0.02 while *M. parvus* (OBBP) only grew at 1 mM 3HP concentration. Isolate 12 grew in the presence of 0.1 mM 3HP but was impeded by 3HP concentration from 1 mM to 100 mM. Growth in the presence of 3HP at all concentrations tested was hindered in methanotrophs (Figure 56).

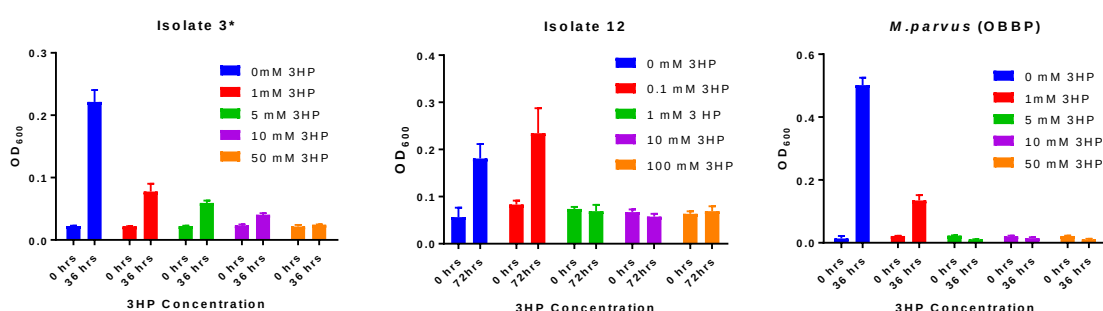


Figure 56: 3HP tolerance of methanotrophs grown in NMS media.

Isolate 12 grown at different concentrations of CH₃OH in the presence of CH₄ showed CH₃OH had no significant effect at the range of concentrations tested (0.5, 1 and 5 mM) (Figure 57). The highest and lowest growth OD₆₀₀ were 0.995 and 0.58 for Isolate 12 grown in NMS with 0.5 mM and 5 mM CH₃OH respectively. There was no significant difference between these values with ($P = 0.76$). No growth was observed for all isolate 12 samples grown without CH₄.

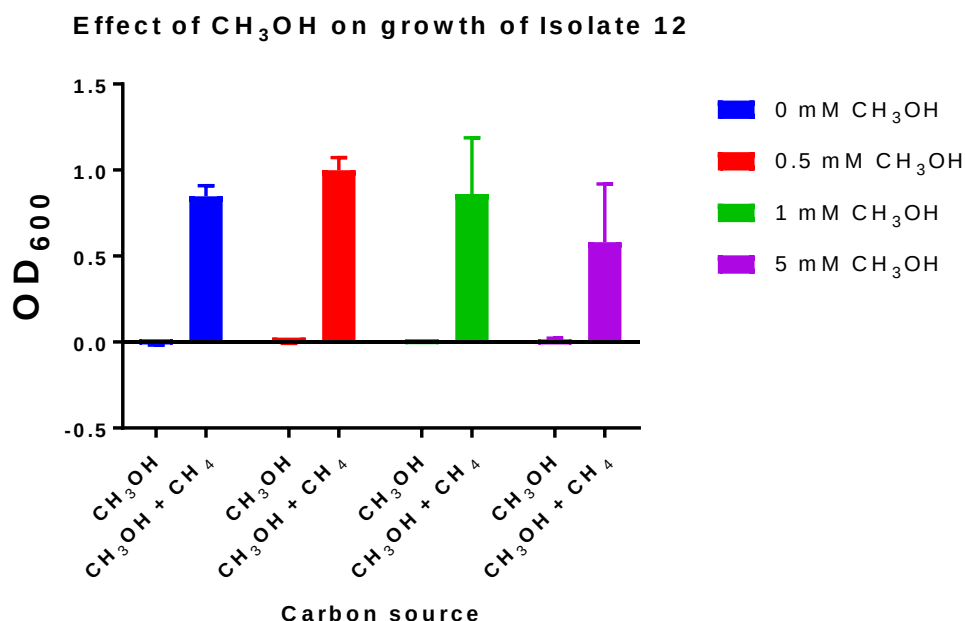


Figure 57: Effect of CH₃OH addition on growth of Isolate 12 with CH₄. CH₃OH had no significant effect on the growth of Isolate 12 ($P = 0.76$). P value measured using one way ANOVA.

Isobutanol hindered the growth of Isolate 12 when added at 0.5, 1 and 5 mM concentrations in the presence of CH₄. Growth OD₆₀₀ values of Isolate 12 in NMS media with 0, 0.5, 1 and 5 mM Isobutanol were 0.827, 0.329, 0.350 and 0.343 respectively. There was significant difference between growth of Isolate 12 in 0 mM Isobutanol and 1 mM Isobutanol ($P < 0.02$) (Figure 58).

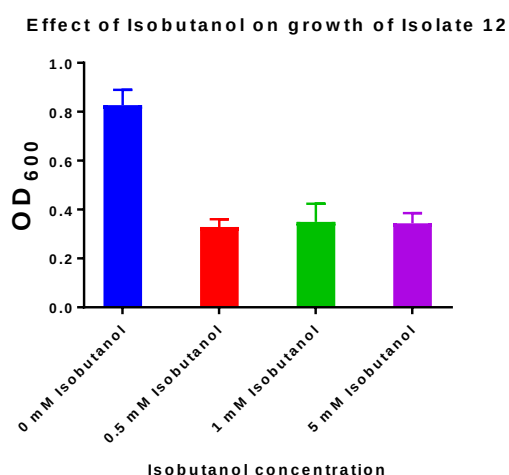


Figure 58: Effect of isobutanol on growth of methanotrophs in NMS media with CH₄. Among the samples grown with 1 isobutanol, Isolate 12 grown with 1 mM Isobutanol had the highest growth OD₆₀₀ (0.35). Growth of Isolate 12 in NMS grown without Isobutanol was significantly higher than growth of Isolate 12 in NMS with 1 mM Isobutanol ($P < 0.02$). P value measured using unpaired t-test.

4.4.9 Effect of NMS components on growth of Isolate 12

Different components such as vitamins, Fe-EDTA and $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ are usually added to NMS to enhance the growth of methanotrophs as suggested by Whittenbury *et al.* [2]. These components were tested at various concentrations to determine the effect they have on growth of methanotrophs.

Vitamins

Isolate 12 was grown for 10 days at different concentrations of vitamins. Growth was highest in NMS media without vitamins (Figure 59).

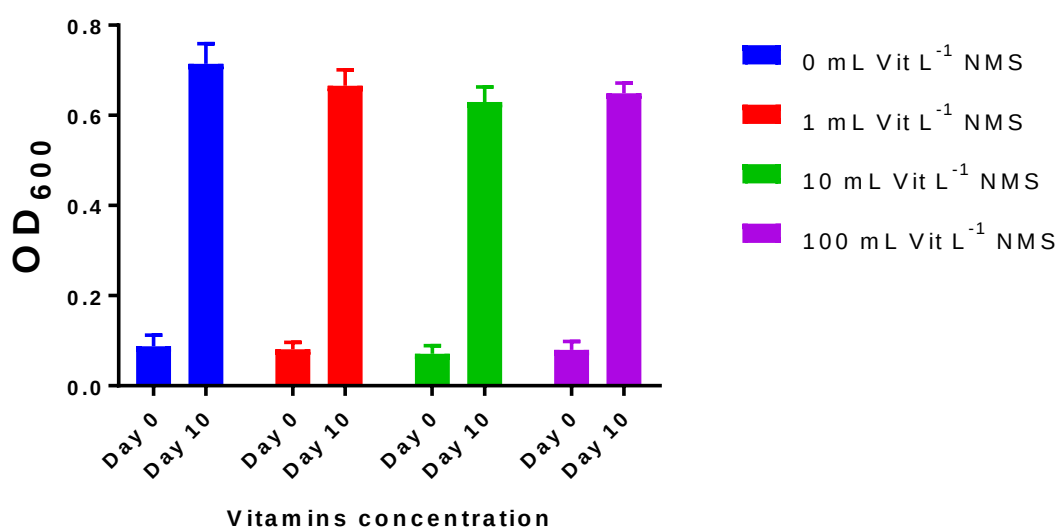


Figure 59: Effect of vitamin concentrations in NMS on growth of Isolate 12. After 10 days, Isolate 12 in NMS without vitamins had the highest growth OD_{600} (0.714) while Isolate 12 grown in NMS with 10 mL of vitamins per litre was the lowest with OD_{600} of 0.629. There was no significant difference between the two values ($P = 0.92$). P value measured using unpaired t-test.

Fe-EDTA

Isolate 12 growth in NMS media was highest when 10 mL Fe-EDTA was added. Sample grown without Fe-EDTA showed the least growth (Figure 60). The lowest and highest growth was 0.58 and 0.685 for 0 and 10 mL of Fe-EDTA per litre of NMS respectively. There was no significant difference between these values ($P = 0.914$).

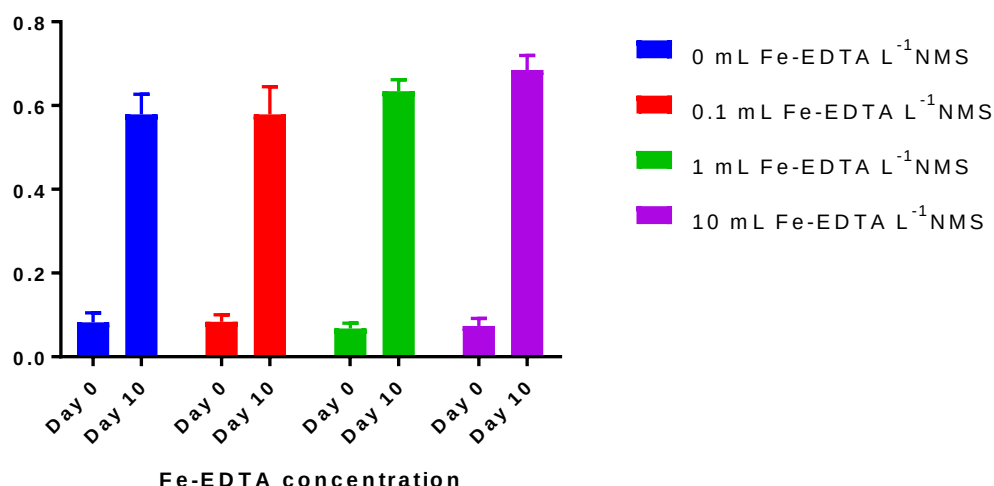


Figure 60: Effect of Fe-EDTA concentration in NMS on growth of Isolate 12. There was no significant difference between the lowest and highest growth ($P = 0.914$). P value measured using unpaired t -test.

CuSO₄·7H₂O

A growth curve was carried out to check the effect of $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ addition to NMS on growth of Isolate 12. 10 mM concentrations hindered the growth of Isolate 12. Peak growth was observed when 100 μM $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ was added (Figure 61).

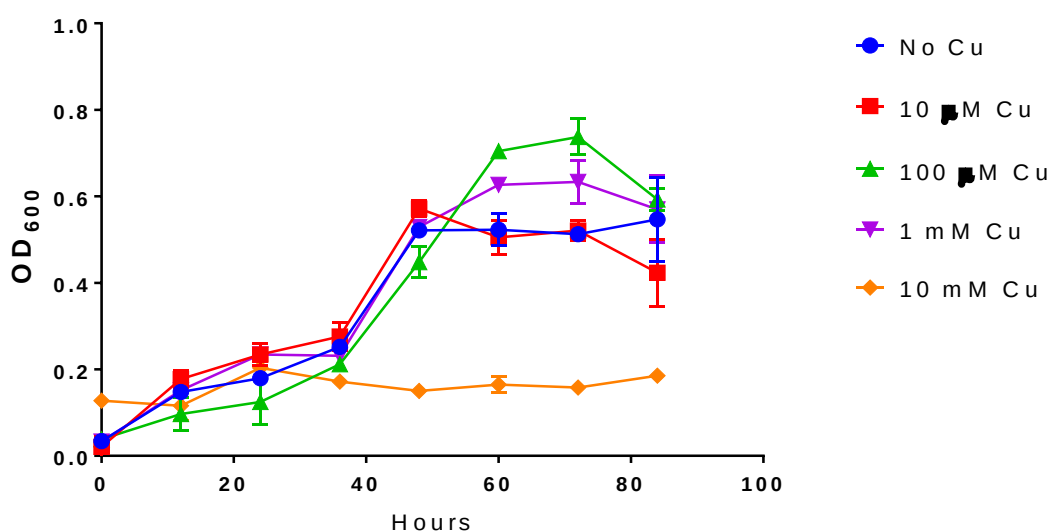


Figure 61: Effect of $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ addition to NMS on growth of Isolate 12. 10 mM Cu led to growth inhibition of Isolate 12. Isolate 12 did not need Cu addition for growth.

Nitrogen source

The effect of two nitrogen sources (nitrate and ammonia) on the growth of Isolate 3* was investigated. Isolate 3* was able to use ammonia and nitrate as source of nitrogen in dilute NMS media. Isolate 3* samples grown with dilute ammonia mineral salt (dAMS) peaked at OD_{600} 0.31. No growth was observed in dilute mineral salt (dMS)

sample which did not contain nitrogen. This media had no nitrogen source. Isolate 3* showed peak growth at OD₆₀₀ 0.64 when nitrate (dNMS) was used as nitrogen source (Figure 62).

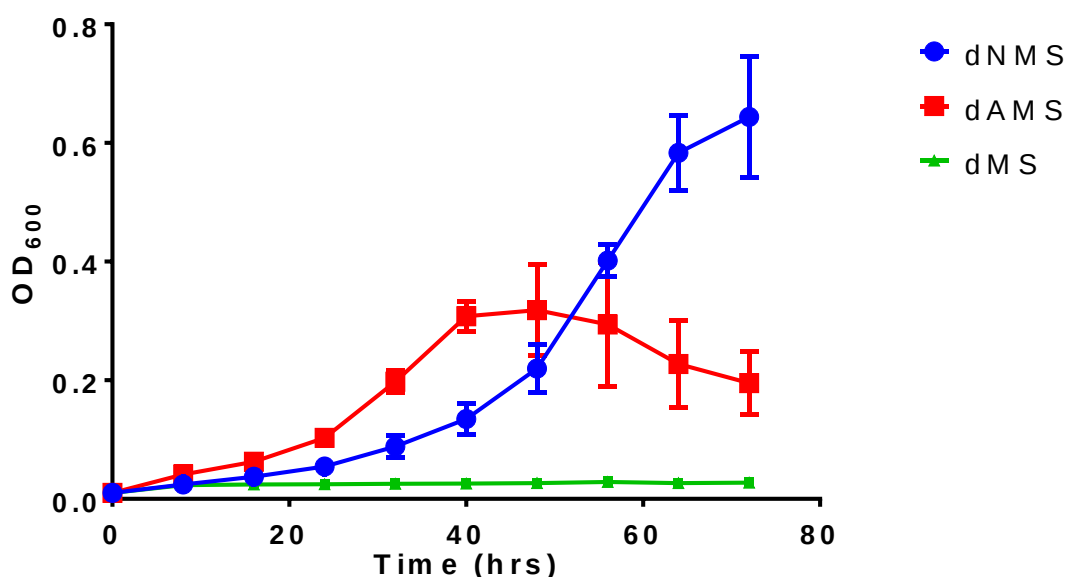


Figure 62: Effect of nitrogen source on growth of Isolate 3*. Total removal of nitrogen stopped growth in Isolate 3*. Isolate 3* grew with ammonia or nitrate acting as nitrogen source.

When 5 mL of CO₂ was added to dNMS, dAMS and dMS, growth of Isolate 3* slowed down. Growth of Isolate 3* in dAMS peaked after 40 hrs whereas addition of CO₂ led to peak growth in dAMS after 55 hrs. Addition of CO₂ did not show significant effect on the growth of Isolate 3* in dNMS. Growth of Isolate 3* with and without CO₂ was not observed in dMS (Figure 63).

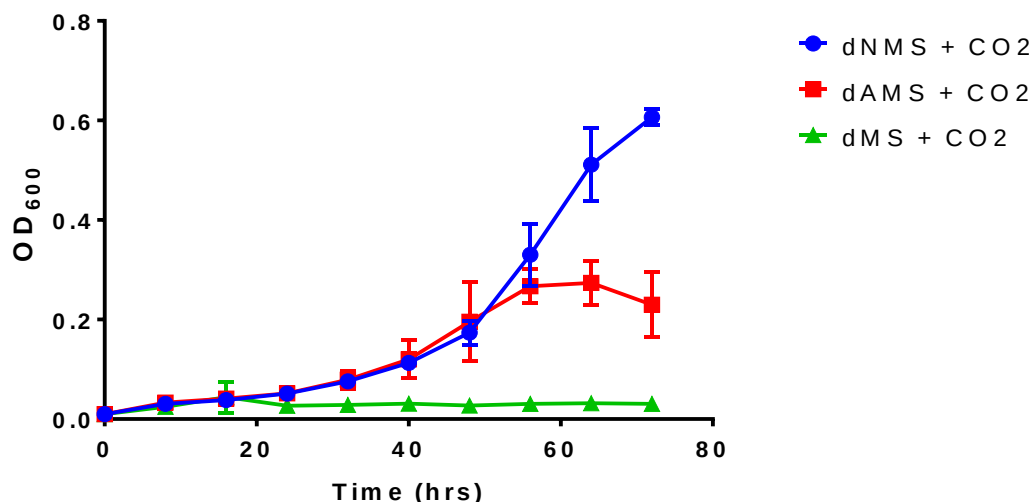


Figure 63: Effect of CO₂ on growth of Isolate 3* in dNMS, dAMS and dMS. The addition of CO₂ did not stop Isolate 3* from growing in either dNMS or dAMS.

Depletion of nitrogen source was suspected for the peak of Isolate 3* growth in dAMS and dNMS media in Figure 62. Additionally, absence of Isolate 3* growth in dMS sample in Figure 62 was attributed to the absence of nitrogen. After addition of 1 mL of nitrogen source in the form of 1 g/L KNO₃, growth of isolate 3* in dAMS resumed. Growth was also observed in dMS. However, Isolate 3* in dNMS kept declining. After 300 hrs of growth, Isolate 3* in dMS and dAMS peaked. Addition of CH₄ led to rise in growth of dAMS and dNMS samples suggesting Isolate 12 growth was suspended due to CH₄ limitation (Figure 64).

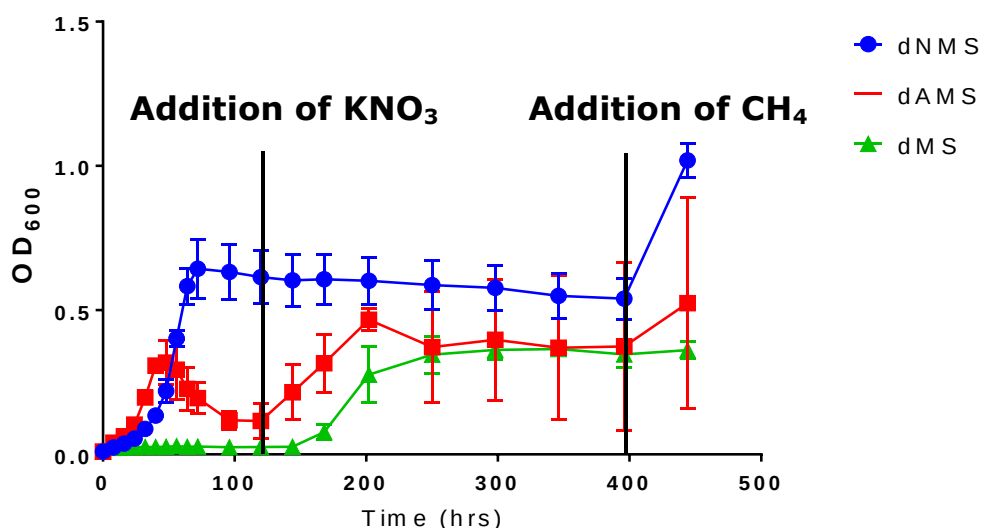


Figure 64: Effect of nitrogen and CH₄ limitation on growth peak of Isolate 3* in dNMS, dAMS and dMS. The first black line represents when nitrogen was added after depletion in dAMS. This led to resumption of Isolate 3* growth in dAMS. It also led to a start in growth of Isolate 3* with dMS which did not have nitrogen. The second black line represents when CH₄ was added after depletion in dAMS and dNMS which also led to resumption in growth of Isolate 3*.

4.4.10 Biogas as a source of methane for methanotrophs

Biogas composition

The bulk composition of Biogas 1 (BG1) and biogas 2 (BG2) was measured using trace GC. BG1 generated from biomass had lower CH₄ concentration (49.47%) compared to BG2 generated from food waste (57.46%), and higher CO₂ concentration (40.64%) compared to BG2 (32.81%) (Figure 65).

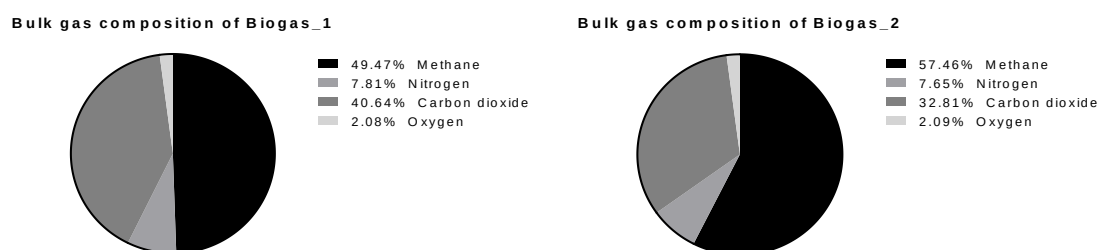


Figure 65 Composition of bulk gases in BG1 and BG2 measured using trace GC.

Trace gases such as siloxanes, NH₃ and H₂S measured by Lucideon, an analytical company, are shown in Table 33.

Table 33: Trace gas composition of BG1 and BG2

Siloxanes (mg/m ³)	Biogas_1	Biogas_2		Biogas_1	Biogas_2
Hexamethylcyclotrisiloxane	NA	<1.0	Ammonia (mg/m ³)	2.9	<0.7
Octamethylcyclotetrasiloxane	NA	1.1			
Decamethylcyclopentasiloxane	0.96	<1.0	Hydrogen Sulphide (ppm)	94	462
Hexamethyldisiloxane	NA	<1.0			
Octamethyltrisiloxane	NA	<1.0			
Decamethyltetrasiloxane	NA	<1.0			
Dodecamethylpentasiloxane	NA	<1.0			
Dodecamethylcyclohexasiloxane	0.75	<1.0			
Trimethyl Silanol (toluene equivalent)	NA	<1.0			
N,N-dimethyl acetamide	2.3	NA			

Growth of methanotrophs in biogas

Methanotrophs grown with CH₄ and biogas were compared. There was no significant difference between the growth of methanotrophs in CH₄ and biogas except the comparison between growth in Isolate 03 in CH₄ and BG2 which had a p value of 0.0311 in favour of Isolate 03 grown with CH₄. Peak OD₆₀₀ 2.123, 2.008 and 1.365 was

seen in Isolate 01, *M. capsulatus* (Bath) and Isolate 01 grown with CH₄, BG1 and BG2 respectively (Figure 66).

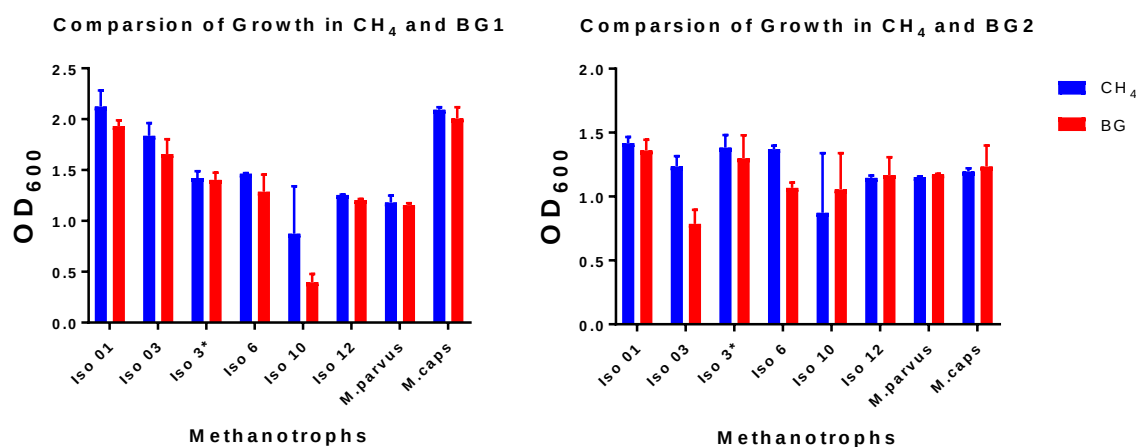


Figure 66: Comparison of methanotrophs grown in CH₄ and BG1 (left) and comparison between methanotrophs grown in CH₄ and BG2 (right).

4.4.10.1 Headspace analysis of methanotrophs grown in CH₄ and biogas

Results from headspace analyses of serum bottles used to grow methanotrophs on CH₄ and biogas are presented below. In the case of oxygen, over 80% of O₂ added to the headspace was consumed by methanotrophs except for isolate 03 and 06 grown with BG2 (Figure 67).

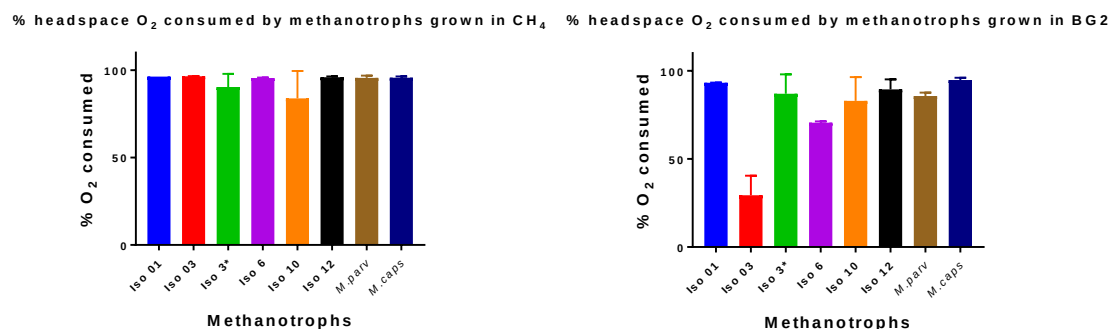


Figure 67: Percentage O₂ consumed by methanotrophs grown in CH₄ (left) and BG2 (right).

In the case of methane, less than 60% of the CH₄ added was consumed by methanotrophs grown in CH₄ whereas most methanotrophs grown in BG2 consumed more than 90% of CH₄ added (Figure 68).

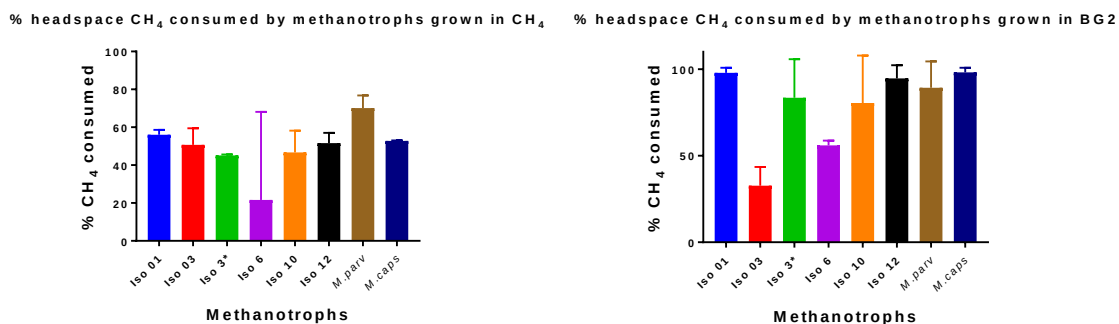


Figure 68: Percentage CH₄ consumed by methanotrophs grown in CH₄ (left) and BG2 (right).

The CH₄ and O₂ consumed led to the generation of CO₂. Figure 69 shows the amount of CO₂ produced as measured in the headspace.

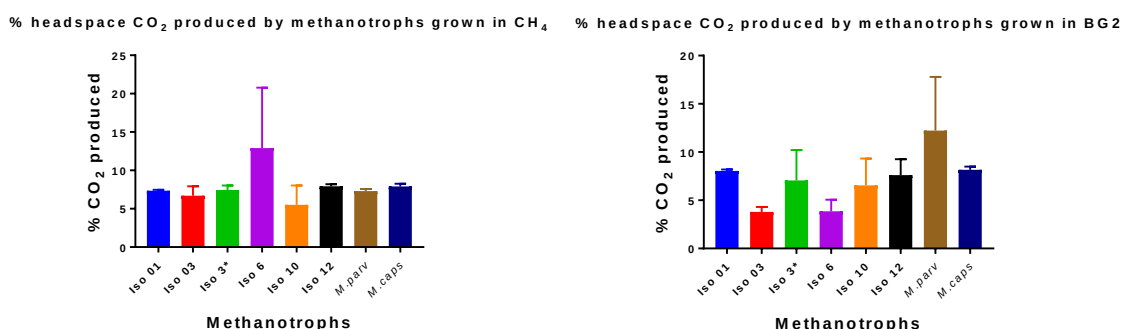


Figure 69: Percentage CO₂ produced by methanotrophs grown in CH₄ (left) and BG2 (right).

The growth of methanotrophs in Figure 70 is shown below to visualise and correlate the consumption and production of headspace gases to growth.

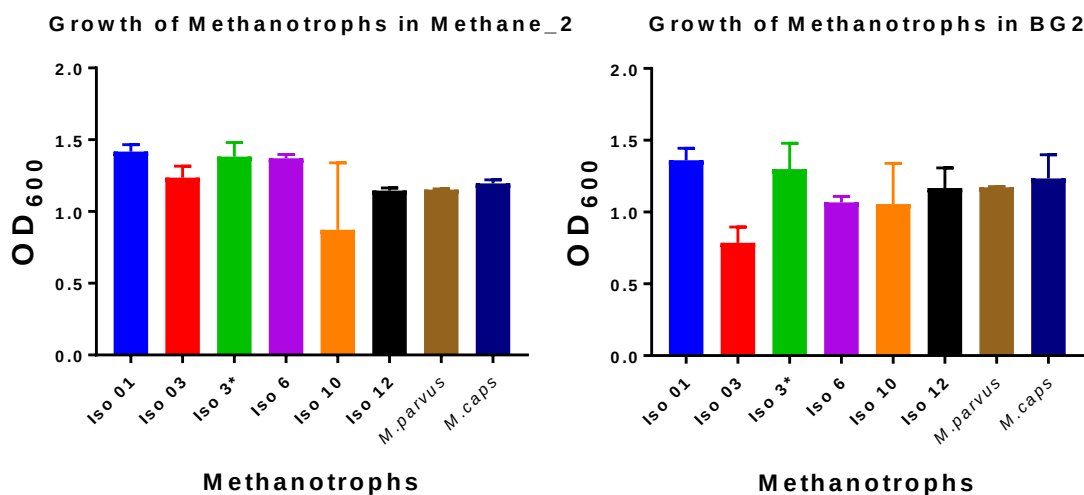


Figure 70: Growth of methanotrophs in CH₄ (left) and BG2 (right).

4.4.10.2 PHB production in methanotrophs using CH₄ and biogas

GC/MS results showed PHB was produced in type II methanotrophs (Isolate 3*, 6, 12 and *M. parvus* (OBBP)). Type I (*Methylocaldum* species) and type X (*M. capsulatus*

(Bath)) methanotrophs did not produce PHB. Only Isolate 12, *M. parvus* (OBBP) and *M. capsulatus* (Bath) were grown with biogas in addition to the samples grown with pure CH₄. Samples grown in biogas produced approximately 10 times more PHB than samples grown in CH₄. Overall Isolate 12 produced more PHB compared to all methanotrophs tested except Isolate 6 (Figure 71).

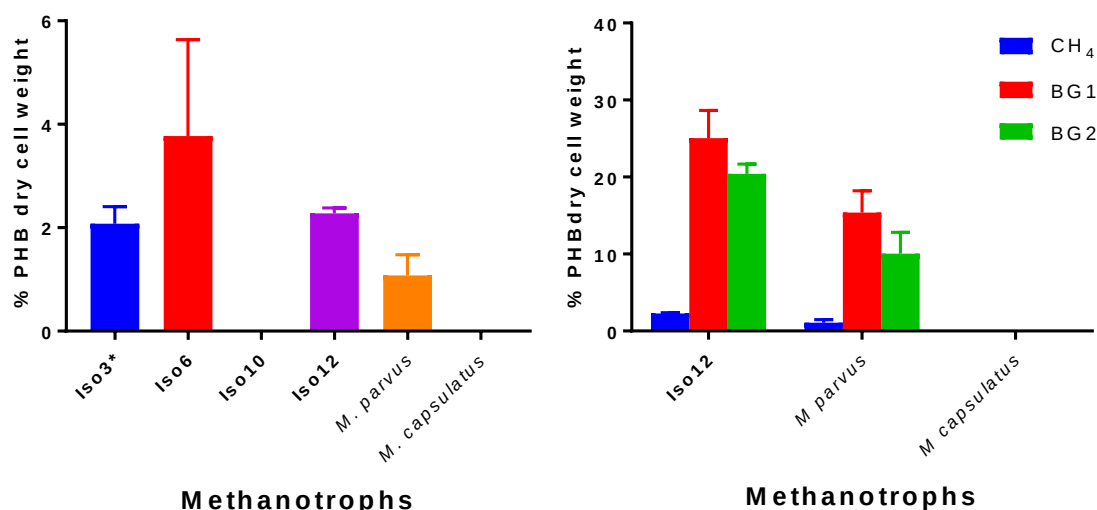


Figure 71: Percentage PHB dry cell weight of methanotrophs grown with CH₄ (left). $P = 0.19$, measured using one way ANOVA. Percentage PHB dry cell weight of methanotrophs grown with CH₄ and biogas (right). $P = 0.23$ when BG1 and BG2 percentage PHB dry cell weight of Isolate 12 were compared. $P = 0.20$ when BG1 and BG2 percentage PHB dry cell weight of *M. parvus* were compared.

4.5 Discussion

Isolation of methanotrophs was carried out in the first chapter of this study which led to nine pure isolates including two type I (*Methylomonas* and *Methylocaldum* species); five type II (*Methylocystis* species) and two type X (*Methylococcus capsulatus*). This chapter investigated the characteristics of these isolates including but not limited to morphology, growth conditions and genome sequences. Preliminary experiments highlighted properties of some species which made them attractive for characterisation in more details than others. For example, there was more focus on Isolate 12 which was selected for developing CRISPR genome editing tools and establishing random transposon mutagenesis using a suicide plasmid, and subsequently intended for TraDIS.

Morphology of isolates

The morphology of isolated strains grown on NMS agar plates were similar in shape and were all opaque. The main difference was seen in colour where some isolates such as 01, 03, 7, 12, 9 and 14 maintained their original colours after prolonged growth on agar plates unlike Isolate 3*, 6 and 10 which changed shortly before attaining full growth. Isolate 3* and 6 changed from cream to pink while Isolate 10 changed from cream to brown. The reason for colour change is not fully understood for Isolate 3* and 6 but the pink colour attained is suspected to be from biosynthesis of carotenoids. Isolate 3* and Isolate 6 have operons in their genes containing *crtBCDFL* and *crtBCDL* respectively, which are genes involved in carotenoid biosynthesis. Isolate 6 also has *crtF* in another genomic region. Further investigation revealed the presence of *crtB*, *crtC*, *crtF* and *crtL* in Isolate 12 although colour change to pink was not observed in Isolate 12. *crtD* was not found in the genome of Isolate 12 neither was a carotenoid biosynthesis operon. The brown pigment acquired in Isolate 10 is suspected to be melanin and it is a common feature of *Methylocaldum* species. The genome of Isolate 10 contains homogentisate 1, 2 – dioxygenase which is involved in melanin biosynthesis [270]. The pink colour in Isolate 7 a *Methylomonas* species is also likely to be from carotenoid biosynthesis which is well documented in *Methylomonas* sp. 16a [84]. Although Isolate 9 appearance under phase contrast microscopy was dominated

by single cocci as well as Isolate 14 under TEM, all the other isolates were predominantly diplococci. However, Isolate 01, 7 and 12 diplococci seemed irregular with one pole slightly longer than the other and more spherical. Regardless of shape and colour, all the cells were opaque.

Transmission Electron Microscope (TEM) carried out revealed internal organs some of which were obvious like the ICM and PHB granules in *Methylocystis* species. The type II ICM arranged continuously around the cell periphery and the type I ICM bundles were similar to those shown by Whittenbury *et al.* [2] Type I methanotrophs are not known for PHB synthesis although numerous PHB biosynthesis genes have been found in Isolate 10. Isolate 10 seems to be covered by an external membrane which has a rough exterior, an important line of investigation this study will look into. An important morphological feature resembling fimbriae was observed around the outer cell membrane of Isolate 3*. This morphological feature which has the appearance of an appendage is unlike any that has been observed in other methanotrophs and provides a compelling argument for considering Isolate 3* as a novel species of methanotroph.

Conjugation efficiency

The high conjugation efficiency of *Methylocystis* species compared to *Methylocaldum* is not fully understood. However, this was an important determinant of which species was selected and used to make a transposon library as organisms with high transformation efficiencies were required. The superiority of pMTL90882 over pMTL71401 was not surprising as ColE1 is a high copy number replicon (approximately 40 copies per chromosome in *E. coli*) compared to pBBR1 which has a low copy number (approximately 5 copies per chromosome in *E. coli*) [271]. The effect of mating time on conjugation efficiency was unexpected. This is because 48-hour mating times were reported in conjugation experiments involving methanotrophs which gave the impression such lengthy mating times were essential to the success of conjugation in methanotrophs [272]. The finding that mating time peaked at 6 hrs is vital to reducing the general time required to carry out conjugations in methanotrophs. However, caution must be taken as the results obtained may not be reproducible in other methanotrophs or even other plasmids.

Electroporation of methanotrophs

Electroporation was also attempted due to its usefulness in cases not suitable for conjugation such as transformation of linear DNA. An example of where linear DNA is introduced into bacteria is seen when single stranded oligo deoxyribonucleotides (ssODN) are used as repair templates after induction of DSB by Cas9 nuclease activity [182]. Additionally, the use of electroporation to generate a transposon library guarantees the absence of contaminating donor cells and genomes that can be encountered during transposon library generation. The difficulty associated with electroporation of methanotrophs cannot be linked to any factors tested such as recovery medium, growth stage at the time cells were made competent and plasmid replicon among others. The RM systems of these strains are not likely responsible for rejecting donor DNA as the same strains have been conjugated successfully. Nonetheless the RM system cannot be completely ruled out as a balance between RM system and physical properties of the cells is required for successful electroporation. However the experiment carried out eliminated likely causes of failure as different growth stages, wash buffer, recovery medium and electroporation cuvettes were investigated.

Phylogenetic tree

Phylogenetic tree showed the close relationship among type I and type II methanotrophs isolated. Isolate 01 and 03 have identical 16S rRNA thus their close relationship was expected. It should be noted that the genomic data of Isolate 01 and 03 is not available and the 16S rRNA region amplified is missing approximately 200 bp due to limitation of Sanger sequencing. Until their complete 16S rRNA is sequenced and further characterisation is carried out, they will be considered as dissimilar species. Although Isolate 3* and Isolate 6 16S rRNA were identical, alignment of their 23S rRNA showed six positions where there were differences in nucleotide base pairs. Additionally, Isolate 3* has a DNA ligase gene that is absent in Isolate 6. The most striking difference between the two species is the absence of fimbriae in Isolate 6 which is the strongest argument pointing towards the two strains being different. One surprising finding is the close relationship between type I methanotrophs isolated and other bacteria compared to type II methanotrophs and vice-versa. This gap can be a

starting point for arguing a different evolutionary ancestry between type I and type II methanotrophs.

Antibiotic minimum inhibitory concentration

The antibiotic MIC data showed relatively higher resistance of gentamicin and streptomycin by type II. Isolate 10 and 14 which are type I and type X respectively share a common sensitivity to kanamycin, streptomycin and gentamicin. Tetracycline is sensitive to light and may degrade over the two-week incubation time required for methanotrophs. Hence the results from tetracycline MIC assay require further investigation. The use of tetracycline in future experiments was avoided due to these conditions. The reason for the resistance by all the methanotrophs to nalidixic acid and their common sensitivity to kanamycin is not well understood. However, it makes it possible to use the same plasmid in all the strains, and this enables a direct comparison of conjugation efficiencies. Observation showed that the values predicted by MIC antibiotic strips were qualitative and not necessarily quantitative. This is because when antibiotics were added to agar plates, methanotrophs were resistant at the concentrations provided in Table 28 but became sensitive at higher concentrations. However, the MIC strips correctly predicted whether or not methanotrophs were resistant to the antibiotics tested. This could be because antibiotics from the MIC strips are more efficient in diffusing to areas around the plates.

Growth on alternative carbon sources

The ability of Isolate 3* to grow on acetate suggested facultative methanotrophy. The slow growth may have resulted from the strain not adapted to growth on acetate. Slow growth rate was not expected as *Methylocella silvestris*, a known facultative methanotroph grew faster and more efficient on acetate than CH₄ [31]. Interest in facultative methanotrophs is increasing and their importance includes potential use in bioremediation in environments inaccessible to CH₄. The inability of methanotrophs to use other carbon sources is still speculated. *Methylococcus capsulatus* for example has all the genes required for sugar metabolism but cannot be cultured on sugars. Type II methanotrophs have all the TCA cycle enzymes but do not grow on organic acids. This limitation could be connected to the absence or dormancy of key enzymes and

membrane transporters required for uptake and metabolism of sugars and organic acids [273, 274].

Growth of methanotrophs on methanol

The growth of Isolate 3*, 12 and *M. parvus* (OBBP) on CH₃OH required more time than growth on CH₄. The same reason responsible for the slow growth on acetate may be at play. The growth of *M. parvus* (OBBP) on 0.01% CH₃OH was not expected as earlier studies showed that *M. parvus* (OBBP) was unable to grow at all concentrations of methanol tested except it was adaptively evolved to growing on CH₃OH by first introducing it in vapour form. Surprisingly, all methanotrophs tested were unable to grow in 0.01% of CH₃OH vapour. However the experimental set up in which *M. parvus* was grown in CH₃OH vapour was different from the one used in this study [275]. Regardless of the slow growth, ability for methanotrophs to grow in CH₃OH has a lot of advantages. One of them is the ability to grow methanotrophs where infrastructure for supplying CH₄ is not available.

Genome sequencing of methanotrophs

The genome sequence data obtained in this study was useful in many areas. One of such areas was the provision of 23S rRNA data used to differentiate Isolate 3* and Isolate 6. Apart from the presence of fimbriae in Isolate 3* which made it morphologically distinct, most of the difference seen between Isolate 3* and Isolate 6 were found in the genome. These include absence of *ligD* in Isolate 6 and absence of *CrtF* in the carotenoid synthesis gene operon of Isolate 6 among others. The genome sequence of Isolate 10 revealed unexpected genes such as the ones required for PHB synthesis. However, the ability of type I methanotrophs to synthesize and accumulate PHB is still speculative [269]. Out of all the genes involved with PHB biosynthesis found in Isolate 3*, 6 and 12 which are PHB producers, only PHB depolymerase C terminus was absent in Isolate 10. This gene is unlikely to be the reason why Isolate 10 does not accumulate PHB as its function is breaking down PHB polymers. One hypothesis is that the PHB genes in Isolate 10 are not expressed under culturing conditions. Further insight into the expression of these genes can be provided by RNAseq experiments. The genome sequences were also useful in guide RNA design of CRISPR-Cas9 targets. There are two main systems of homologous recombination in bacteria – *recBCD* and

recF. The information obtained from genome sequence aided the selection of strains for CRISPR-Cas9 genome editing as both types of homologous recombination systems were targeted. Isolate 10 and 14 unlike the rest recombined using the *recBCD* pathway. Finally, the inability to electroporate all the methanotrophs tested may be solved by exploiting the RM system of these organisms.

Chemical tolerance tests for methanotrophs

3-hydroxypropionic (3HP) significantly impeded the growth of methanotrophs even at 0.1 mM concentrations. 3HP is an organic acid and pH-effect was first suspected as the reason. Measurement of media pH showed that NMS containing 5 mM 3HP even in the presence of phosphate buffer has a pH of 5.34 which can be tolerated by methanotrophs. Therefore, the toxicity has an underlying cause that requires further investigation. The inability of methanotrophs to grow in the presence of 3HP suggests they will be poor candidates to genetically engineer for the synthesis of 3HP. Isolate 12, the only methanotroph tested for CH₃OH toxicity in the presence of CH₄, was tolerant up to 5mM. Earlier results demonstrated the ability of Isolate 12 to grow on CH₃OH. Hence the relatively high tolerance is advantageous as Isolate 12 can be grown in CH₃OH as carbon and energy source for long durations without the need for replenishment. Although the growth of Isolate 12 in the presence of isobutanol was hindered, the tolerance at 5 mM which is relatively high means it can be potentially engineered for isobutanol production. Additionally, attempts can be made to adaptively evolve it to grow better at 5 mM or higher concentrations.

Effect of NMS components on methanotroph growth

Nitrate Mineral salt widely used for culturing methanotrophs was reported by Roger Whittenbury [2]. This study showed that vitamins, Fe-EDTA and CuSO₄.7H₂O were not essential for the growth of one methanotroph – Isolate 12. Elimination of these components especially vitamins can result in significant cost savings in industrial processes. Elimination of vitamins can also reduce the risk of contaminating methanotroph cultures. Contamination risks can further be reduced by adding high concentrations of copper to NMS which Isolate 12 can tolerate above many bacteria [276]. Nitrogen source in NMS is also crucial to the growth of methanotrophs and this

study showed the limitation of growth that can be encountered if the wrong nitrogen source is used as seen in Isolate 3*.

Growth of methanotrophs on biogas

Biogas and landfill gas are two viable sources of CH₄, especially in locations far away from fossil fuel-based natural gas. Hence the feasibility of using of biogas to culture methanotrophs is crucial. This study showed no significant difference overall between the growth of methanotrophs in biogas and pure CH₄. Further studies with biogas revealed biogas increased PHB accumulation in type II methanotrophs when used as a source of CH₄. The reason for increased accumulation of PHB in methanotrophs grown with biogas is unknown. One reason could be increased CO₂ from biogas may decrease the pH of the growth media thereby inducing stress conditions which favour PHB accumulation. Additionally, *Cuprividus necator* is known to be able to fix CO₂ for PHB accumulation [277]. Although there is no data to suggest the same is true in methanotrophs, it is an interesting prospect worth investigating. The correlation between O₂ and CH₄ consumption to CO₂ release by methanotrophs was shown in this study and the data gave an estimate of the O₂ and CH₄ concentrations required for growth to stationary phase. This information will be very important in cutting culturing costs of methanotrophs during industrial process as wastage can be minimised by supplying the right amount of O₂ and CH₄.

The results of growth experiments with CH₄ and biogas showed methanotrophs reached different maximum growth OD₆₀₀ in different experiments. This is usually due to the environment and volumes they are grown in. For example, growth of Isolate 12 in 10 mL NMS using a 165 mL serum bottle will peak approximately at OD₆₀₀ 1, while the same growth experiment using a 60 mL serum bottle will see Isolate 12 growth peak at OD₆₀₀ 0.7. Growth of methanotrophs in 24, 48 and 96 well plates will all peak at different OD₆₀₀. Additionally, the growth time has effects on the final growth OD₆₀₀. Usually, growth in liquid media peaks in day 2 – 3 if the starting OD₆₀₀ is 0.02 or higher. Sometimes growth tends to decline after stationary phase which may be as a result of pH change or accumulation of toxic by-products from methanotrophic growth. This information is crucial to understanding why peak growths are different in different experiments carried out in this study.

A case for novel isolates

One main reason why characterisation of methanotrophs was carried out was to determine which isolates are novel. The criterion for determining novelty of isolates was mainly based on 16S rRNA sequence similarity. Stackebrandt and Goebel assigned $\geq 97\%$ threshold for species to be regarded the same [278]. However the study was carried out in 1994 when few 16S rRNA sequences were available. After a review in 2018 by Robert C. Edgar, 99% was regarded to be a more accurate value for classifying species [279]. Nonetheless several publications used to announce new species have focused on morphological, physiological and biochemical characteristics [26]. It is based on the direction of these papers and the revised 16S rRNA sequence similarity threshold that a case is made for Isolate 3* and Isolate 10. Isolate 3* possess fimbriae, a very distinct morphological feature observed with Transmission Electron Microscopy (TEM) which is absent in its closest relative *Methylocystis rosea* [1].

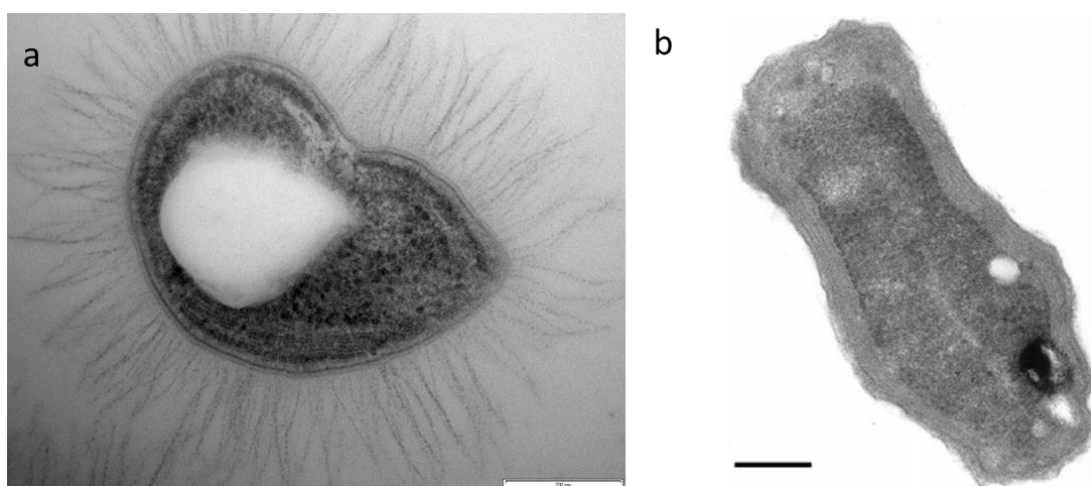


Figure 72: Comparison of Isolate 3* (a) and *Methylocystis rosea* (b) morphology. Isolate 3* possesses fimbriae absent in *M. rosea*. The scale for the image is 5000 nm at 4200X magnification for Isolate 3* and 200 nm for *M. rosea* [1].

Isolate 10 on the other hand has several characteristics which distinguishes it from its closest 16S rRNA relative – *Methylocaldum marinum* isolated by Takeuchi et al[26]. These include inability to grow on methanol, growth temperature, salt requirement, growth of colonies on plate and absence of sMMO (Table 34) [26].

Table 34: Comparison of *M. marinum* and Isolate 10 characteristics

Characteristic	<i>M. marinum</i>	Isolate 10
Temperature range (°C)	20–47	30-42
Motility	–	–
DNA G+C content (mol%)	59.7	58.1
Cell shape	Rod/coccus	coccus
Cell size (µm)	0.8–1.7×1.0–2.8	1.2-2.3 X 2.4 - 3.1
NaCl requirement	+	–
NaCl tolerance (%)	5	5
Colony formation on agar medium	–	+
Growth on methanol	+	–
sMMO	+	–
Colour of colonies	Brown to dark brown	Brown to dark brown
Heat resistance	–	NA
Desiccation resistance	+	NA
Chain formation	+	NA
Major quinone	Methylene Q-8	NA
Major fatty acids (% of total)		NA
C _{16:0}	59.2	NA
C _{16:1}	39.7	NA

5 Chapter 5: Transposon mutagenesis in Methanotrophs

5.1 Chapter Introduction

Transposable elements (TEs) are defined as specific DNA segments capable of repeatedly inserting into a few or many sites in a genome [280]. There are two classes of transposons – Class I and Class II. Here, the focus will be on Class II transposons which in bacteria are DNA based and composed of a transposase gene between two flanking Terminal Inverted Repeats (TIRs). In derivatives of class II transposons such as Tn5, information in the form of DNA sequences such as antibiotic resistance genes can also be packaged between the TIRs [281]. TIRs are also referred to as Inside and Outside Ends (IE and OE) [282]. The combination of TIRs and foreign DNA can be transferred from a plasmid vector into the genome of a target organism which results in genomic integration of foreign DNA. This process underpins transposon mutagenesis. With Tn5 transposon, insertion occurs randomly in the genome. Another type of Class II transposons, Tn7, has bias for certain A-T (Adenine-Thymine) DNA sequence regions referred to as *attn7* [230].

The random nature of transposon insertion can be leveraged to provide useful information regarding gene function and essentiality. This is because current genome sequencing technologies enable high throughput tracking of transposon insertion locations from DNA samples such as genomic pools of transposon mutant libraries. In addition to gene essentiality, gene function and genes that confer fitness under designed growth conditions can be determined. This information has different significance in every day molecular biology and genetic studies. For example, metabolic engineering studies constantly involve knocking out genes. Sometimes gene knockout experiments are carried out without prior knowledge of gene essentiality. Attempts to knock out essential genes often fail as cells do not survive the process. However, these attempts sometimes provide misleading or false positive results which can explain the difficulty encountered while trying to reproduce gene knockout experiments. Prior knowledge of gene essentiality can prevent these problems.

A sequencing technology used to translate genome insertion location into gene essentiality data is TraDIS. TraDIS has been applied in *Salmonella typhi* to determine

genes essential for growth under standard laboratory conditions [35]. In the SBRC Nottingham, unpublished data showed the use of TraDIS to provide insight into genes responsible for tolerance of high 3HP concentrations in *Cuprividus necator*. In both studies, Tn5 derivatives were used. Although a Tn5 based plasmid has been used in type I methanotrophs (*Methylobomonas* species) [97], the study focused on finding optimal expression sites for carotenoid biosynthesis genes. This study on the other hand focuses on establishing random transposon insertion using a suicide plasmid replicon.

One crucial step to creating a transposon library is making sure each mutant has only one transposon insertion. The link between phenotype and transposon insertion becomes unreliable if more than one transposon insertion is present in a mutant. To ensure only one transposon insertion in the transposon mutants made in this study, suicide plasmid vectors were used to carry transposons into recipient cells. Suicide vectors are incapable of replicating in recipient cells as the recipients lack host factors essential for the plasmids to replicate. One example of suicide plasmid replicon is R6K which is known to replicate if the plasmid host has the *pir* gene such as *E. coli* S17-1 λ *pir* strain. The *pir* gene codes for pi protein which is the replication initiator of R6K [283]. Although Isolate 12 the methanotroph selected for use in making a transposon library lacks the *pir* gene, the first step of making a transposon library was to confirm the inability of R6K to replicate in Isolate 12. The inability of R6K to replicate in Isolate 12 implies that there will be no plasmid background during sequencing of transposon library genomic pool. Transposition with Tn5 transposon is a cut-and-paste event and in the case of the Tn5 derivative (miniTn5kan_R6K) used in this study, only the transposon carrying the kanamycin resistance gene is moved from plasmid to Isolate 12 genome. This leaves behind the transposase gene in the plasmid. It is emphasised again that unlike in the original Tn5 where the transposase is in between the TIRs, the transposase gene is adjacent to the transposon in miniTn5kan_R6K (Figure 73). Another significant difference between the original Tn5 discovered in *E. coli* and the one used in this study (miniTn5kan_R6K) is the DNA sequence of the TIRs (Figure 74). The ME_I and ME_O used in (miniTn5kan_R6K) are mosaics derived from both the IE

and OE DNA sequence of the *E. coli* Tn5 transposon. The mosaic was used as it is reported to be hyperactive thereby increasing transposition efficiency [284].

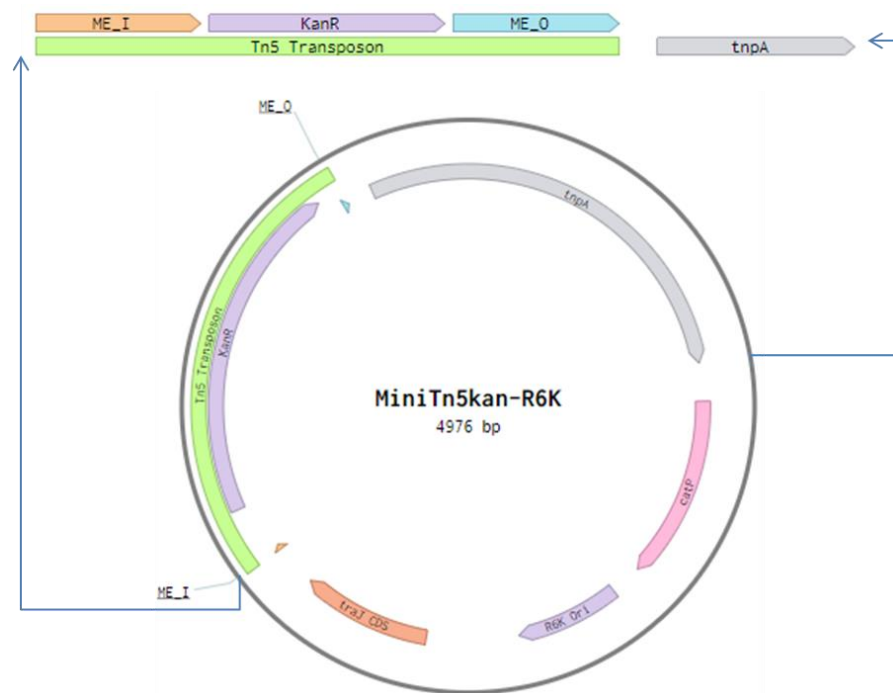
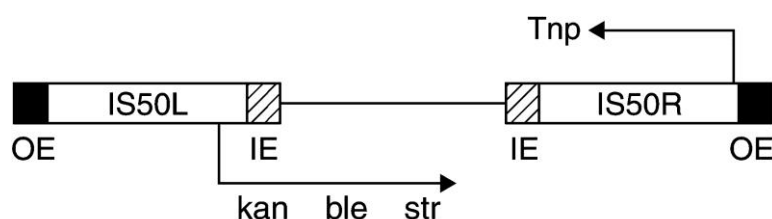


Figure 73: Arrangement of transposon and transposase in transposon plasmid used in this study. MiniTn5kan_R6K, a Tn5 transposon derivative consists of mosaics of Inside and Outside Ends of the original Tn5. Kanamycin resistance gene is in between the mosaic ends all of which experience transposition. The transposase gene *tnpA* is outside the mosaic ends and therefore does not move from the plasmid during transposition events. Sizes of different components described are not to scale.

Once the Tn5 transposon jumps from the plasmid to the genome, no other transposition event occurs due to the absence of adjacent transposase in the genome that can initiate further transposition. The single mutant with the transposon multiplies thereby all its offspring carry the same transposon insertion. However, because of the absence of *pir* gene in Isolate 12, the plasmid does not replicate and remains only in the original parent cell. So in reality the plasmid is not lost but its presence is obscured by the number of offspring that arise from the original parent cell.



OE: CTG**A**CTCTT**A**TACACA**A**GT
 IE: CTG**T**CTCTT**G**ATCAG**A**TCT
 MOSAIC: CTGTCTCTT**A**TACACA**T**CT

Figure 74: Tn5 is composed of two insertion sequences, IS50L and IS50R. IS50R codes for Tn5 transposase. Each insertion sequence is flanked by 19-bp end sequences termed outside end and inside end. The black box represents the OE and the striped box represents the IE. The OE and IE differ by 7 bases (shown in boldface). A hyperactive, hybrid end sequence, the mosaic end (MOSAIC) was used in this study. The boldface letters in the mosaic end sequence are outside end sequences, whereas the lightface letters are inside end sequences [284].

Tn5 transposition is tightly regulated hence transposition efficiencies tend to be low [218]. The practicality of making a library with hundreds of thousands of mutants depends on the transposition efficiency. To put this into perspective, a transposition efficiency that results in 100 mutants per NMS agar plate will require 10000 plates to make a library of 1000000 mutants which is labour intensive. In addition to the labour intensive work this requires, it increases risks of contamination and other sources of error. Hence it is important to have transposition efficiencies that will enable practical execution of these experiments which is why the transposition efficiency of miniTn5kan_R6K was determined.

After making a mutant library, further experiments investigated the nature of mutant library growth under various conditions. The outcome of these experiments will potentially provide information about the genes that are required for tolerance to these conditions.

5.2 Specific Research Aims

The aim of this study was to establish methods for carrying random transposon mutagenesis in Isolate 12, a type II methanotrophs using a suicide vector. This will enable the determination of essential genes across the whole genome without plasmid background in the mutant DNA pool.

5.3 Hypotheses

Detection of transposon DNA sequence by PCR amplification of Isolate 12 conjugated with *E. coli* carrying transposon plasmid suggests transposon insertion in Isolate 12 genome.

Inability to detect DNA sequences outside the transposon region of miniTn5_R6K plasmid in transconjugants using PCR amplification suggests plasmid loss and supports the hypothesis of R6K being a non-replicative replicon.

Inverse PCR products showing different insertion location of transposons in transconjugants' genome suggests Tn5 transposition in Isolate 12 is random.

5.4 Materials and methods

5.4.1 Strains

Table 35: Strains

Strain	Description	Reference
DH5α	<i>F'</i> <i>endA1 hsdR17</i> (<i>r_k⁻,m_k⁻</i>) <i>supE44 thi-1 recA1 gyrA</i> (Nal ^r) <i>relA1Δ(lacIZYA-argF)</i> U169 <i>deoR</i> (ϕ 80dLac Δ(<i>lacZ</i>)M15)	[239]
DH5α (High Efficiency from NEB)	<i>fhuA2 Δ(argF-lacZ)</i> U169 <i>phoA glnV44 Φ80 Δ(lacZ)</i> M15 <i>gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	[240]
S17-1 λ pir	<i>E. coli</i> S17-1 λpir strain contains the pir gene (λpir). This strain has conjugal transfer functions (RP4 transfer functions) integrated into the chromosome. The genotype of the strain is <i>TpR SmR recA, thi, pro, hsdR-M+RP4: 2-Tc:Mu: Km Tn7 λpir</i> .	[242]
XL-1 Blue	XL-1 cells are tetracycline resistant. XL1-Blue cells are endonuclease (<i>endA</i>) deficient, which greatly improves the quality of miniprep DNA, and are recombination (<i>recA</i>) deficient, improving insert stability. The <i>hsdR</i> mutation prevents the cleavage of cloned DNA by the <i>EcoK</i> endonuclease system. The <i>lacIqΔM15</i> gene on the F' episome allows blue-white color screening. The genotype is: <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacIqΔM15 Tn10</i> (Tetr)]. (Genes listed signify mutant alleles. Genes on the F' episome, however, are wild-type unless indicated otherwise)	[243]
Isolate 12	Methanotroph isolated from Moseley bog	This study

5.4.2 Primers

Table 36: Primers

Name	Sequence	Description
R6K_for_PmeI2	TACAAGCAGTTTAAACGCAGTTCAACTGTTGATAGTAC	Forward primer used for amplification of R6K from pUC18R6K-mini-Tn7T-Gm
R6K-rev4_SacII	TAGATACCGCGGTTGTCTAGCCGTTAAGTG	Reverse primer used for amplification of R6K from pUC18R6K-mini-Tn7T-Gm
TnpaR	CGTTCAGGGTAATGTTGC	For sequencing miniTn5kan-R6K plasmid
TnpaF	GCAAAGCAAAGTGGATGGC	For sequencing miniTn5kan-R6K plasmid
R6K_F	GTACGTACTAAGCTCTCATG	For sequencing miniTn5kan-R6K plasmid
R6K_R	GAACACTTAACGGCTGACA	For sequencing miniTn5kan-R6K plasmid
Kan_IG_for	GAACAAGATGGATTGCACGC	Forward primer used for screening colonies to check if there was transposon insertion
Kan_IG_rev	CGTAAAGCACGAGGAAGCG	Reverse primer used for screening colonies to check if there was transposon insertion
ChI_IG_for	ATGGTATTTGAAAAATTG	Forward primer used for screening colonies to check for loss of plasmid after transposon insertion
ChI_IG_rev	CAATTCCTGCAATTCGTTTAC	Reverse primer used for screening colonies to check for loss of plasmid after transposon insertion
Kan_F	CTTCTATCGCCTTCTTGAC	For sequencing inverse PCR products to determine location of transposon insertion
Kan_IV_For_KOD	GATTTCGAGCGCATCGCCTTCTATCGCCTTCTTGAC	Forward primer for inverse PCR to determine genomic insertion location
Kan_IV_Rev_KOD	GCCATCCAGTTTACTTTGCAGGGCTTCCCAACTTAC	Reverse primer for inverse PCR to determine genomic insertion location
Kan_Tn5_F	CGATCGGGCCCTGCAGGGGCTGTCTTATACAC	Forward primer for Transposon region used for NEB HiFi assembly of miniTn5kan-R6K3
Kan_Tn5_R	CGGACCATCCCTGGGTGAAGTCTGTCTCTTATACACA	Reverse primer for Transposon region used for NEB HiFi assembly of miniTn5kan-R6K3
Pals_Tn5_F	GTATAAGAGACAGACTAGTTCAACCCAGGGATGGTCCG	Forward primer for medium strength transposase promoter region used for NEB HiFi assembly of miniTn5kan-R6K3
Pals_Tn5_R	GCAGTGCACTGGTAATCATGCTTCGGCCCTCTTACT	Reverse primer for medium strength transposase promoter region used for NEB HiFi assembly of miniTn5kan-R6K3
Tnpase_F	GTAAAGAGGGGCCGAAGCATGATTACAGTGCACTGC	Forward primer for Transposase region used for NEB HiFi assembly of miniTn5kan-R6K3
Tnpase_R	GCCCCCTTTTCGACGGGACTGGCCGCCAAAAAAG	Reverse primer for Transposase region used for NEB HiFi assembly of miniTn5kan-R6K3
Phps_Ery_F	TTTTTTTGGCCGCGCAGTCCCTGCGAAAGGGGG	Forward primer for medium strength erythromycin promoter region used for NEB HiFi assembly of miniTn5kan-R6K3
Phps_Ery_R	GAATATTTTATATTTTGTTCATGAGATCTCCTCCGATCAAAAAA	Reverse primer for medium strength erythromycin promoter region used for NEB HiFi assembly of miniTn5kan-R6K3
Ery_Tn5_F	CTTTTTTATCGGAGGAGATCTCATGAACAAAATATAAAATATTCTCAAACT	Forward primer for erythromycin region used for NEB HiFi assembly of miniTn5kan-R6K3
Ery_Tn5_R	CAACAGGTTGAAGTGCCTTTGTTTAAACACATTCCTTTAGTAAC	Reverse primer for erythromycin region used for NEB HiFi assembly of miniTn5kan-R6K3
90882_Tn5_F	AACGGGAGGAAATAAGAGTTTAAACGCCGGA	Forward primer for Transposon region used for NEB HiFi assembly of miniTn5kan-R6K2
90882_Tn5_R	CAGTAGCTGACATGGTTTGCCTGCAGGGGT	Reverse primer for Transposon region used for NEB HiFi assembly of miniTn5kan-R6K2
909882_KanTn5_F	ACCCCTGCAGGCAACCATGTCAGCTACTGGG	Forward primer for Transposon region used for NEB HiFi assembly of miniTn5kan-R6K2
90882_EryTn5_R	CCGGCGTTTAAACTCttatttctccgttaaataatag	Reverse primer for Transposon region used for NEB HiFi assembly of miniTn5kan-R6K2

5.4.3 Plasmids

Table 37: Plasmids

Name	Description	Source
pMTL82221	SBRC modular plasmid carrying P15a and traJ origin of replication	SBRC culture collection
pMTL70115	SBRC modular plasmid carrying Tn5 transposon in the form of tetracycline resistant cassette flanked by an inner (ME_I) and outer (ME_O) mosaic ends. The plasmid also carries the Tn5 transposase and a chloramphenicol antibiotic resistant gene for checking plasmid loss. This plasmid uses ColEI as its origin of replication which is known to replicate plasmids in methanotrophs. Hence the need to replace it with R6K which cannot replicate in Isolate 12. Therefore it can be used to make a suicide plasmid which can only deliver a transposon into a cell but cannot replicate, leading to the desired single transposition event per cell	SBRC culture collection
pUC18R6K-mini-Tn7T-Gm	Plasmid carrying R6K origin of replication. R6K cannot replicate in Isolate 12, hence it was used for making suicide plasmid needed for transposon library generation	SBRC
miniTn5Tet_P15a	Plasmid made by digesting P15a and traJ region of pMTL82221 with SbfI and PmeI and ligating it into pMTL70115 backbone digested with the same enzyme set	This study
miniTn5Tet_R6K	Plasmid made by replacing P15a region of miniTn5Tet_P15a with R6K region of pUC18R6K-mini-Tn7T-Gm. miniTn5Tet_P15a was digested with PmeI and SacII to remove the origin of replication and replace with R6K PCR product flanked with PmeI and SacII	This study
miniTn5Kan_P15a	The tetracycline antibiotic cassette flanked by inner and outer mosaic ends in miniTn5Tet_P15a was replaced with kanamycin	This study
miniTn5Kan_R6K	The tetracycline antibiotic cassette flanked by inner and outer mosaic ends in miniTn5Tet_R6K was replaced with kanamycin. This plasmid was used for generating a transposon library. Flanking kanamycin on the left and right side are mosaic ends which confer the ability to move to random genomic locations. <i>E. coli</i> S17-1 λ pir carrying this plasmid was used to conjugate Isolate 12. R6K origin does not replicate in Isolate 12. Therefore cells that were not successfully conjugated failed to survive. Hence only cells that were conjugated and experienced a transposition event were present in the library created	This study
miniTn5Kan_ColEI	This plasmid was made using HiFi assembly. A ColEI rep was assembled into it. More importantly, the plasmid transposase was driven by a Pal promoter	This study
miniTn5Kan_R6K2	Here R6K and traJ was used to substitute the ColEI origin of replication in miniTn5Kan_ColEI	This study

5.4.4 Conjugation of Isolate 12 with transposon plasmid

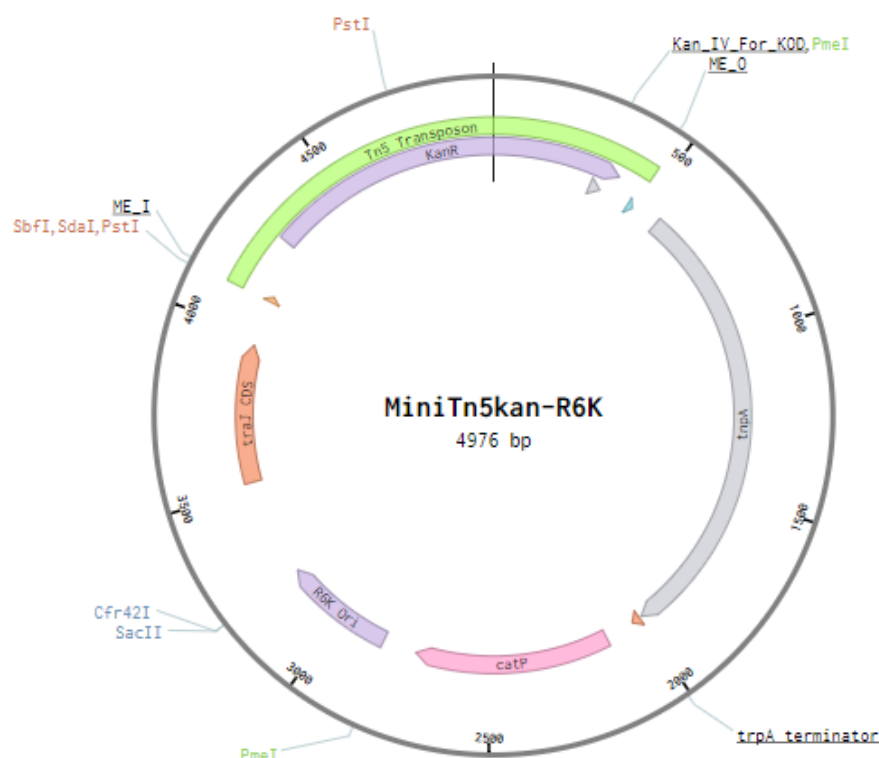


Figure 75: MiniTn5kan-R6K plasmid used for making transposon library. Plasmid carries R6K origin of replication which makes it a suicide plasmid in methanotrophs; a kanamycin resistant cassette as its transposon and a Tn5 transposase.

E. coli S17-1 λ pir was used for conjugation of Isolate 12 with miniTn5Kan_R6K. Mating was carried out for 48 hrs after which mating spot was resuspended in 100 μ L NMS and spread on NMS BA containing kanamycin 50 μ g/mL and Nalidixic acid 25 μ g/mL. Colonies that appeared after two weeks were subject to PCR screen and replica plated on NMS kanamycin 50 μ g/mL and NMS chloramphenicol 20 μ g/mL plates. Inverse PCR was also carried out. PCR was used to screen colonies for two reasons – confirmation of transposon insertion in the genome and loss of plasmid after transposon insertion.

Transposon insertion

Primer set Kan_IG_for and Kan_IG_rev was used to carry out PCR with Dreamtaq polymerase to check for presence of kanamycin gene in the transconjugants. The detection of kanamycin suggests three things. The first is the desired outcome which is that transposition occurred and the kanamycin was detected from a genomic location and not from the plasmid which was expected to be lost during cellular replication. The

second possibility is undesirable, which is the kanamycin detected by PCR is coming from the plasmid and hence the plasmid replicated and was not lost. This implies that due to multiple plasmids, more than one transposition event will occur which will affect quality of data obtained from the transposon library. The third possibility is that kanamycin detected came from both the plasmid and the genome which equally affect the transposon library data. A second PCR screen was therefore carried out to verify plasmid loss.

Plasmid loss

Plasmid loss screen was carried out using primer set Kan_IG_for and TnpaR. The region this primer set amplifies is within the transposon plasmid but outside the kanamycin transposon and this region is not expected to transpose into the genome. Hence detection of bands with this screen suggests the presence of plasmid in the cell. Additionally, individual colonies were streaked on two different NMS plates: NMS chloramphenicol 20 µg/mL and NMS kanamycin 50 µg/mL. Growth on kanamycin will suggest either the presence of plasmid or occurrence of transposition. Growth on chloramphenicol plate will suggest the presence of plasmid which will confer antibiotic resistance and enable the cells grow.

5.4.5 Inverse PCR of transposon mutants

Inverse PCR determines the location of transposon insertion in the genome. Therefore it can also reveal the DNA sequence adjacent the transposon and can be indirectly be used to determine plasmid loss. Inverse PCR was carried out as described in Chapter 2. In all cases, colonies were grown, picked with sterile loops and dipped in NMS media containing kanamycin 50 µg/mL and Nalidixic acid 25 µg/mL. After 4 – 7 days of growth (time taken to reach exponential phase varies due to different colony sizes), OD₆₀₀ of samples were taken, gDNA is extracted and used for inverse PCR using KOD Hot Start Master Mix Polymerase and primer set Kan_IV_For_KOD and Kan_IV_Rev_KOD. Inverse PCR products were run on 1% agarose gel. DNA from gel was extracted and sent to Source Bioscience for sequencing with primer Kan_F to determine region of insertion. Benchling was used to visualise data.

5.4.6 Efficiency of transposition

Efficiency of transposition was carried out to determine the mating time with the highest transposition efficiency. It was also carried out to investigate the duration of mating that minimizes cell division without compromising transposition efficiency. During conjugation, mating was carried out for 3, 6, 12, 24 and 48 hrs. Afterwards cells were spread on antibiotic plates till colonies appeared. Colonies from each mating time were grown in liquid NMS with kanamycin 50 µg/mL and Nalidixic acid 25 µg/mL. Extraction of gDNA and inverse PCR was also carried out to determine location of insertion. It is important to mention that although this protocol is very similar to conjugation efficiency protocol, there is a technical difference. In conjugation efficiency, transconjugants per donor is measured based on the number of transconjugants that acquire a plasmid from a donor cell. Transposition efficiency in this case however is measured based on the number of transconjugants that acquire the Tn5 transposon which has an antibiotic marker (kanamycin) from the donor *E. coli* cell.

5.4.7 Mutant library preparation

After calculating the transposition efficiency of different mating times, 12hr mating was selected. Transposon mutant library was prepared by conjugating Isolate 12 with *E. coli* S17-1 λ *pir* carrying miniTn5Kan_R6K. Mating was carried out for 12hrs and mating suspensions (100 µL) was spread on antibiotic plates. This was carried out in batches and the process lasted for more than three months as 1 million mutant library was targeted. The long duration necessitated storage of each batch which was done by pipetting 0.5 – 1 mL of NMS containing antibiotics and 15% glycerol on each plate and scraping the colonies. The cell suspension from each plate was collected in a 50 mL falcon tube. After scraping colonies from a batch, the number of mutants was estimated, the falcon tube was shaken to mix the mutants and 100 µL/50 000 mutants was redistributed from the falcon tube to several 2 mL screw cap tubes and stored at -80°C. The same 2 mL screw caps were used to collect cell suspensions from other batches so that each 2 mL screw cap tube contained all batches of mutants.

5.4.8 Library processing

The library of transposon mutants stored at -80°C was revived in NMS with kanamycin 50 µg/mL and nalidixic acid 25 µg/mL. To carry out TraDIS in duplicate, two tubes containing approximately 600, 000 mutants each were thawed on ice for 30 minutes. Half the volume of each tube was pelleted and stored at -80°C for gDNA extraction and TraDIS. From the other two tubes, 100 µL (50, 000 mutants) was transferred into 40 mL of NMS with antibiotics and grown for 3 – 4 days. The cultures grown in 40 mL were pulled together in 1 L of Duran bottle for each duplicate and mixed thoroughly by shaking to ensure all the mutants are uniformly mixed. The content were transferred into falcon tubes, spun at 8000 rpm for 10 minutes and resuspended in 3 mL NMS with antibiotics. A calculated amount from the 3 mL was transferred into 40 mL of NMS with antibiotics under selected growth conditions. The calculated amount was such that the starting OD₆₀₀ in 40 mL NMS with antibiotics is 0.02. From the 3 mL, dilution was made to 10⁻⁶ and plated on NMS with antibiotics to approximate the number of mutants per mL.

5.4.9 Mutant library growth conditions

To investigate the fitness of Isolate 12 and gene essentiality under selected conditions, the pulled mutants were grown in NMS with antibiotics containing the following: NMS only, 5 mM CuSO₄·7H₂O, 5 mM Isobutanol, 5 mM methanol and 0.1 mM 3HP. The library was also grown in NMS with antibiotics and Na₂CO₃/NaHCO₃ to a pH of 7.98 and NMS with antibiotics and HCl to a pH of 5.47 to investigated growth under basic and acidic conditions respectively. All the above were grown with CH₄ except the final condition which was mutant library in NMS antibiotics grown with biogas (BG2).

Samples grown in the listed conditions were subcultured after 3 – 5 days in 40 mL NMS with antibiotics and their respective conditions. Dilutions of the growth culture were made to 10⁻⁷ to approximate the number of mutants growing per mL. 3 passages in duplicate were made for each condition and the starting OD₆₀₀ was kept at approximately 0.02. After growth and subculture of each condition, 35 mL was pelleted in falcon tubes and stored at -80°C for gDNA extraction and TraDIS.

5.5 Results

5.5.1 Transposon insertion and plasmid loss

The conjugation of Isolate 12 with *E. coli* S17-1 λ *pir* carrying miniTn5Kan_R6K transposon plasmid led to the formation of colonies ranging from 200 – 2000 per NMS antibiotic plate. The variation in the number of colonies varied due to several reasons that will be revisited in the discussion section. To investigate transposon insertion in the genome, PCR was carried out to amplify the expected transposon insertion region using primer pair that amplify within the kanamycin region of the transposon. Part of the kanamycin gene which makes up the transposon corresponding to 729 bp was detected (Figure 76). Plasmid loss was also investigated with primer pair that amplify regions in and out of the transposon region. This is important as only the transposon plasmid contains the template for both primers, hence PCR product will only form if plasmid is not lost. No bands around 1824 bp were detected except in positive control suggesting that the plasmid did not replicate in the genome of Isolate 12 and was therefore lost (Figure 76).

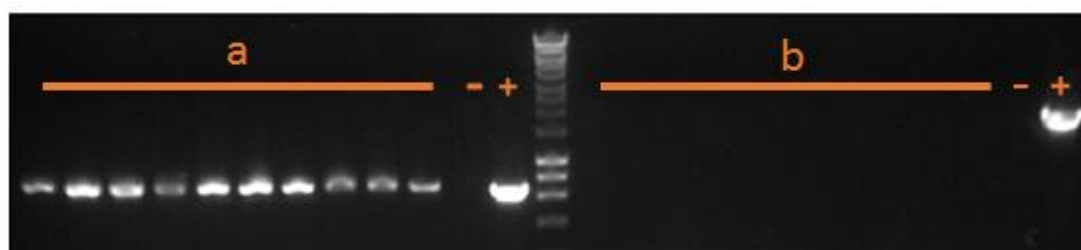


Figure 76: Transposon insertion detection using PCR amplification with primer pair Kan_IG_for and Kan_IG_rev (left). Plasmid loss investigation using primer pair Kan_IG_for and TnpA_R (right). Colonies 1 – 10 screened for transposon insertion are represented by (a) and the same colonies screened for plasmid loss are represented by (b). Isolate 12 colonies were used as negative (-) control while transposon plasmid was used as positive (+). Bioline 1 kb ladder was used in the gel image.

An additional experiment was carried out to determine loss of plasmid. This involved streaking the same transposon mutant colonies on two different plates. The first plate was NMS BA with kanamycin 50 μ g/mL and nalidixic acid 25 μ g/mL and the second plate was the same plus additional chloramphenicol 20 μ g/mL added to it representing the antibiotic selection marker on the backbone of miniTn5Kan_R6K. When transposon mutants were streaked on both plates, no growth was observed on chloramphenicol plate but growth was observed on plate without chloramphenicol which suggested

plasmid loss. This is because if plasmid was present which carried a chloramphenicol resistant marker, growth would have been observed on chloramphenicol plates. However in subsequent experiments, Isolate 12 with plasmid carrying a chloramphenicol resistant plasmid did not grow in NMS with chloramphenicol 20 µg/mL which suggested the chloramphenicol resistant antibiotic cassette did not work for Isolate 12 and its use in this test was invalid. However, the PCR in (Figure 76) and the inverse PCR provided enough evidence to show plasmid loss.

5.5.2 Inverse PCR

To determine the location of transposon insertion, inverse PCR was carried which detected only one transposon insertion location among all the colonies screened. Its location was around 400000 base pairs in the genome (Figure 77). Inverse PCR involves a genomic DNA digest, ligation and PCR stage. *SacI* was used initially in the digest stage which resulted in one PCR products (Figure 77). The reason for this maybe that *SacI* cuts Isolate 12 genome and mega plasmids at 1315 sites, which is relatively low and may have led to digests products too long to be PCR amplified after self-ligation. Hence another restriction enzyme (*EcoRI*) was used which cuts Isolate 12 genome and its two mega plasmids at 1633 sites thereby likely to

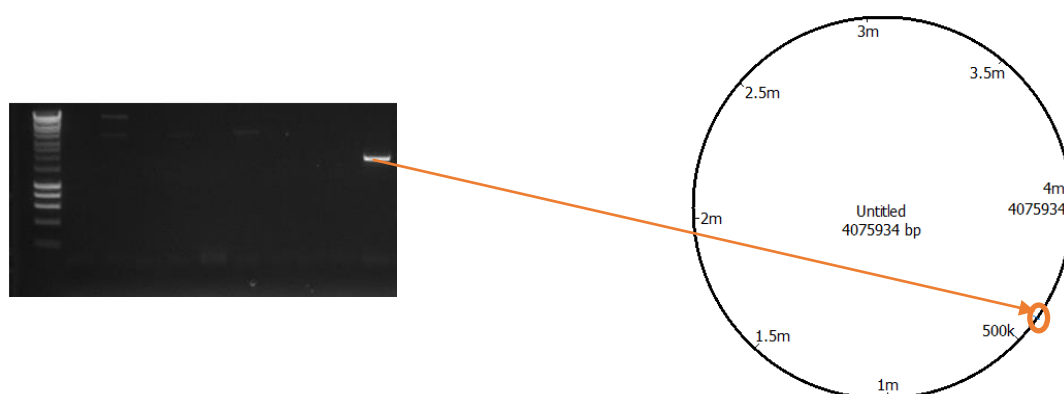


Figure 77: Inverse PCR band size of genomic DNA digested with *SacI* (left) and Transposon insertion location in the genome of Isolate 12. By sequencing the only band obtained, it was possible to determine location of transposon insertion which was around 400000 base pairs. Bioline 1kb ladder was used in the gel image.

produce shorter genomic DNA fragments more suitable for inverse PCR. Indeed, Figure 78 shows that all 10 inverse PCR carried out except one resulted in visible bands when *EcoRI* was used as restriction digest enzyme for preparing inverse PCR samples. When DNA from the bands were sequenced, transposon insertions in bands 1 – 5 were in the

same genomic location (870737 bp). The same was observed for bands 6, 8 and 10 (1051377 bp). Band 7 genomic insertion location was 2546031 bp.

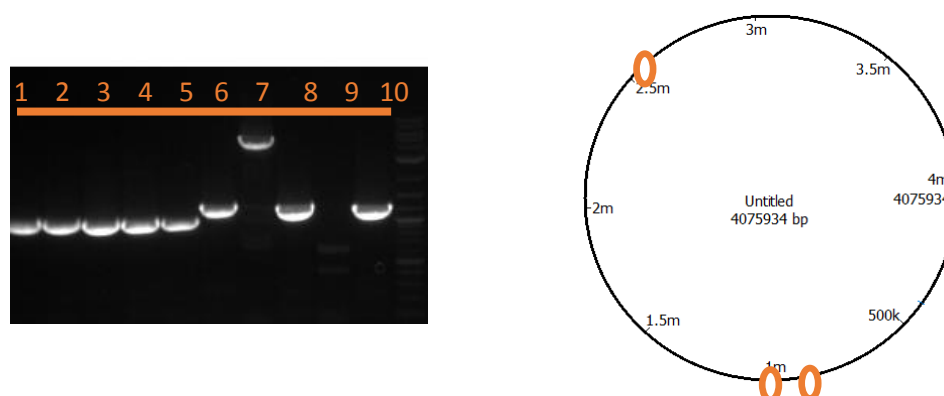


Figure 78: Inverse PCR band sizes of genomic DNA digested with EcoRI (left) and transposon insertion locations in the genome of Isolate 12. Bands 1 – 5 were in the same genomic location (870737 bp). Bands 6, 8 and 10 were in genomic location (1051377 bp). Band 7 genomic insertion location was 2546031 bp. The genomic insertion locations are represented by orange circles. NEB 2 log DNA ladder was used in the gel image.

Multiple insertion of transposons in one location is not desirable in a transposon mutant library. In addition to plasmid replication, it may also suggest a bias for insertion in a preferred genomic location which could mean transposon insertion is not global. However, the absence of transposon plasmid replication was already shown. To explain this apparent bias which led to multiple insertions in one genomic location, it was hypothesized that colonies which had the same transposon insertion location were related and may have been offspring of the same ancestor. This is highly likely considering mating time lasts 48 hours, enough time for cells that mutated to divide multiple times. This problem, in addition to the need to find out the most suitable mating time led to the determination of transposition efficiency at different mating times. Although transposition efficiency protocol was very similar to that of conjugation efficiency, it is important to understand that in

Table 38: Transposition efficiency of Isolate 12 at different mating times. Transposition Efficiency calculated as number of number of transconjugants with transposon insertion in genome.

Mating time (hrs)	Transposition efficiency
3hrs	2.25×10^{-4}
6hrs	4.43×10^{-4}
12hrs	1.67×10^{-4}
24hrs	1.48×10^{-4}
48hrs	9.05×10^{-5}

conjugation efficiency, transconjugants per donor is measured based on the number of transconjugants that acquire a plasmid from a donor cell. Transposition efficiency in this case however is measured based on the number of transconjugants that acquire the Tn5 transposon from *E. coli*. The Tn5 transposon has a kanamycin antibiotic cassette. Efficiency of transposition peaked at 6 hours 4.43×10^{-4} (Table 38). The least conjugation efficiency was seen in samples incubated for 48 hrs after mating (9.05×10^{-5}). Inverse PCR was carried out on colonies used to determine conjugation efficiency. Figure 81 shows the bands obtained, from which DNA was extracted and sent for sequencing to determine the location of chromosomal insertion. The location of chromosomal insertions was mapped to Isolate 12 genome and mega plasmids and these locations were distributed all over the genome (Figure 79) and mega plasmid 1 (Figure 80). Mapping the chromosomal and mega plasmid location of transposon insertion in Isolate 12 genome illustrated the spread and randomness of these insertions. In total 36 insertion locations were identified and there were two instances when two different cells had insertions in the same position. The first one was during the 3 hrs mating in which bands from lane 15 and 16 provided information that showed identical insertion location in the genome. Secondly, during the 6 hrs mating, lane 10 and 16 had the same insertion location. The location of insertions from 12 and 24 hrs mating were all random and not identical.

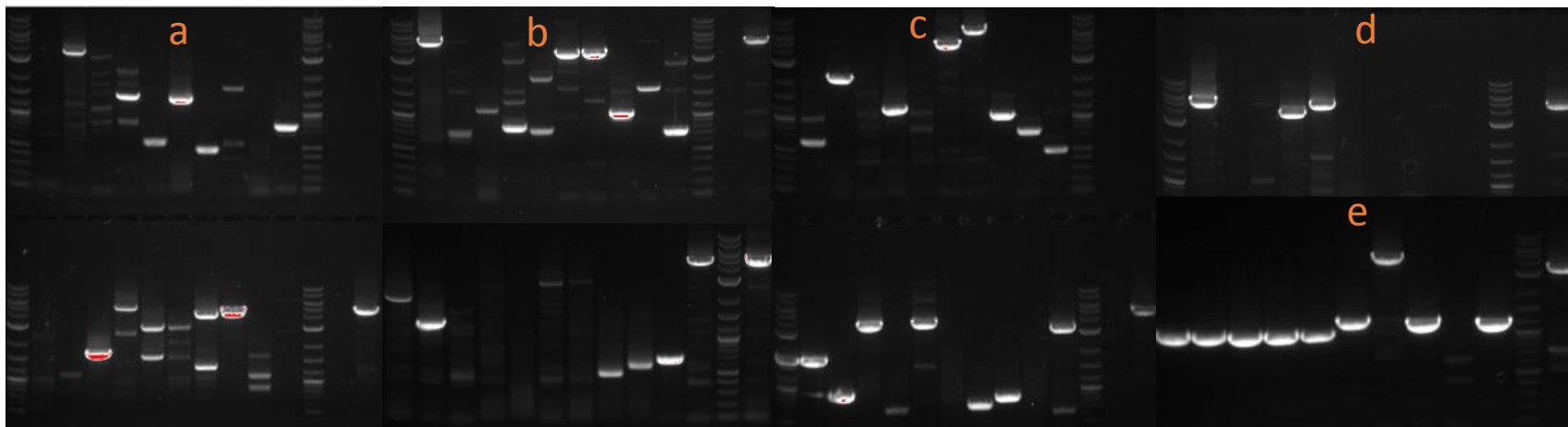


Figure 81: Inverse PCR bands for miniTn5-R6K transconjugants from mating plates incubating for 3 hrs (a), 6 hrs (b), 12 hrs (c), 24 hrs (d) and 48 hrs (e). NEB 2 log DNA ladder was used in the gel image. Bands sequenced and used to identify genomic location of transposon insertion.



Figure 79: Location of transposon insertion around different regions of Isolate 12 chromosome.

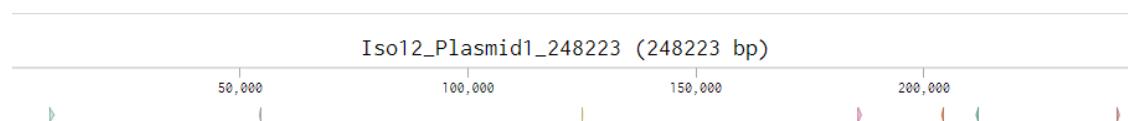


Figure 80: Location of insertion around different regions of Isolate 12 mega plasmid 1.

5.5.3 Mutant library preparation

After determining the conjugation efficiency of different mating times, 12 hrs mating time was selected for making transposon library even though it did not have the highest conjugation efficiency. The reason was because logistically, 12 hrs was more practical as it allowed for mating experiments and plating on antibiotic plates to be done on different days. This is crucial as the experiments involved long hours due to the high throughput nature and was more practical if done in two days. Additionally, inverse PCR data of 12hrs mating showed more specific bands and hence was suspected to have less chances of double mutant insertion (Figure 81c).

Making the transposon library for this study lasted more than three months and was therefore made in batches. After conjugation experiments for a batch, transposon mutant colonies were scraped from NMS agar antibiotic plates using 0.5 – 1 mL NMS with antibiotics containing 15% glycerol into a Falcon tube. The volume used depended on the number of colonies on plates as sometimes 1 mL is what is required to scrape off all colonies effectively. After harvesting transconjugants and estimating the number of mutants harvested, 100 – 200 μ L was transferred from the Falcon tube containing the mutants into a 2 mL screw cap tube. Approximately 576664 transposon mutants pooled from different conjugation batches and stored at -80°C in 2 mL screw cap tubes were processed further for TraDIS.

To carry out the experiments in duplicates, the growth OD_{600} of two 2 mL screw cap tubes each containing approximately 576664 mutants were measured after thawing from -80°C . The measured values 0.811 (sample a) and 14.23 (sample b) were used to calculate the amount to add to 40 mL NMS with antibiotics to have a starting growth OD_{600} of 0.02. 90 μ L of sample a was added to 10 different 40 mL NMS with antibiotics in 250 mL serum bottles. 56 μ L of sample b was added to 13 different 40 mL NMS with antibiotics. They were grown in many serum bottles to prevent a situation whereby a group of mutants dominated one serum bottle. These were grown for four days. After growth, all 250 mL serum bottles for each sample were pulled together in a 1 L Duran bottle, shook and half the volume was transferred to Falcon tubes in which they were pelleted. The pellets were resuspended in 3 mL NMS with antibiotics and pooled

together. From the pool, a calculated amount of sample a and sample b was transferred to 40 mL serum bottles containing NMS, antibiotics and additional growth conditions at a starting growth OD₆₀₀ of 0.02. The samples were grown for 3 – 5 days. After a maximum of five days, subcultures were made in 40 mL NMS media with antibiotics under specified growth conditions. This was repeated till four passages were available. Table 39 provides growth OD₆₀₀ reading under different conditions tested.

Table 39: Growth OD₆₀₀ of transposon mutant library in four successive passages under different growth conditions. The readings not available (NA) was due to contamination.

Growth condition	First passage		Second passage		Third passage		Fourth passage	
	Sample a	Sample b	Sample a	Sample b	Sample a	Sample b	Sample a	Sample b
NMS	0.474	0.511	0.263	0.257	0.415	0.334	0.424	5.08
Biogas (BG2)	0.223	0.138	0.091	0.153	0.236	0.282	NA	0.485
Acidic condition	0.264	0.321	0.34	0.369	0.162	0.442	0.229	0.432
Alkaline condition	0.454	0.352	0.122	0.121	0.208	0.401	0.364	0.391
3HP	0.491	0.432	0.323	0.332	0.445	0.430	0.501	0.408
Isobutanol	0.337	0.19	0.254	0.145	0.137	0.378	0.356	0.414
Methanol	0.415	0.23	0.338	0.498	0.170	0.250	NA	NA
Copper	0.378	0.305	0.431	0.384	0.631	0.525	0.606	0.249

The transposon mutant library grown under methanol conditions during the third passage showed clear signs of contamination and was discontinued. The same applied to sample a of biogas in the third passage. In addition to growing transposon mutants in different conditions, passage 1, 3 and 4 were also diluted to 10^{-6} and 10 μ L of each dilution was spotted on NMS BA with antibiotics to estimate the number of mutants in 40 mL of NMS media growing in each passage. Table 40 gives average values of colonies that were counted in the highest dilutions for each condition. To calculate the number of mutants grown in 40 mL NMS media with antibiotics under each condition, the values obtained from Table 40 were multiplied by 4000 and the dilution factor. This is because 10 μ L was spotted on NMS agar plates and to determine the number of cells in 40 mL NMS media, the number of CFUs was multiplied by 4000 and the dilution factor (Table 41). While counting colonies on media plates, it was observed that more than half the cells growing had different physical appearance from Isolate 12. Two types of colony appearances were observed. The first type was transparent and flat on NMS agar plates unlike

Isolate 12 which is known to be concave and opaque. The second type of contamination also appeared transparent and flat, except it had an opaque centre. This was the first clear indication that the library processed got contaminated. The first suspicion of contamination arose from the relatively low growth rate and maximum growth OD₆₀₀ of liquid cultures. However, the strange growth behaviour in liquid cultures was attributed to change in phenotype caused by transposon mutation. When liquid cultures growing on NMS media were diluted and restreaked on LB agar kanamycin (50 µg/mL) and nalidixic acid (25 µg/mL), two colony appearances were observed (Figure 82). A yellow coloured concave shaped colony and a smaller cream coloured concave shaped colony.

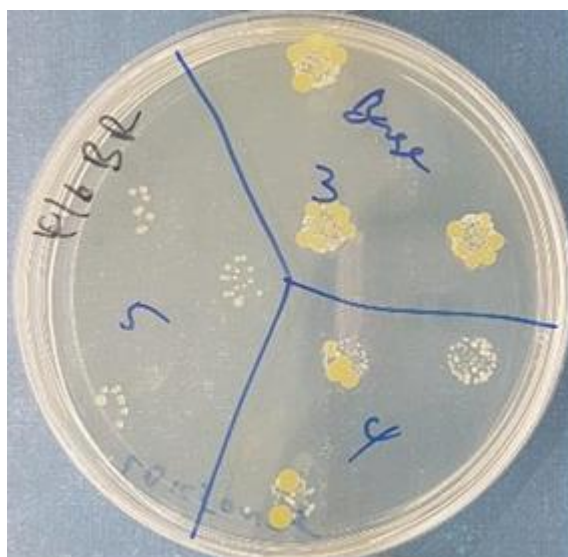


Figure 82: Contaminants present in transposon libraries as they appear on LB agar with kanamycin 50 µg/mL and nalidixic acid 25 µg/mL. The yellow colonies were identified as *Brevundimonas* species and the cream colonies were identified as *Microbacterium* species.

Colony PCR was carried out on the two colonies types using F515_F and F515_R primer pairs to amplify 16S rRNA genes. DNA extracted from the PCR product revealed the yellow colony morphology was *Brevundimonas* species while the other contaminant was a *Microbacterium* species. The presence of these contaminants, especially at the high proportion found had a negative impact on quality of the transposon library created. After trying other transposon mutant library batches stored at -80°C, it was clear that the contamination was in other batches tested. The source of the contamination remained unknown. Another round of transposon mutant library was planned.

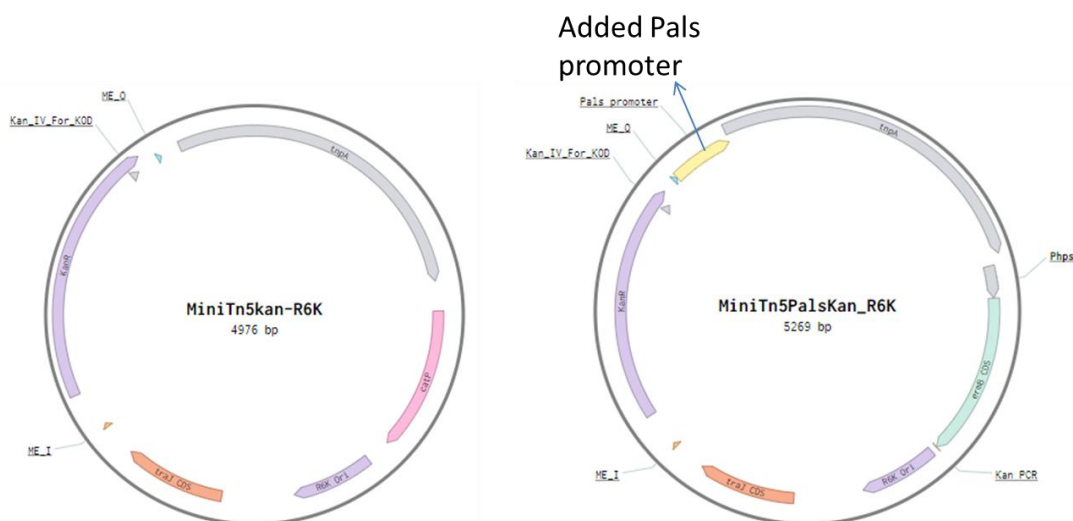


Figure 83: Pals promoter introduced to control transcription of transposase in new plasmid. The chloramphenicol antibiotic cassette was also replaced with erythromycin cassette.

To improve the conjugation efficiency of miniTn5Kan_R6K used in the first transposon library used, a second plasmid miniTn5Kan_R6K2 was designed. In the new plasmid, the transposase gene was under the transcriptional control of Pals promoter shown to work in Isolate 12 with medium strength (Figure 83). Additionally, the chloramphenicol antibiotic resistant cassette which was suspected not to work in miniTn5kan_R6K was replaced with an erythromycin antibiotic cassette. When the conjugation efficiency of miniTn5Kan_R6K2 was tested (1.83×10^{-8}), it was significantly less than miniTn5Kan_R6K (1.67×10^{-4}) $P < 0.0001$. There are many possible reasons why a strong promoter for the transposase gene would result in lower conjugation efficiency and will be discussed in the next section. Due to time limit of the author's PhD programme, the experiments on transposon library preparations were suspended.

Table 40: Dilution of Transposon mutants grown under different conditions

Dilution	Dilution of Transposon mutants grown under different conditions													
	1st Passage													
	CH ₄		Biogas		Acid		Base		Isobutanol		Methanol		Copper	
10 ⁻⁴ (methanotrophs)			25											
10 ⁻⁴ (contaminants)														
10 ⁻⁵ (methanotrophs)					1						1		4	2
10 ⁻⁵ (contaminants)													2	1
10 ⁻⁶ (methanotrophs)	6	3		1		1	1	1	1	1		1		
10 ⁻⁶ (contaminants)	13	7	1	4	3	3	1	3	2	11	5	5		

Table 41: Estimated number of transposon mutants growing in 40 mL of NMS media and antibiotic under specified conditions

	Estimated number of transposon mutants growing in 40 mL of NMS media and antibiotic under specified conditions													
	1st Passage													
	CH ₄		Biogas		Acid		Base		Isobutanol		Methanol		Copper	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Number of methanotrophs in 40 mL media	2.4 × 10 ¹⁰	1.2 × 10 ¹⁰	1 × 10 ¹¹	4 × 10 ⁹	4 × 10 ⁹	4 × 10 ⁹	4 × 10 ⁹	4 × 10 ⁹	4 × 10 ⁹	4 × 10 ⁹	4 × 10 ⁹	4 × 10 ⁹	1 × 10 ¹⁰	8 × 10 ⁹
Number of contaminants in 40 mL media	5.2 × 10 ¹⁰	2.8 × 10 ¹⁰	4 × 10 ⁹	1.6 × 10 ¹⁰	1.2 × 10 ¹⁰	1.2 × 10 ¹⁰	4 × 10 ⁹	1.2 × 10 ¹⁰	8 × 10 ⁹	4.4 × 10 ¹⁰	2 × 10 ¹⁰	2 × 10 ¹⁰	8 × 10 ⁹	4 × 10 ⁹

5.6 Discussion

Transposons have been used to carry out mutations in bacteria in a process called transposon mutagenesis [285]. The combination of high throughput DNA sequencing technologies and transposon mutagenesis is a useful tool in forward genetics that can be used to determine gene essentiality. This study selected TraDIS as the tool for transposon mutagenesis analyses in Isolate 12, a methanotrophic bacteria. Tn5 was selected as the transposon used to carry out transposon mutagenesis for making a transposon mutant library. The Tn5 used in this study differed from the Tn5 isolated from *E. coli* [218, 225]. These differences include the rearrangement of transposon and transposase gene on the plasmid used (Figure 73); the use of Tn5 IE and OE mosaic (ME_I and ME_O) instead of the native *E. coli* IE and OE as TIR; and the presence of only kanamycin between the TIRs instead of kanamycin, bleomycin and streptomycin found in the native *E. coli* Tn5 transposon (Figure 74). Some of these changes were essential for TraDIS applications whereas others such as using IE and OE mosaics were to improve transposition efficiencies.

The early stages of this study focused on confirming the inability of R6K replicon to replicate in Isolate 12 in order to ensure plasmid loss after transposon insertion using miniTn5kan_R6K. Experiments (Figure 76 and Figure 81) were used to investigate this problem. The first one which involved amplifying regions of the plasmid did not detect plasmid presence after transposon insertion. The second experiment identified 36 different locations of transposon insertion and none was found in the plasmid. This would have been unlikely if the plasmid replicated. However the main aim of the second experiment was to determine the randomness of transposon insertion. R6K is a medium copy number replicon, and if it had replicated the plasmid carrying the transposon, the probability of detecting the plasmid would have been high. In the absence of plasmid detection in both experiments, it will be safe to assume the lack of plasmid replication. The third experiment which involved patch-plating transposon mutants on NMS kanamycin BA plates with and without chloramphenicol was inconclusive.

Relatively high conjugation efficiencies are required for making a transposon mutant library. The efficiency of miniTn5Kan_R6K transposition peaked at 6 hours mating time between Isolate 12 and *E. coli* S17-1 Δ *pir*. This agreed with results obtained from measuring the conjugation efficiency of pMTL90882 described in Chapter 4 of this study which reaffirms the unnecessary prolonged mating time of 48-hour carried out in other studies with methanotrophs [272, 286]. The use of 12 hours mating time for making transposon library even with the knowledge that transposition efficiency peaked at 6 hours mating was based on practicality. The preparation process that leads to mating is time consuming. This added to the 6-hour wait for mating to occur, in addition to long hours required to dry and spread NMS antibiotic plates will require > 14 hours, a task that may lead to mistakes if done under stress after a long day in the lab. Therefore 12-hour mating times which can be carried out overnight were selected, and this permitted the execution of the experiment for each batch of transposon library to be carried out in two separate days.

One of the most crucial parts of this study was making the mutant library. From the earlier conjugation experiments, variation was observed in the number of colonies growing on NMS antibiotic plates. The range observed growing on plates was between 200 – 2000 colonies. The quality of the anaerobic jars used to grow methanotrophs was among the reasons for this fluctuation as some of the container seals were likely worn out. Additionally, the size of the jars ranged between 2.5 – 8.5 Litres. Fewer colonies were observed in the 8.5 L jars which could be due to lower gas pressure as a result of high volume that could have resulted in reduced mass transfer between CH₄ and methanotrophs. It should be noted that CH₄ was flushed into the anaerobic jars, therefore it was difficult to maintain a uniform 50:50 CH₄:air ratio across all jars which may have introduced another source of variation. During the period of library preparation, the dishwasher broke down. This resulted in washing serum bottles manually which affected the growth of Isolate 12 in liquid media as sometimes traces of detergent were hard to completely remove. This may have been another source of variation in number of colonies. Finally, making transposon mutant library was carried out over duration of 3 months. This may have resulted in slight variation in media preparation which could have led to variation in the number of colony growth.

Contamination was a major hindrance during this study. Different sources of contamination were anticipated. These include Isolate 12 cultures, *E. coli* cultures, anaerobic jars, plate drying and spreading environments among others. Steps were taken to ensure pure cultures of Isolate 12 were conjugated by testing for growth on rich media plates in parallel with NMS plates. It was more difficult to ensure the purity of conjugation donor *E. coli* as it grew on rich media and will be hard to differentiate from growth of contaminants. Therefore extra care was taken such as using fresh LB media to make sure *E. coli* cultures were not contaminated. Wiping anaerobic jars with ethanol is known to reduce contamination of methanotrophs. Consequently, anaerobic jars were constantly wiped with ethanol to avoid contamination from prolonged incubation in anaerobic jars. Normally, plates were dried in fume cupboards. Unexpectedly, the fume cupboard in the methanotroph lab broke down around the time of making transposon library. As a result, plates were dried in a 37°C room where most plates from other research groups were dried. This environment for drying plates was never reported to result in contamination. Therefore NMS antibiotic plates were dried there. In hindsight people from the other groups were mostly working with *Clostridia* species and *E. coli* which grew relatively quicker than methanotrophs. Isolate 12 requires two weeks of growth on NMS plate, enough time for contaminants to outgrow Isolate 12 if present. Regardless of contamination source, the proportion of contaminants to methanotrophs was unexpectedly high. This was likely due to faster growth rate of contaminants compared to Isolate 12 when mutant library stored at -80°C were revived. In future experiments, batches of transposon mutants will be stored separately so that possible contaminations can be compartmentalised.

The conditions used to grow methanotrophs were based on experiments which showed certain conditions impeded Isolate 12 growth. Such conditions included Isobutanol and 3HP. Other conditions such as methanol, biogas, $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$, acidic and alkaline conditions were to see if certain genes were under or overexpressed when Isolate 12 was exposed to such conditions. For example, growing *C. necator* in the presence of 3HP gave insight into genes required for 3HP tolerance. Contamination of the mutant library would have invalidated any conclusions as regards which genes

were over or under expressed at certain growth conditions so no further investigation was carried out.

The attempt to design a plasmid with higher transposition efficiency (miniTn5PalsKan_R6K2) resulted in a plasmid with lower conjugation efficiency. The most crucial alteration in the new plasmid was putting the transposase gene under the transcriptional control of the Pals promoter shown to be of medium strength in Isolate 12. This was based on a hypothesis that expression of transposase at that transcriptional level will increase conjugation efficiency. The opposite was found to be the case. Reports from Tn5 studies support the observation recorded. One of them is related to how transposase gene *tnp* and transposase inhibitor gene (*inh*) are arranged. These two genes have the same reading frame but are under the transcriptional control of different promoters (Figure 84). The T-10 and T-35 expresses *tnp* and I-10 and I-35 expresses *inh*. However, it was reported that T-10 and T-35 in addition to expression of *tnp* also expresses *inh* [218, 287]. This suggest stronger expression of *tnp* will lead to stronger expression of its inhibitor *inh*. Hence when the Pals promoter was introduced, it may have increased both *tnp* and *inh* expression. In addition, *inh* would have gained additional expression from its own promoter which does not express *tnp*.



Figure 84: Regulator elements controlling *tnp* and *inh* expression: *tnp* is only expressed by T-10 and T-35, *inh* is expressed by T-10, T-35, I-10 and I-35. *Tnp* is the gene that codes for the transposase while *inh* codes for the inhibitor protein that regulates the transposase [225].

Additionally, overexpressing transposase gene may lead to increased oligomerisation of transposase protein with itself which reduces its ability to bind to Inside and Outside mosaic ends thereby reducing the efficiency of transposition [218]. With the above in mind, future plasmid design should focus more on altering the transposase gene. Replacing glutamate with lysine in position 110 and 345 of the amino acid codon were shown to increase transposition efficiency by 6 – 16 fold [220].

5.7 Conclusion

The Tn5 derivative miniTn5Kan_R6K containing the R6K non-replicative replicon is capable of inducing transposon mutagenesis at random genomic locations of Isolate 12, a *Methylocystis* species, without plasmid replication. Transposition efficiency was highest when Isolate 12 and *E. coli* S17-1 Δ *pir* mating was carried out for 6 hours.

6 Chapter 6: CRISPR genome editing in methanotrophs

6.1 Chapter Introduction

CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. This refers to repeats often found in genomic sequences of bacteria and archaea which are regularly interspaced with DNA sequences of previously encountered viruses and bacteriophages. CRISPR-Cas9 on the other hand refers to the complex formed between Cas9 which is an endonuclease, and a guide RNA which is associated with the repeats found in the CRISPR DNA sequences. The use of this complex as a tool for targeted genome engineering gained wide attention after early demonstration of its specificity, efficiency, ease of design and application across a wide range of organisms [168-178].

Cas9 is one of the most studied Cas proteins, particularly the one found in *Streptococcus pyogenes*. It has been used for genome editing in eukaryotes, bacteria and archaea [167, 170, 180]. Cas9 genome editing entails creating potentially lethal double strand breaks in target DNA sequences [113, 114]. The process of inducing a double strand break (DSB) is initiated when there is homology between the guide RNA seed sequence in CRISPR-Cas9 complex and the target DNA sequence. This homology directs Cas9 to the target site where it exerts its nuclease activity. DSBs occur as a result of disruption of the phosphodiester backbone on both strands of the DNA double helix. In living organisms, DSB are caused by several other factors including ionising radiation, meiotic recombination and transposition of certain mobile elements [288]. In mammals, at least 10 spontaneous DSBs occur daily [289]. After potentially lethal DSBs are formed, four possible options known to this author are available for rescuing cells. These include Non-Homologous End Joining (NHEJ) and Homology Directed Repair (HDR) which are well known and have been widely studied. However, Single Strand Annealing (SSA) [288] and Alternative End-Joining (A-EJ)[290] have also been implicated in DSB repair. SSA and A-EJ will not be discussed here as they have been covered in a recent review on DSBs [291].

NHEJ is an error prone DSB repair pathway that does not require a repair template. It is mostly found in Eukaryotes although it is present in some prokaryotes. In Eukaryotes

such as mammals and yeast, NHEJ is carried out by a core set of proteins which include the DNA end binding proteins (Ku70/Ku80), ATP-dependent DNA ligase IV and its associated XRCC4 protein. The process entails Ku binding to the DNA ends and aligning them which suggest it may plays a role in DNA damage detection. It then prepares them for ligation and protects them from degradation before they are annealed by DNA ligase [292-295]. In bacteria, NHEJ was only fully discovered in 2002 [296]. Prior to that, *ykoU* and *ykoV*, genes of two proteins with relative homology to Ku70/Ku80 and DNA ligase IV respectively were speculated to be involved in NHEJ [297-299]. In bacteria, Ku interacts directly with DNA ligase IV to promote NHEJ [288].

Homology Directed Repair (HDR) is another pathway involved with DSB repair and it is known to occur with high fidelity. In eukaryotes, DNA repair by HDR involves the interaction between DSB end and either sister chromatid or homologous template and this is mediated by Rad51 and associated proteins [300]. In prokaryotes, HDR occurs either through RecBCD, RecE or RecF pathways [301].

The understanding of DSB repair played a significant role in the experimental design of this study. Intriguingly, methanotrophs isolated in this study have different DSB repair mechanisms as observed in their genome annotation (Table 32: Comparison of genes shared by isolates sequenced in this study). With regards to HDR, all the methanotrophs sequenced (Isolate 3*, Isolate 6, Isolate 10, Isolate 12 and Isolate 14) have the RecF pathway for HDR. In addition to RecF, Isolate 10 and Isolate 14 have the RecBCD pathway. This informed the selection of Isolate 10 as a candidate for CRISPR-Cas9 gene knockout as it may have advantage during DNA repair after Cas9-induced DSB. With regards to NHEJ, Isolate 3* and Isolate 6 have putative *ykoU* and *ykoV* genes and the involvement of these genes in NHEJ was reported in *Bacillus subtilis* [302]. Finally, only Isolate 12 was found to possess genes for ATP-dependent DNA ligase IV and Protein Kinase (Ku) which were also reported to carry out NHEJ in *Mycobacterium smegmatis* and are the key proteins for NHEJ in eukaryotes [303-305]. In addition, *M. parvus* (OBBP) was also found to possess ATP-dependent DNA ligase IV and Protein kinase (Ku) and was therefore included in the CRISPR-Cas9 gene knockout study.

Anecdotal reports suggested promoter strength to play a role in the success of CRISPR-Cas9 gene knockout. As a result, this line of enquiry was investigated. Promoters of different strength were selected to control the transcription of Cas9 and guide RNAs in the CRISPR-Cas9 plasmids used in this study. In this regard, three sets of plasmids were planned for use. The first set included plasmids with Cas9 under the transcriptional control of a moderately strong promoter and a weak promoter for guide RNA. This constitutes the 6-series of plasmids (3P6 and 12P6). The second plasmid set was Cas9 under the transcriptional control of a weak promoter and a moderately strong promoter for guide RNA. This constitutes the 11-series (3P11, 10E11, 12P11 and 12L11). The last plasmid set included a weak promoter for Cas9 and a very strong promoter for guide RNA which constituted the 12-series (10E12, 12P12 and 2.12P12). Initial experiments were carried out to investigate expression levels of different promoters in the candidate methanotroph selected for CRISPR-Cas9 gene knockout. These promoters include those found in *M. parvus* (OBBP), *M. capsulatus* (Bath) and other promoters made by Naglis Malys in SBRC Nottingham.

The candidate genes selected as targets for knockout were also important considerations in this study. One of the most important concerns when selecting genes for knockout is their essentiality. However, no data was available to inform this decision. Nonetheless, the candidates selected all had Poly-3-Hydroxybutyrate synthesis genes (*phaC*). *phaC* has been knocked out in *C. necator* (H16) which suggests it is not essential in that organism [306]. As a result *phaC* was selected as a gene target in Isolate 3*, 12 and *M. parvus* (OBBP). For Isolate 10, *efe* was selected as gene target. The ethylene forming gene *efe* was selected in Isolate 10 in order to better understand its function in making ethylene, a chemical used for making plastic. Another gene selected for knockout was the ATP-dependent DNA ligase IV (*ligD*) gene. Studies have shown increased efficiency of homologous recombination when the activity of *ligD* was suppressed by Scr7 pyrazine [183, 184]. Therefore in addition to attempts made to suppress *ligD* by addition of Scr7, *ligD* was selected as a knockout target. Additionally, it was shown that NAD-dependent DNA ligase (LigA) complemented the NHEJ function of LigD in a *ligD* mutant [291]. Hence it was hypothesized that even though *ligD* plays

important roles in the cell DNA repair system, *ligA* will complement the function of *ligD* in *ligD* knockout mutant.

Other miscellaneous factors considered were repair template position in the plasmid. This resulted in a two plasmid system with one plasmid carrying Cas9 and guide RNA while the other plasmid carried the homology arm (Figure 106). It was hypothesized in this case that having a two plasmid system can increase the likelihood of homologous recombination and consequently gene knockout efficiency. A minor variation of the two plasmid system used in this study was employed for CRISPR-Cas9 gene knockout in *Clostridium acetobutylicum* [307]. In the study, Cas9 under the transcriptional control of anhydrotetracycline promoter was in one plasmid while guide RNA and repair template was carried by a second plasmid.

Finally, a study showed that guide RNAs are more effective if they anneal to the template DNA strand of transcribed genes [186]. As a result guide RNAs that anneal to template and non-template strands were tested in this study.

Understanding the controls used for all the experiments in this study is crucial. In essence a set of three plasmids were used for each plasmid type tested, including two control plasmids and a test plasmid. The test plasmid transconjugants were the only ones used for CRISPR-Cas9 gene knockout screening. The first control plasmid often denoted with C4 as the prefix was included to show the level of conjugation efficiency when a plasmid carrying Cas9 only was conjugated into methanotrophs. High conjugation efficiency was expected for this plasmid because of guide RNA absence which prevented Cas9 from targeting and binding to any region thus preventing lethal DSB. The second plasmid type was the one which carried Cas9 and guide RNA and was denoted by C5 prefix in the plasmid name. The conjugation efficiency of this plasmid was expected to be low due to the absence of repair template and occurrence of Cas9 binding to target region thus inducing lethal DSB. This control plasmid demonstrates if the guide RNA used is functional. The last plasmid type which includes a guide RNA and repair template (left and right homology arms) served as the test plasmid and is expected to induce double strand break and repair the double strand breaks with the HAs. The conjugation efficiency of this plasmid is unpredictable as the number of cells

that survive will depend on the efficiency of homologous recombination. However the conjugation efficiency is expected to be relatively lower compared to the first control plasmid (C4).

6.2 Specific Research Aims

The aim of this chapter is to develop a CRISPR/Cas9 system in methanotrophs to carry out gene knock out, using guide RNAs to target the gene of interest, Cas9 to induce DSB at the target gene location and HAs of the target gene to repair and recombine at the point of DSB consequently deleting the target gene at the point of DSB.

6.3 Hypothesis

Homologous recombination will occur using left and right homology arms as repair templates after Cas9-induced DSB thereby deleting target gene.

There is an optimum Cas9:guide RNA ratio and transcriptional level required for efficient CRISPR-Cas9 gene knockout in methanotrophs.

6.4 Materials and methods

6.4.1 Promoter probe assays

The promoters in Table 42 were tested for activity in *E. coli* S17-1 λ *pir* and *M. parvus* (OBBP). Most of the promoters were derived by cloning DNA regions upstream the start codon of genes in *M. parvus* (OBBP) and *M. capsulatus* (Bath). Regions approximately 300 base pairs from the start codon of selected genes were run through three promoter prediction softwares (Promoter Prediction by Neural Network, BPROM and PePPER) [308]. The promoter sequences with average scores predicted by at least two softwares were sent to Twist Bioscience for synthesis. After synthesis, these promoters along with other promoters were cloned upstream of Enhanced yellow fluorescent protein (EYFP) which served as the reporter and transformed into *E. coli* S17-1 λ *pir*. *E. coli* carrying plasmids with different promoters were conjugated with *M. parvus* (OBBP).

The activity of these promoters was tested using fluorescence assays. In *E. coli*, overnights of different promoter cultures were grown and diluted to approximately OD₆₀₀ 0.5. Quadruplicates of each were pipetted (100 μ L) into Black Greiner 96 Well Flat Bottom (Chimney well) plates. Culture harbouring the control plasmid (plasmid with only EYFP reporter) was pipetted (100 μ L) in 12 replicates. The media was also pipetted (100 μ L) into 12 different wells. The plate was inserted into the Tecan M1000 for measuring fluorescence. The following parameters were used:

- Excitation wavelength – 495 nm
- Emission wavelength – 530 nm
- Optical Density wavelength – 600nm
- Mode – Read from bottom
- Gain – 70
- Shaking frequency – 408 rpm
- Kinetic cycles 5 – 10

6.4.2 Promoters

Table 42: List of promoters

Promoter	Full name	origin
Pact	Apolipoprotein acyl transferase	<i>M. parvus</i> (OBBP)
Pcah	Carbonic anhydrase	<i>M. parvus</i> (OBBP)
Phik	Histidine Kinase	<i>M. parvus</i> (OBBP)
Ppmmo	particulate Methane monooxygenase	<i>M. parvus</i> (OBBP)
Path	Amino acyl trna hydrolase	<i>M. parvus</i> (OBBP)
Psat	Serine acetyl transferase	<i>M. parvus</i> (OBBP)
Pfdh	Formate dehydrogenase subunit alpha CDS	<i>M. parvus</i> (OBBP)
Pshd	Shikimate dehydrogenase	<i>M. parvus</i> (OBBP)
Pmdh	Methanol dehydrogenase	<i>M. parvus</i> (OBBP)
Phps	3-Hexulose-6-phosphate synthase	<i>M. capsulatus</i> (Bath)
Pals	acetolactate synthase	<i>M. capsulatus</i> (Bath)
Pphac	Polyhydroxyalkanoate promoter	<i>C. necator</i> H16
Npro1	Synthetic promoters	Naglis Malys (SBRC Nottingham)
P13	Synthetic promoters	Naglis Malys (SBRC Nottingham)
P3	Synthetic promoters	Naglis Malys (SBRC Nottingham)

After factoring the differences in OD₆₀₀ of all samples, fluorescence from the media and the YFP control were subtracted from the fluorescence of cultures having both reporter and promoter.

A second experiment tested the activity of selected promoters (Phik, Pmdh, Pals, Phps, Pphac and P3) in four different strains of methanotrophs (Isolates 3*, Isolate 10, Isolate 12 and *M. parvus*)

6.4.3 Strains

Table 43: *E. coli* strains

Strain	Description	Reference
DH5α	<i>F'</i> <i>endA1 hsdR17</i> (r_k^- , m_k^-) <i>supE44 thi-1 recA1 gyrA</i> (Nal ^r) <i>relA1Δ(lacIZYA-argF)</i> U169 <i>deoR</i> (ϕ 80dLac Δ(<i>lacZ</i>)M15)	[239]
DH5α (High Efficiency from NEB)	<i>fhuA2 Δ(argF-lacZ)</i> U169 <i>phoA glnV44 Φ80 Δ(lacZ)</i> M15 <i>gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	[240]
S17-1	<i>Thi, pro hsdR, hsdμ⁺, recA</i> ; integrated plasmid <i>RP4-Tc::Mu-Kn::Tn7</i>	[241]
S17-1 λ pir	<i>E. coli</i> S17-1 λpir strain contains the pir gene (λpir). This strain has conjugal transfer functions (RP4 transfer functions) integrated into the chromosome. The genotype of the strain is <i>TpR SmR recA, thi, pro, hsdR-M+RP4: 2-Tc:Mu: Km Tn7 λpir</i> .	[242]
XL-1 Blue	XL-1 cells are tetracycline resistant. XL1-Blue cells are endonuclease (<i>endA</i>) deficient, which greatly improves the quality of miniprep DNA, and are recombination (<i>recA</i>) deficient, improving insert stability. The <i>hsdR</i> mutation prevents the cleavage of cloned DNA by the <i>EcoK</i> endonuclease system. The <i>lacIqZΔM15</i> gene on the F' episome allows blue-white color screening. The genotype is: <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacIqZΔM15 Tn10</i> (Tetr)]. Genes listed signify mutant alleles. Genes on the F' episome, however, are wild-type unless indicated otherwise	[243]

6.4.4 Methanotroph strains

Table 44: Methanotroph strains

Strain	Description	Reference
<i>M. parvus</i> (OBBP)	Type II <i>Methylocystis</i> species wild type	NCIMB
Isolate 3*	Type II <i>Methylocystis</i> species isolated from peat bog	This study
Isolate 10	Type I <i>Methylocaldum</i> species isolated from cow manure	This study
Isolate 12	Type II <i>Methylocystis</i> species isolated from peat bog	This study

6.4.5 Primers

Table 45: Primers (table continued in next page)

Name	Sequence	Description
PhaC_LHA_for2	ATATATGCGATCGCCGGCGAGCTGTTGAACAGG	Forward primer for <i>M. parvus</i> (OBBP) and Isolate 12 <i>phaC</i> gene left HA SOE-PCR
PhaC_LHA_R	GGAGCGCCGATGACCGAAGGGCTGACCTCAGCC	Reverse primer for <i>M. parvus</i> (OBBP) and Isolate 12 <i>phaC</i> gene left HA SOE-PCR
PhaC_RHA_F	GGCTGAGGTCAGCCCTTCGGTCATCGGCGCTCC	Forward primer for <i>M. parvus</i> (OBBP) and Isolate 12 <i>phaC</i> gene right HA SOE-PCR
PhaC-RHA_rev	ATATATGGCGCGCCCTCTTCGTTACGCTGTCGC	Reverse primer for <i>M. parvus</i> (OBBP) and Isolate 12 <i>phaC</i> gene right HA SOE-PCR
Phac_LHA_Hifi_F1	TTCTTATTTTATGCGATCGATCGTCATCGCAGGCA	Forward HiFi primers for amplification of <i>M. parvus</i> (OBBP) and Isolate 12 <i>phaC</i> gene left HA
Phac_LHA_Hifi_R1	CAACAATTCCGGCTGAGGGGCCAGAATCCTGACCGG	Reverse HiFi primers for amplification of <i>M. parvus</i> (OBBP) and Isolate 12 <i>phaC</i> gene left HA
Phac_RHA_Hifi_F2	CCGTCAGGATTCTGCCCCCTCAGCCGAATTGTTG	Forward HiFi primers for amplification of <i>M. parvus</i> (OBBP) and Isolate 12 <i>phaC</i> gene right HA
Iso12Phac_RHA_R3	GCCGGCCAGTCGGCGCAGGAAGCGGTGATACATC	Reverse HiFi primers for amplification of <i>M. parvus</i> (OBBP) and Isolate 12 <i>phaC</i> gene right HA
Iso12_sgR5-3_2	ATAAGCTATTAATTAAGAAGGGACGACGCGTCGGC GTTTTAGAGCTAGAAATAGCAAG	Forward primer with 5'-3' facing <i>phaC</i> seed gRNA overhang for amplification of gRNA
PhaC_LHAF_OHA	ACTTTGGCCAAGACCTTC	Reverse primer right outside HA for screening <i>M. parvus</i> (OBBP) and Isolate 12 <i>phaC</i> gene knockouts
Phac_RHA_OHA2	GACGAGACGCCTGATTCG	Forward primer outside left HA for screening <i>M. parvus</i> (OBBP) and Isolate 12 <i>phaC</i> gene knockouts
LigDFor-sgRNAPrm1	ATAAGCTATTAATTAAGGAAGCGGGCTGTCCAATCG GTTTTAGAGCTAGAAATAGCAAG	Forward primer with <i>ligD</i> seed gRNA overhang for amplification of gRNA
LigD_LHA-FWD	AGGCTTCTTATTTTATGCGATTTTCTTTTTCGTATCTCCGGC	Forward HiFi primers for amplification of <i>ligD</i> left HA
LigD_LHA-REV	GCCGCGCAACGCCTCGCAATGTGGATCAAGCCCGGCGCGCGTT	Reverse HiFi primers for amplification of <i>ligD</i> left HA
LigD_RHA-FWD	AACGCGCCCGGGGCTTGATCCACATTGCGAGGCGTTGCGCGGC	Forward HiFi primers for amplification of <i>ligD</i> right HA
LigD_RHA_Rev2	GCCGCCCCAGTCGGCGCGGGCGCAAGGAGCTGGACCTCGC	Reverse HiFi primers for amplification of <i>ligD</i> right HA
LigD_LHA_OHA-F	CCCGTCAACCTTCGATCCG	Forward primer outside left HA for screening <i>M. parvus</i> (OBBP) and Isolate 12 <i>ligD</i> gene knockouts
LigD_LHA_OHA-R	CGCTCTTTGACCTCATCTAC	Reverse primer right outside HA for screening <i>M. parvus</i> (OBBP) and Isolate 12 <i>ligD</i> gene knockouts

Table 46: Primers

Name	Sequence	Description
Iso3_Phbc1-sgr1	ATAAGCTATTAATTAACAGAGAACCTTACGGCTACG GTTTTAGAGCTAGAAATAGCAAG	Forward primer with Isolate 3* <i>phaC</i> seed gRNA overhang for amplification of gRNA
Iso3PhbC1_KA-F3	AGGCTTCTTATTTTATGCGATAGTTCGCCGAGCAGGGCTGTT	Forward HiFi primers for amplification of Isolate 3* <i>phaC</i> gene left HA
Iso3PhbC1_KA-R3	ATGAGTGGCGCGTCCCTTCGCGCAACGTCCTTTAAGCCGAGGCG	Reverse HiFi primers for amplification of Isolate 3* <i>phaC</i> gene left HA
Iso3PhbC1_KA-F4	CGCCTCGGCTTAAAGGACGTTGCGCAAGGACGCGCCACTCAT	Forward HiFi primers for amplification of Isolate 3* <i>phaC</i> gene right HA
Iso3PhbC1_KA-R4	CATGGCCGCCCCAGTCGGCGCTTCATTTATCGGACGTTCCGGCG	Reverse HiFi primers for amplification of Isolate 3* <i>phaC</i> gene right HA
Iso3PHBC1_OHA_F	CGGTCTACAACACGCTCAAC	Forward primer outside left HA for screening Isolate 3* <i>phaC</i> gene knockouts
Iso3PHBC1_OHA_R	CGAAATCGCTGCTCATCGTC	Reverse primer outside right HA for screening Isolate 3* <i>phaC</i> gene knockouts
EFE_sgRNA1	ATAAGCTATTAATTAATCGATGAAGCGCTTCAGGAC GTTTTAGAGCTAGAAATAGCAAG	Forward primer with Isolate 10 <i>efe</i> seed gRNA overhang for amplification of gRNA
109EFE_LHA-F	AGGCTTCTTATTTTATGCGATAAGCTGAAAGACATGATTTACGCG	Forward HiFi primers for amplification of Isolate 10 <i>efe</i> gene left HA
110EFE_LHA-R	ATTCACGTCTAGGACGCCATTTGAAACCTCCGAGGCGGCTGC	Reverse HiFi primers for amplification of Isolate 10 <i>efe</i> gene left HA
111EFE_RHA-F	GCAGCCGCTCGGGAGGTTTCCAAATGGCGTCCTAGACGTGAAT	Forward HiFi primers for amplification of Isolate 10 <i>efe</i> gene right HA
112EFE_RHA-R	CATGGCCGCCCCAGTCGGCGCGGTTGATTGAACGGCGCGGTGGC	Reverse HiFi primers for amplification of Isolate 10 <i>efe</i> gene right HA
Iso10EFE_OHA_F	CCAGAAAACCAAGATCCGC	Forward primer outside left HA for screening Isolate 10 <i>efe</i> gene knockouts
Iso10EFE_OHA_R	CTGCGTGAACGCATTGGAG	Reverse primer outside right HA for screening Isolate 10 <i>efe</i> gene knockouts
Asisi_fdxterm-Rev	ATTACAGTGCATCGCATAAAAAATAAGAAGCCTGCAATGCAG	Reverse primer for amplification of all guide RNAs used in this study
TWIST_F	GAAGTGCCATTCCGCCTG	Forward primer for amplification of Twist Bioscience gene strings
TWIST_R	CACTGAGCCTCCACCTAGC	Reverse primer for amplification of Twist Bioscience gene strings

6.4.6 Plasmid design

6.4.6.1 *Cas9*

The success of CRISPR-Cas genome editing depends on plasmid design. Variables including the type of Cas9, guide RNA and promoters must be of the right type. In this study, plasmids were designed for use in four species of methanotrophs (Isolate 3*, 10, 12 and *M. parvus* (OBBP)). Two types of Cas9 genes were used, native *Streptococcus pyogenes* Cas9 (*spCas9*) and Cas9 nickase (Cas9n) both obtained in plasmids available at the SBRC Nottingham. Whereas *spCas9* has both its endonucleases (HNH and RuvC) active and can cause double strand breaks, Cas9n has only one active nuclease (HNH) and can only cut one strand to create gene knockouts. It is emphasized here that the difference between *spCas9* and Cas9n is one base pair difference at position 72 that is a thymine in *spCas9* and an adenine in Cas9n. The change from thymine to adenine in Cas9n resulted in a frameshift mutation that abolished the function of RuvC.

6.4.6.2 *Guide RNA and Guide RNA seeds*

Guide RNA seeds are responsible for directing Cas9 to the target cut site in the genome and are specific for each gene. However, the guide RNA (gRNA) itself can be used for any gene target and it is involved in the formation of the complex between Cas9 and gRNA seed. This complex constitutes the active and programmed form of Cas9 capable of making cuts in desired regions of the genome. The gRNA seeds were designed using Benchling online software and synthesized as oligos from Sigma-Aldrich. The synthesized oligos constituted a region of homology with the gRNA, the seed sequence was in the form of an overhang flanked by a *PacI* restriction site and extra base pairs to enable restriction enzyme digestion. This acted as forward primer while *AsiSi_fdxterm-Rev* was used as reverse primer which had *AsiSi* as restriction site. The plasmid *pCas9_Empty_fdx+term* served as the template used for PCR amplification as it contained a guide RNA region. After PCR amplification, guide RNAs containing seeds were digested with *PacI* and *AsiSi* and ligated into their respective CRISPR-Cas plasmids. Unless specified, all the gRNAs containing seeds (sgRNAs) were

cloned into their respective plasmids by PCR amplification of the gRNA from plasmid pCas9_Empty_fdx+term.

6.4.6.3 Homology arms (repair templates)

Homology arms were designed to act as templates for homologous recombination (HR). Successful HR will lead to the removal of the target gene. The working hypothesis is that cells that have undergone HR will not be subject to Cas9 nuclease activity due to the absence of the target gene which will be lost if HR occurred. All other cells will still have the target gene and will therefore not survive the lethal nuclease attack from Cas9. Therefore in the end only cells that have lost the target gene (knockouts) will grow. To design homology arms, 1kb of DNA flanking the left and right target gene region were assembled using NEB HiFi assembly kit. The genes were first PCR amplified with NEB HiFi primers from the genome of methanotrophs before assembling into the desired plasmid with NEB HiFi assembly. However, HA of plasmids pMTL90882_Cas9For3 and pMTL90882_Cas9For6 were assembled using SOE-PCR before cloning into the plasmids.

6.4.6.4 Promoters

Another important component of the CRISPR-Cas plasmids investigated in this study was the effect of promoters driving the expression of Cas9 and sgRNAs on the outcome of CRISPR-Cas genome editing in methanotrophs. It was hypothesised that the right promoter combination will result in the desired genome editing efficiency by maximising the elimination of cells that did not undergo HR. This will make it easy to screen for knockouts that successfully recombined with HA. Three promoter sets were tested: Moderately strong promoter for Cas9 (Pals) and weak promoter for sgRNA (Pmdh); weak promoter for Cas9 (Pmdh) with moderately strong promoter for sgRNA (Pals); and very weak promoter for Cas9 (Phik) with very strong promoter for sgRNA (P3). These three promoter sets were designated as P6, P11 and P12 respectively for all three *Methylocystis* species investigated and E6, E11 and E12 for the *Methylocaldum* species. For the plasmid used to knockout DNA ligase IV (LigD) in *M. parvus* (OBBP) and Isolate 12, only one promoter set was tested and it was designated L11. These designations can be seen as suffixes in

plasmid names. For example the P6 at the end of pMTL90882_C4-12P6 means that the plasmid was carrying a moderately strong promoter for Cas9 (Pals) and a weak promoter for sgRNA (Pmdh).

6.4.6.5 Controls

The controls used in this experiment were designed to provide insight into the activity of Cas9 after conjugation. Control 1 designated as C4 for historical reasons is the CRISPR-Cas control plasmid that has neither an sgRNA nor homology arm (HA) and it is expected to have the efficiency of conjugation of a regular plasmid like pMTL90882. This is because absence of sgRNA implies that Cas9 will not be directed to a PAM site where it can attach and exhibit its nuclease activity.

The second control designated as C5 contains Cas9, sgRNA but lacks HA. Therefore it was expected that in this scenario most if not all the cells will not survive Cas9 nuclease activity as there is no means of evading nuclease attack from Cas9, or fixing strand breaks after the attack due to absence of HA. Control 4 and 5 are represented by the first two characters after 90882 in the plasmid names. It is emphasized here that the first set of plasmids have control 1 and 2 instead as the plasmids were carrying Cas9n and not *sp*Cas9.

6.4.7 *M. parvus* (OBBP) and Isolate 12 CRISPR-Cas9 plasmids

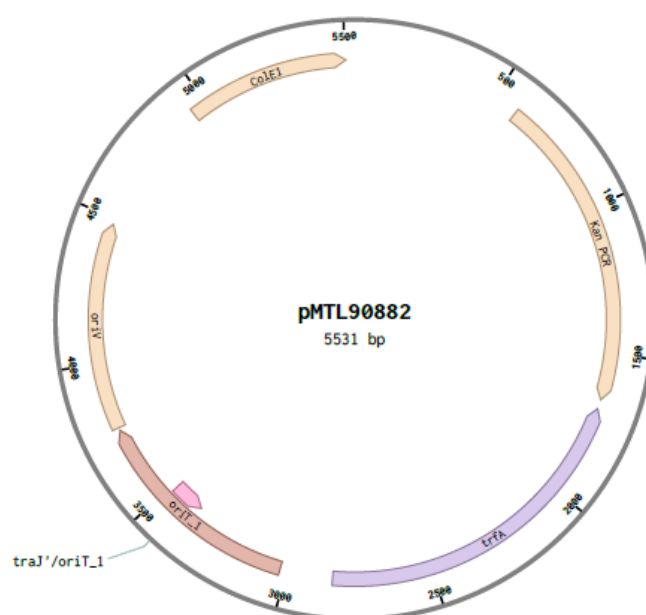


Figure 85: pMTL90882 plasmid used as backbone for cloning CRISPR modules. Plasmid designed in this study and has a multiple cloning site, kanamycin resistant cassette and ColEI replicon.

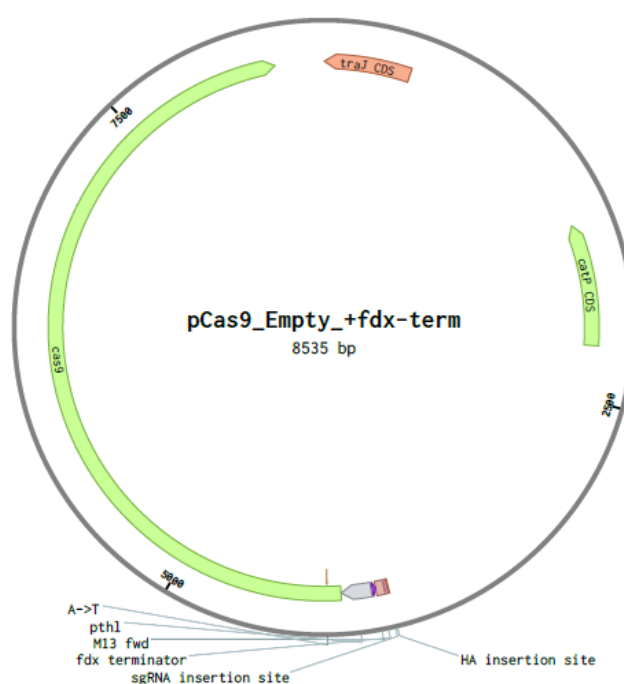


Figure 86: pCas9_Empty_+fdx-term carrying Cas9 without guide RNA and HA. Plasmid available in SBRC Nottingham culture collection.

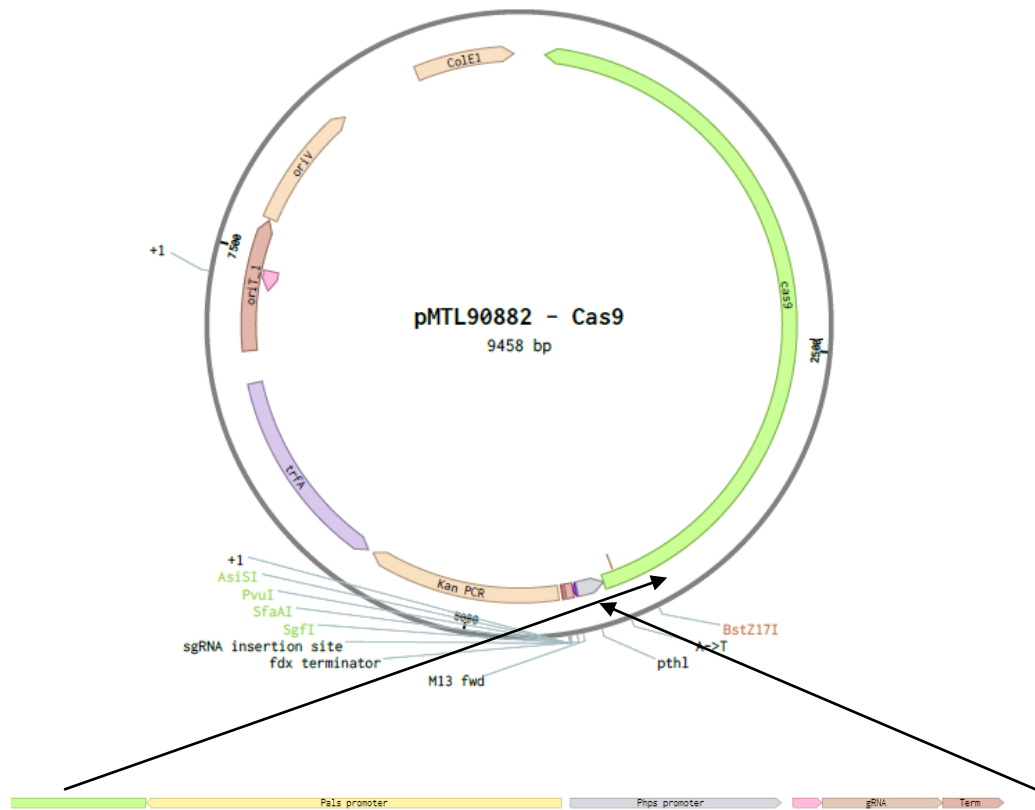


Figure 87: pMTL90882-Cas9 plasmid carrying Cas9 with synthesized CRISPR module made of Pals promoter for Ca9 and Phps promoter for sgRNA and terminator.

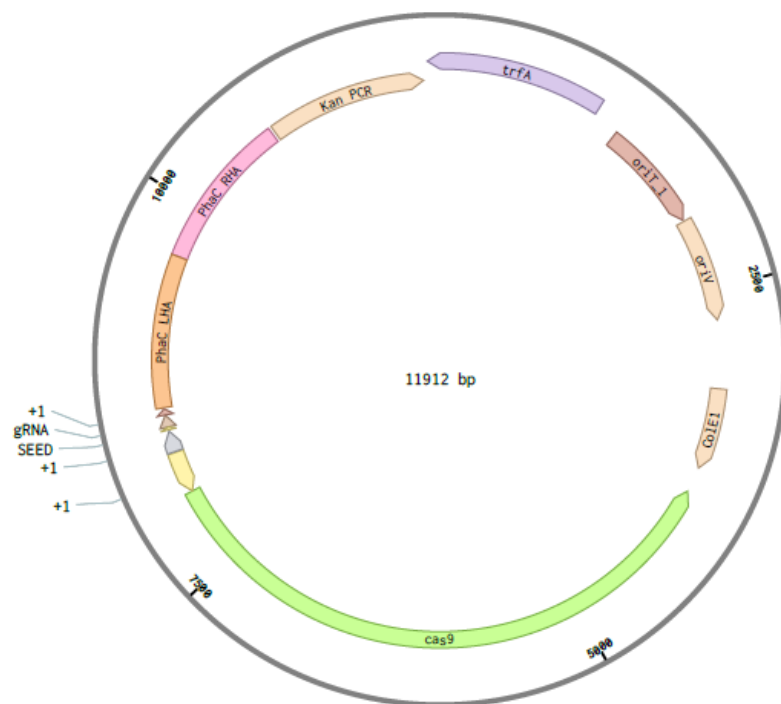


Figure 88: pMTL90882_Cas9For3 plasmid with promoters that work in methanotrophs and sgRNA for *phaC* in Isolate 12 and *M. parvus* (OB BP).

6.4.7.1 *pMTL90882_Cas9For1,pMTL90882_Cas9For2and pMTL90882_Cas9For3*

This set of plasmids were designed for poly-3-hydroxybutyrate (PHB) gene knockout in *M. parvus* (OBBP) and Isolate 12 as they both have identical gene target sequences. pCas9_Empty_+fdx-term plasmid obtained from the SBRC culture collection contained Cas9 nickase (Cas9n) gene with no guide RNA and homology arm. This was digested with SbfI and Ascl to remove the Cas9 module. The Cas9 module was ligated into pMTL90882 to give pMTL90882-cas9n. pMTL90882-Cas9n was digested for ligation with two synthetic Cas9 modules. The first module contained the first 215 base pairs of Cas9n fused to Pals promoter while the second module contained the first 215 base pairs of Cas9 fused to the Pals promoter, Phps promoter for the guide RNA, the *phaC* gRNA seed and a terminator. To attach these modules, Bstz171 and XbaI were used to digest pMTL90882-Cas9n for the first module. For the second module, pMTL90882-Cas9 was digested with Bstz171 and AsiSi. Ligation of digested pMTL90882-Cas9 was carried out with digested module 1 (Bstz171/XbaI) and module 2 (Bstz171/AsiSi). Transformation was carried out in *E. coli* XL-1 Blue. Module one became the first control which is Cas9n without gRNA and it was called Cas9For1. Module 2 became the second control which is Cas9 with gRNA and it was designated Cas9for2. To obtain Cas9 plasmid with sgRNA and HA(repair template) capable of gene knockout, Cas9For2 was digested with AsiSI and Ascl. The HA of the target gene were amplified and assembled by SOE-PCR, then ligated into Cas9For2. This resulted in Cas9For3.

6.4.7.2 *pMTL90882_Cas9For4,pMTL90882_Cas9For5 and pMTL90882_Cas9For6*

This set of plasmids are identical to the set described in 6.4.7.1 except they contain the *spCas9* instead of Cas9n. Additionally, the Phps promoter driving the sgRNA for the Cas9n set of plasmids was replaced with the Pmdh promoter. To clone these set of plasmids, genes synthesized by Twist Bioscience were amplified with TWIST_F/TWIST_R primers and digested with Bstz171 and XbaI. pMTL90882_Cas9For1 was digested with the same set of restriction enzymes as

the module and both fragments were subsequently ligated and transformed to give pMTL90882_Cas9For4. To clone pMTL90882_Cas9For5 and pMTL90882_Cas9For6, pMTL90882_Cas9For2 and pMTL90882_Cas9For3 were digested with the same Bstz171/PacI restriction enzymes. These digests were ligated with synthesized DNA from Twist Bioscience made of spCas9 modules and Isolate 12 *phaC* sgRNA.

6.4.7.3 pMTL90882_C4-12P6, pMTL90882_C5-12P6 and pMTL90882_12P6

These set of plasmids are identical to the set in 6.4.7.2 except that NEB HiFi was used to assemble the homology arms in pMTL90882_12P6 instead of SOE-PCR. This led to the loss of Ascl and AsiSi restriction sites flanking the homology arms.

6.4.7.4 pMTL90882_C4-12P11, pMTL90882_C5-12P11 and pMTL90882_12P11

This plasmid set is identical to the ones described in 6.4.7.3 except the promoters for Cas9 and sgRNA were interchanged. Therefore Cas9 was driven by Pmdh and sgRNA by Pals.

6.4.7.5 pMTL90882_C4-12P12, pMTL90882_C5-12P12 and pMTL90882_12P12

This plasmid set is identical to the one described in 6.4.7.3 except the relative promoter strength. Here Cas9 was given a weak promoter (Phik) while sgRNA was driven by a strong promoter (P3)

6.4.7.6 pMTL90882_C4-2.12P12, pMTL90882_C5-2.12P12 and pMTL90882_2.12P12

This plasmid set is identical to the one described in 6.4.7.5 except the difference in gRNA seed. In this plasmid set, the guide RNA seed is for the coding strand and not the template strand like in the plasmid sets listed.

6.4.7.7 pMTL90882_C5-12P11 and pMTL70E01_PhaC

The above plasmids were co-conjugated in a two plasmid system for delivery of CRISPR-Cas. pMTL70E01_PhaC had a pBBR1 origin of replication and HA of Isolate 12 *phaC* gene. It also carried an erythromycin resistant cassette while the remaining Cas9 module which includes the sgRNA was carried by pMTL90882_C5-12P11. During mating, both samples of *E. coli* S17-1 λ *pir* carrying either plasmid

were halved and mixed together to ensure a 1:1 ratio of *E. coli* and methanotrophs.

Another CRISPR experiments with Isolate 12 and *M. parvus* (OBBP) aimed to find the effect of DNA ligase IV inhibitor which has been shown to increase the efficiency of HR in eukaryotes. Although not often found in bacteria, Isolate 12 and *M. parvus* (OBBP) possess Ku protein and DNA ligase IV (LigD) genes which can carry out DNA strand break repair using NHEJ. To test the effect of Scr7 pyrazine on methanotrophs, 100 μ M of Scr7 was added to the mating mixture of *E. coli* S17-1 λ *pir* carrying pMTL90882_12P11 and the two methanotrophs mentioned above. Everything else was unchanged. It is reemphasized here that Isolate 12 and *M. parvus* (OBBP) have the same DNA sequence for *phaC*, *ligD* and the homology arms of these genes

6.4.8 Plasmid sets for Isolate 3*

6.4.8.1 pMTL90882_C4-3P6, pMTL90882_C5-3P6 and pMTL90882_3P6

These set of plasmids were designed to knockout the *phaC* gene in Isolate 3* so they carry the guide RNA for *phaC* gene. A moderately strong promoter (Pals) drives the Cas9 gene while a weak promoter (Pmdh) drives the sgRNA. The plasmids were derived from the set in 6.4.7.32 by substituting the sgRNAs and HA. This was done for all subsequent plasmids described.

6.4.8.2 pMTL90882_C4-3P11, pMTL90882_C5-3P11 and pMTL90882_3P11

The plasmid sets here are similar to those in 6.4.8.1 except the promoter of Cas was interchanges with that of the sgRNA. Therefore Cas9 was driven by Pmdh while and sgRNA by Pals.

6.4.9 Plasmid sets for Isolate 10

6.4.9.1 pMTL90882_C4-10E6, pMTL90882_C5-10E6 and pMTL90882_10E6

This plasmid set was designed to knock out the gene for 2-oxoglutarate dependent ethylene-forming enzyme (*efe*) in Isolate 10 which is a *Methylocaldum* species and a type I methanotroph unlike the others which are type II methanotrophs. The sgRNA used was synthesized with forward primer EFE_sgRNA1. A weak promoter

(Pals) in Isolate 10 drives the Cas9 gene while a very weak promoter (Pmdh) drives the sgRNA. It should be noted that the promoter expression in Isolate 10 differs from their expression in other *Methylocystis* species tested.

6.4.9.2 *pMTL90882_C4-10E11, pMTL90882_C5-10E11 and pMTL90882_10E11*

The plasmid set here is identical to that in 6.4.9.1 except the promoter of Cas was interchanged with that of the sgRNA. Therefore Cas9 was driven by Pmdh while and sgRNA by Pals.

6.4.9.3 *pMTL90882_C4-10E12, pMTL90882_C5-10E12 and pMTL90882_10E12*

The plasmid set here is identical to that in 6.4.9.1 except the promoter of Cas9 and sgRNA were changed. Cas9 was driven by a very weak promoter (Phik) while sgRNA was driven by a very strong promoter (P3).

6.4.10 Conjugation of CRISPR plasmids into methanotrophs

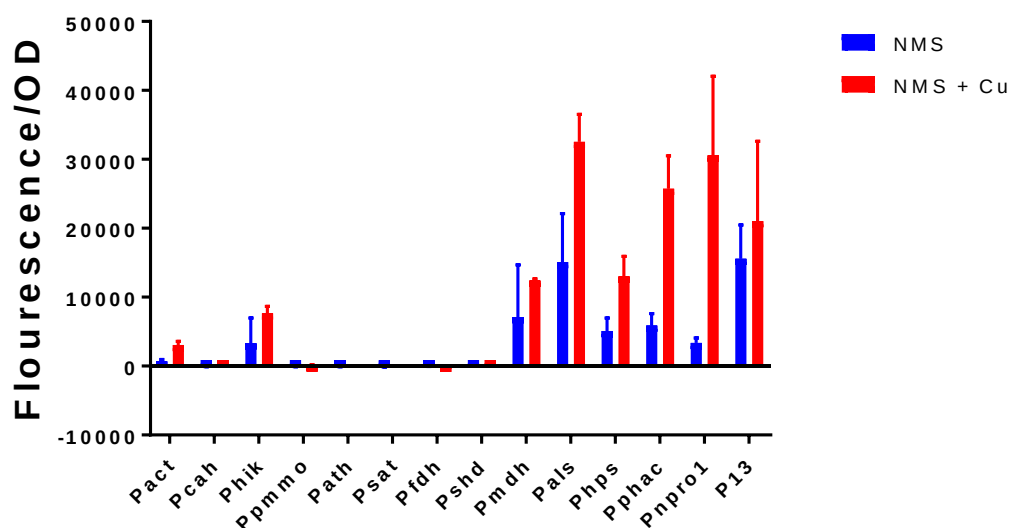
All CRISPR-Cas plasmids were cloned into *E. coli* XL-1 Blue. After growing liquid cultures of *E. coli* XL-1 Blue, the respective plasmids were extracted using miniprep and transformed into *E. coli* S17-1 λ pir which was grown overnight and used for conjugation with methanotrophs. Conjugation was carried out to determine the conjugation efficiency of each plasmid. Mating was carried out for two days and dilutions of mating spots were pipetted on LB kanamycin 50 μ g/mL and NMS Kanamycin 50 μ g/mL and Nalidixic acid 25 μ g/mL. About 850 μ L of the neat suspension of the mating spot was resuspended in 100 μ L and spread on NMS Kanamycin 50 μ g/mL and Nalidixic acid 25 μ g/mL plates for additional screening of knockouts. The mating suspension of the two-plasmid delivery system (pMTL90882_C5-12P11 + pMTL70E01_PhaC) was plated on NMS Kanamycin 50 μ g/mL, Erythromycin 50 μ g/mL and Nalidixic acid 25 μ g/mL. Samples were flushed with CH₄ gas and incubated at 30°C (or 37°C for isolate 10) for three weeks. LB plates were incubated at 37°C overnight. Afterwards, colonies were counted and conjugation efficiency of each plasmid was determined. Images of plates were also taken with the Biorad Molecular Imager GelDoc XR+ Imaging System to visualise the efficiency of conjugation. Colonies were screened for knockouts using primers that bind outside the target gene HA in Table 45 and Table 46.

6.5 Results

6.5.1 Finding promoter candidates for CRISPR-Cas9 plasmid construction

The principal aim of this chapter was to implement CRISPR-Cas9 genome editing in methanotrophs. Emphasis was laid on investigating the effect of Cas9 and guide RNA promoter strength on the efficiency of Cas9 gene knockout. However a lot of the strains tested were isolates from this study which have not been characterised earlier. Therefore it became pertinent to determine which promoters worked in different species and quantify their expression.

Firstly, promoters were tested on a type strain of methanotroph before testing on isolates chosen for CRISPR cas9 gene knockouts. Expression of promoters in *M. parvus* (OBBP) grown in NMS and NMS + Cu was tested. Pact, Pcah, Pmdh, Pals, Phps, Pphac, Pnpro1 and P13 were expressed with P13 being the most highly expressed in *M. parvus* (OBBP) grown with NMS and Pals in *M. parvus* (OBBP) grown in NMS + Cu. Pact was the least expressed promoter in *M. parvus* grown in both media. Comparing the relative strength of promoters in *M. parvus* (OBBP) grown with NMS and NMS + Cu, results showed that Pals, Phps, Pphac and Pnpro1 were significantly different in levels of expression in favour of *M. parvus* (OBBP) grown in NMS + Cu with P values 0.0204, 0.0025, 0.0147 and 0.0165 respectively using unpaired t-test (Figure 89).



Promoters

Figure 89: Comparison of promoter expression in *M. parvus* (OBBP) grown in NMS and NMS + Cu. Fluorescence/OD of Pals, Phps, Pphac and Pnpro1 was significantly when *M. parvus* (OBBP) was grown in NMS + Cu with P values 0.0204, 0.0025, 0.0147 and 0.0165 respectively using unpaired t-test.

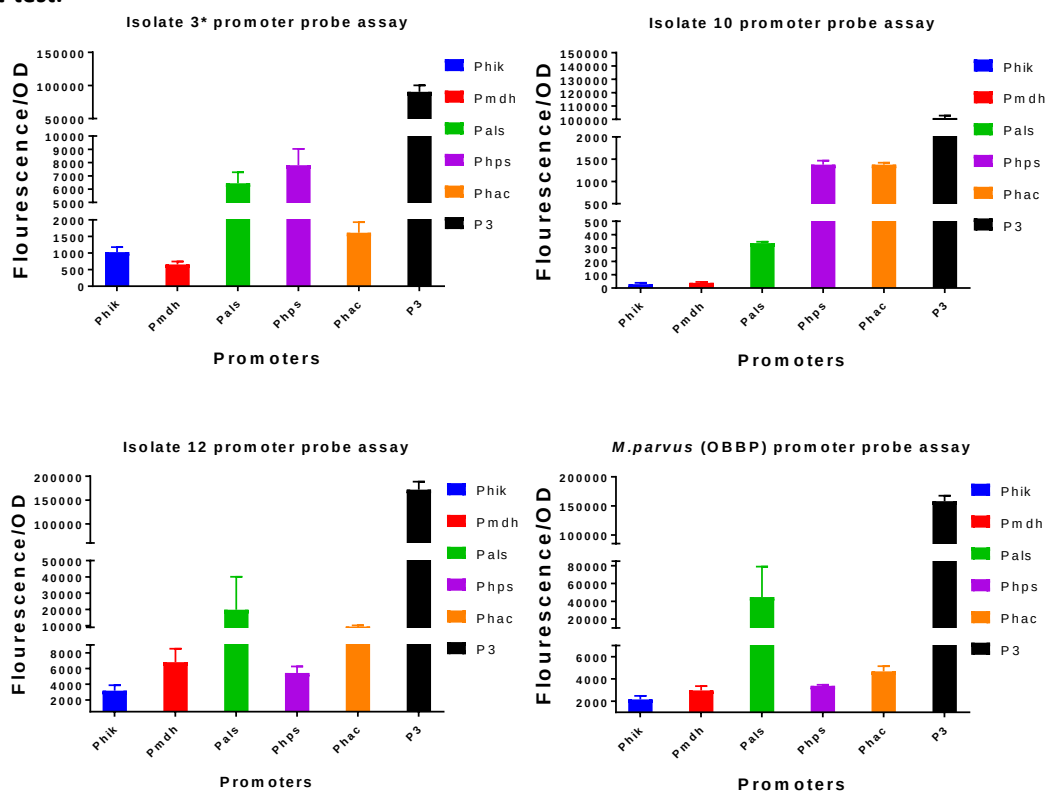


Figure 90: Promoter expression in Isolate, 3*, 10, 12 and *M. parvus* (OBBP) measured by Fluorescence/OD. P3 was the strongest promoter in all isolates while PhiK was the weakest.

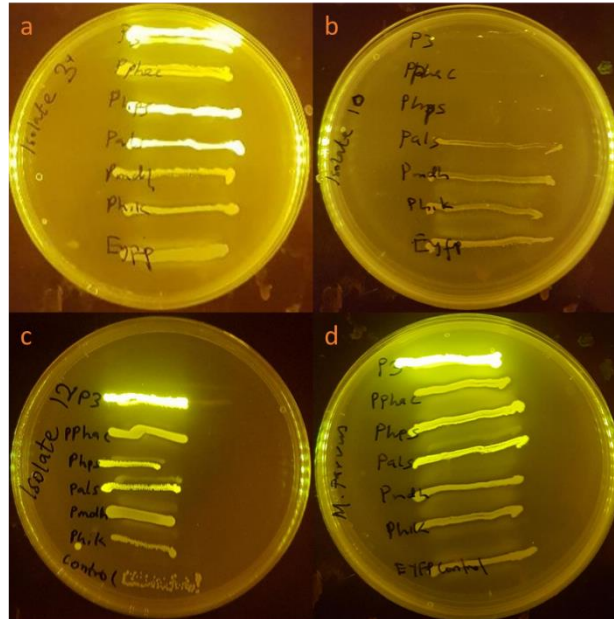


Figure 91: Visual observation of fluorescence in Isolate 3*(a), 10 (b), 12 (c) and *M. parvus* (OBBP) (d) on NMS BA plates under blue light. From top to bottom on each plate: P3, Pphac, Phps, Pals, Phik and EYFP control.

To visually observe the fluorescence of Isolates on agar plates, streaks were made on NMS agar plates and viewed under blue light (470 nm) using Syngene blue light illuminator. Figure 91 showed that P3 fluoresced higher than all other promoters. Pals and Phps fluorescence can also be observed although this was obscured by P3. For Isolate 10, P3, Phps and Pphac streak did not grow well and will have to be repeated.

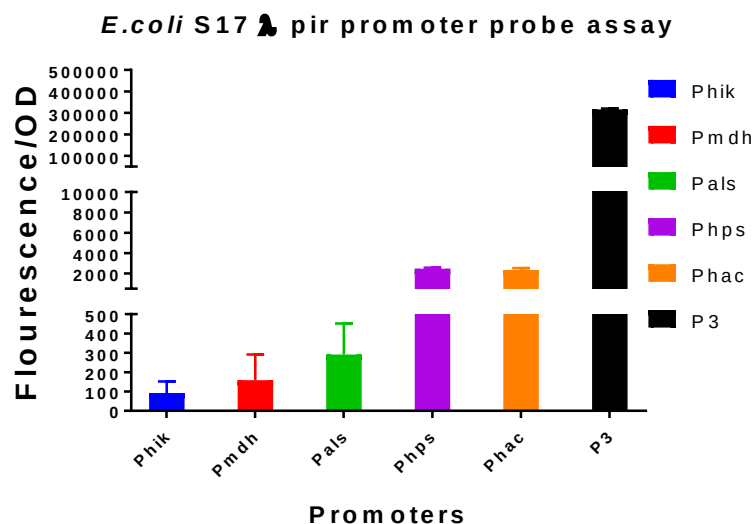


Figure 92: Promoter expression in *E. coli* S17 λ pir. P3 was the most expressed promoter while Phik was the least.

In addition to P3 which was not tested earlier, five promoters (Phik, Pals, Pmdh, Phphs and Pphac) shown to be expressed in *M. parvus* (OBBP) were tested in four other organisms (Figure 90) and *E. coli* S17 λ pir (Figure 92). P3 was found to be the strongest promoter in all the methanotrophs tested. The highest expression of P3 was in Isolate 12 (171883) while the lowest expression was seen in Isolate 3* (90476). Pmdh which was expressed at very low levels in *E. coli* S17 λ pir showed more than 100 fold expression in *M. parvus* (OBBP).

6.5.2 Conjugation of methanotrophs with CRISPR plasmids

pMTL90882_Cas9For1*, *pMTL90882_Cas9For2* and *pMTL90882_Cas9For3

After testing, selected promoters were used for designing CRISPR-Cas9 plasmid. In the first set of plasmids, Cas9 nickase was used (nCas9). nCas9 differs from native *Streptococcus pyogenes* Cas9 because it has a mutation that limits its nuclease activity to single strand breaks. The target in this experiment was the gene responsible for PHB accumulation (*phaC*).

pMTL90882_Cas9For1, the control plasmid carrying Cas9 nickase (nCas9) but without guide RNA and HA was transformed into Isolate 12. No nCas9 activity was expected due to the absence of guide RNA which finds the target gene thereby initiating nCas9 activity. The efficiency of conjugation was 1.69×10^{-1} . This was significantly higher than the conjugation efficiency of *Cas9For3* (2.96×10^{-5}) which is the test plasmid carrying nCas9, guide RNA and homology arms (HA) (Figure 93a). P value obtained was <0.0001 . The nCas9 and guide RNA in this plasmid were under the transcriptional control of *Pals* and *Phps* respectively. After screening 12 colonies, no nCas9 knockout was obtained as shown in Figure 93b. Except the gel image below where Bioline 1 kb ladder was used, NEB 2 log ladder was used in all subsequent gel images.

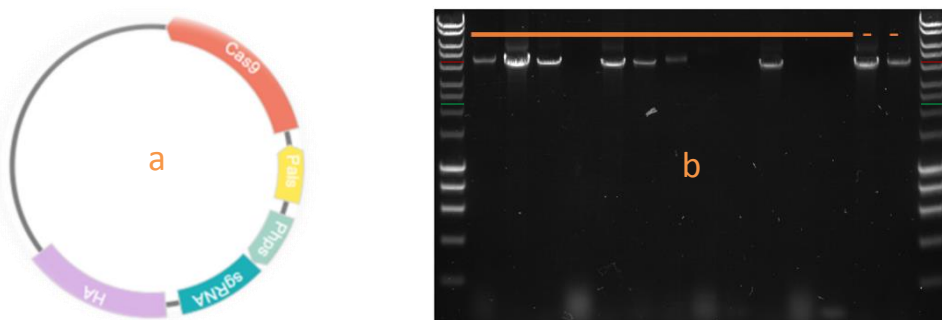


Figure 93: Screen for *phaC* knockouts in Isolate 12 after transforming with *Cas9For3* (a). Isolate 12 KOs were expected to have band size of 2324 bp (green streak) instead of 4208 bp of wild type shown by red streak on DNA ladder (b). The orange bar represents 12 colonies screened. The negative signs are wild type Isolate 12 colonies used as negative controls. Bioline 1 kb ladder was used in the gel image.

pMTL90882_Cas9For4*, *pMTL90882_Cas9For5* and *pMTL90882_Cas9For6

In this experiment, nCas9 was substituted for native *Streptococcus pyogenese* Cas9 which should carry out DSB. *pMTL90882_Cas9For4*, the control plasmid carrying Cas9 but without guide RNA and homology was transformed into Isolate 12. Here

also, no Cas9 activity was expected due to the absence of guide RNA which finds the target gene. The efficiency of conjugation was 1.65×10^{-1} . This was significantly higher than the conjugation efficiency of Cas9For6 (6.8×10^{-5}) which is the test plasmid carrying Cas9, guide RNA and HA (Figure 94a). P value obtained was <0.0001 . The Cas9 and guide RNA in this plasmid were under the transcriptional control of Pals and Phps respectively. After screening 40 colonies, no Cas9 knockout was obtained as shown in Figure 94b.

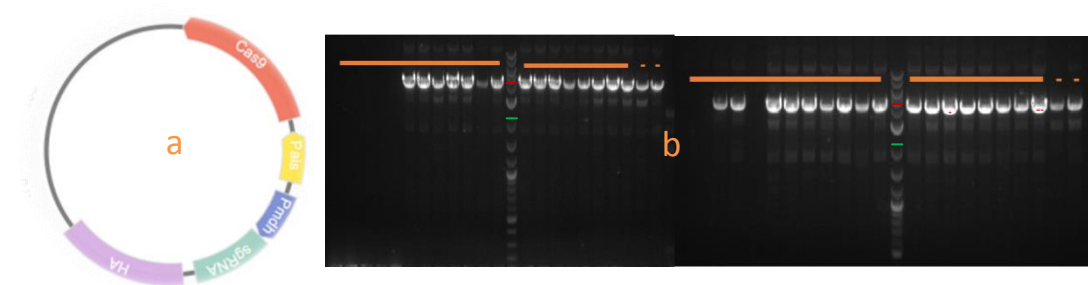


Figure 94: Screen for *phaC* knockouts in Isolate 12 after transforming with Cas9For6 (a). Isolate 12 KOs were expected to have band size of 2324 bp (green streak) instead of 4208 bp of wild type shown by red streak on DNA ladder (b). The orange bar represents 40 colonies screened. The negative signs are wild type Isolate 12 colonies used as negative controls.

The above were the first set of CRISPR-Cas9 knockout experiments carried out in this study. The next set of experiments involved similar plasmids to Cas9for6. However, this phase involved using different sets of promoters for Cas9 and guide RNA. In addition to Isolate 12, three new species were used (Isolate 3*, 10 and *M. parvus* (OBBP). Native *Streptococcus pyogenes* Cas9 was used throughout subsequent experiments.

6.5.3 Isolate 3* CRISPR-Cas9 plasmid conjugation

pMTL90882_C4-3P6, pMTL90882_C5-3P6 and pMTL90882_3P6

pMTL90882_C4-3P6, the control plasmid carrying Cas9 but without guide RNA and HA was transformed into Isolate 3*. The conjugation efficiency of this plasmid was expected to be relatively high as no Cas9 activity was expected due to the absence of guide RNA on the plasmid. The efficiency of conjugation was 1.2×10^{-2} . This was significantly higher than the conjugation efficiency of *pMTL90882_C5-3P6* (1.25×10^{-5}) which is another control plasmid carrying only Cas9 and guide RNA (P <0.0001). *pMTL90882_C5-3P6* was included in the experiment to highlight the

conjugation efficiency obtained in the absence of repair template (HA) after DNA cleavage by Cas9. This control demonstrates the activity of guide RNA. The conjugation efficiency of pMTL90882_C4-3P6 was also compared to pMTL90882_3P6 (test plasmid) (Figure 95a). The Cas9 and guide RNA of pMTL90882_3P6 were under the transcriptional control of Pals and Pmdh respectively. pMTL90882_C4-3P6 was significantly higher than pMTL90882_3P6 (1.67×10^{-5}) with $P < 0.0001$ (Figure 96). The significant difference and the low conjugation efficiency suggests Cas9 carried out double strand break. After screening 20 colonies of pMTL90882_3P6 conjugation, no Cas9 knockout was obtained as shown in Figure 97a.

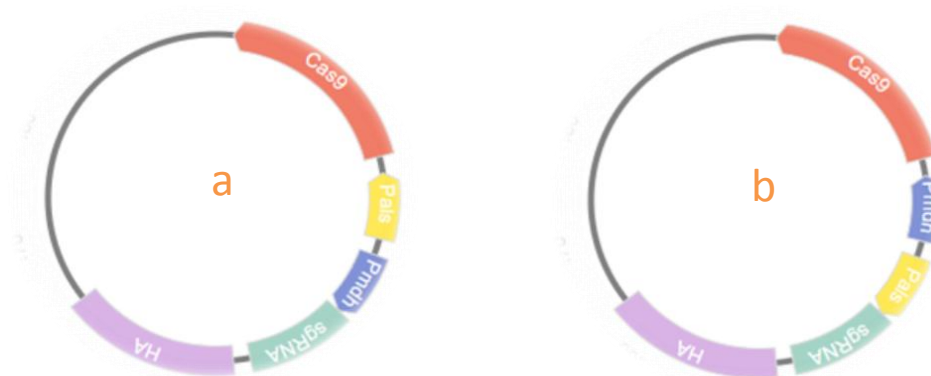


Figure 95: Plasmid design of pMTL90882_3P6 (a) and pMTL90882_3P11 (b) used for CRISPR_Cas9 genome editing of Isolate 3*. With the exception of Cas9 and guide RNA promoters, both plasmids are identical.

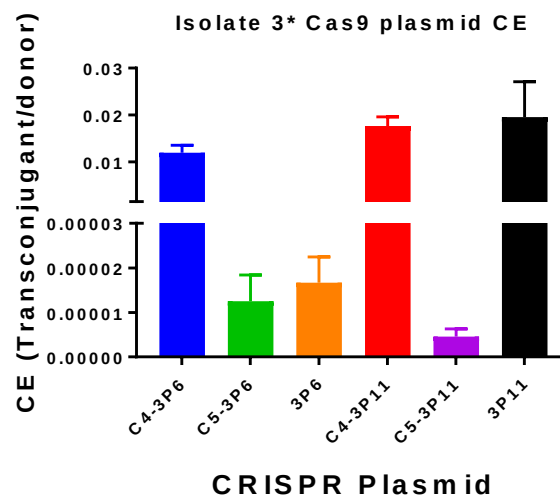


Figure 96: Conjugation efficiency of CRISPR-Cas9 plasmids used for Isolate 3* gene knockout. Unpaired t test was used to compare the differences between control plasmids and test plasmid. There was significant difference between pMTL90882_C4-3P6 and pMTL90882-3P6 ($P < 0.0001$). There was no significant difference between pMTL90882_C4-3P11 and pMTL90882_3P11 ($P = 0.6386$).

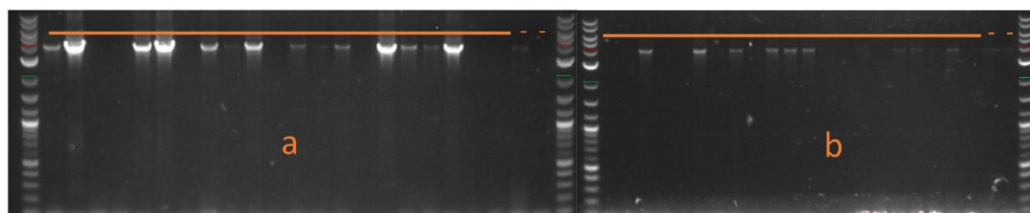


Figure 97: Screen for *PhaC* knockouts in Isolate 3* after transforming with pMTL90882_3P6 (a) and pMTL90882_3P11 (b). Isolate 3* KOs were expected to have band size of 2070 bp (green streak) instead of 3954 bp of wild type shown by red streak on DNA ladder (b). The orange bar represents 20 colonies screened for each plasmid. The negative signs are wild type Isolate 3* colonies used as negative controls.

It is likely the cells that survived escaped Cas9 activity due to a mutation on *phaC* gene homologous to the guide RNA seed.

pMTL90882_C4-3P11, pMTL90882_C5-3P11 and pMTL90882_3P11

Isolate 3* was conjugated with another set of plasmids which only differed in the promoters used to transcribe Cas9 and guide RNA. Pmdh and Pals used in the first set of plasmids were switched; this time around Pmdh was used to transcribe Cas9 while Pals was used for guide RNA transcription (Figure 95b). The conjugation efficiency of pMTL90882_C4-3P11 was 1.76×10^{-2} . This was significantly higher than the conjugation efficiency of pMTL90882_C5-3P11 (4.54×10^{-6}) which was the control plasmid carrying only Cas9 and guide RNA ($P < 0.0001$). The conjugation efficiency of pMTL90882_C4-3P11 was also compared to pMTL90882_3P11 (test plasmid). There was no significant difference between pMTL90882_C4-3P11 and pMTL90882_3P11 (1.96×10^{-2}) with $P = 0.6386$ (Figure 96). This suggested that Cas9 double strand break did not occur in pMTL90882_3P11. Several reasons could have caused this and will be detailed in the discussion but one possibility is that the plasmid mutated either during cloning in *E. coli* or after transfer into Isolate 3* thereby compromising the nuclease activity of Cas9. After screening 20 colonies of pMTL90882_3P11 conjugation, no Cas9 knockout was obtained as shown in Figure 97b.

6.5.4 Isolate 10 plasmid

pMTL90882_C4-10E11, pMTL90882_C5-10E11 and pMTL90882_10E11

The conjugation efficiency of Isolate 10 with Cas9 plasmids was low and no colonies were obtained when the experiment was conducted with the protocol used for Isolate 3* and Isolate 12 was applied. After modifying the protocol which involved spreading all the mating plate resuspension on antibiotic plates, 106, 246 and 16 colonies were obtained for plasmid pMTL90882_C4-10E11, pMTL90882_C5-10E11 and pMTL90882_10E11. 108, 29 and 11 colonies were obtained for plasmids pMTL90882_C4-10E12, pMTL90882_C5-10E12 and pMTL90882_10E12 respectively (Figure 98a and b). These colonies were screened for *efe* knockouts (Figure 99). No PCR product of the transconjugants screened gave band at 2155 bp which was the *efe* knockout PCR product was expected when run on agarose gel. Instead bands were obtained near 3177 bp which was the expected wild type band size. Other non-specific bands were also present. DNA extraction was carried out on the first three bands of pMTL90882_10E11 and pMTL90882_10E12 which were close to the expected knockout size. Sanger sequencing of these DNA products did not yield quality sequences which suggest they bands were from non-specific DNA binding.

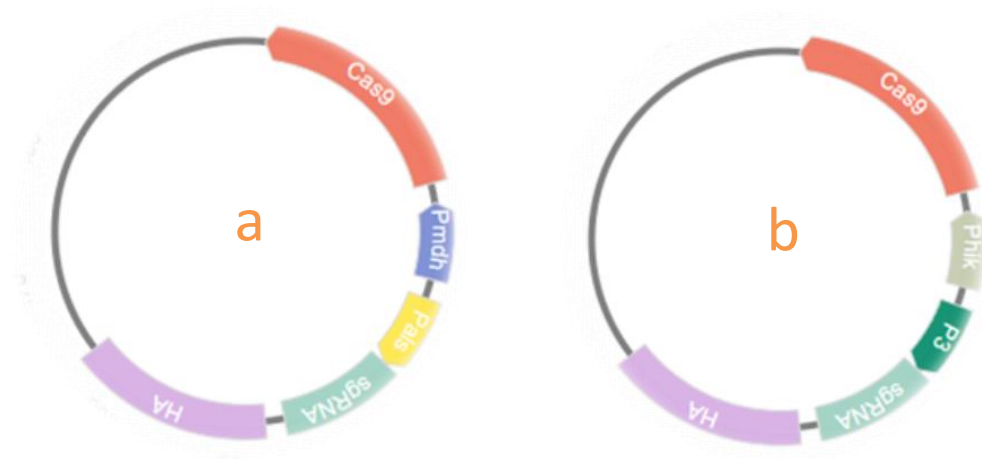


Figure 98: Plasmids pMTL90882_10E11 with Pmdh and Pals promoter (a), and pMTL90882_10E12 with Phik and P3 promoters (b) used for CRISPR-Cas9 knockout in Isolate 10.

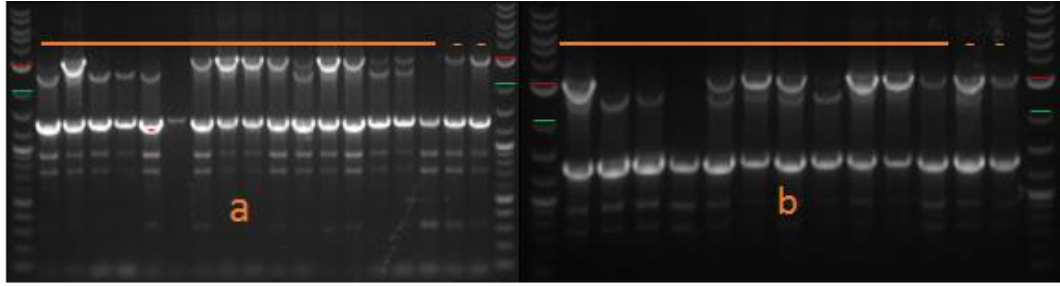


Figure 99: Screen for *ege* knockouts in Isolate 10 after transforming with pMTL90882_10E11 (a) and pMTL90882_10E12 (b). Isolate 10 KOs were expected to have band size of 2155 bp (green streak) instead of 3177 bp of wild type shown by red streak on DNA ladder (b). The orange bar represents 16 and 11 colonies screened for pMTL90882_10E11 (a) and pMTL90882_10E12 (b) respectively. The negative signs are wild type Isolate 10 colonies used as negative controls.

6.5.5 *M. parvus* plasmid (P6 and P11)

pMTL90882_C4-12P6, pMTL90882_C5-12P6 and pMTL90882_12P6

pMTL90882_C4-12P6, the control plasmid carrying Cas9 but without guide RNA and HAs was transformed into *M. parvus* (OB BP). This plasmid design was similar to the one used for Isolate 3* which is essentially Cas9 with Pmdh promoter. Just as suspected in Isolate 3*, the conjugation efficiency of this plasmid in *M. parvus* (OB BP) was expected to be relatively high as no Cas9 activity was expected due to the absence of guide RNA on the plasmid. The efficiency of conjugation was 2.6×10^{-3} . This was significantly higher than the conjugation efficiency of pMTL90882_C5-12P6 (7.25×10^{-7}) which has only Cas9 and guide RNA but no HAs ($P < 0.0001$). pMTL90882_C5-12P6 was included in the experiment to investigate the conjugation efficiency obtained in the absence of repair template (HA) after DNA cleavage by Cas9. The conjugation efficiency of pMTL90882_C4-12P6 was also compared to pMTL90882_12P6 (Figure 100a). The Cas9 and guide RNA of pMTL90882_12P6 were under the transcriptional control of Pals and Pmdh respectively. pMTL90882_C4-12P6 conjugation efficiency was significantly lower than pMTL90882_12P6 (1.77×10^{-2}) with $P < 0.0001$ (Figure 101). The CE was expected to be lower if Cas9 carried out DSB. After screening 10 colonies of pMTL90882_12P6 conjugation, no Cas9 knockout was obtained as shown in Figure 102a.

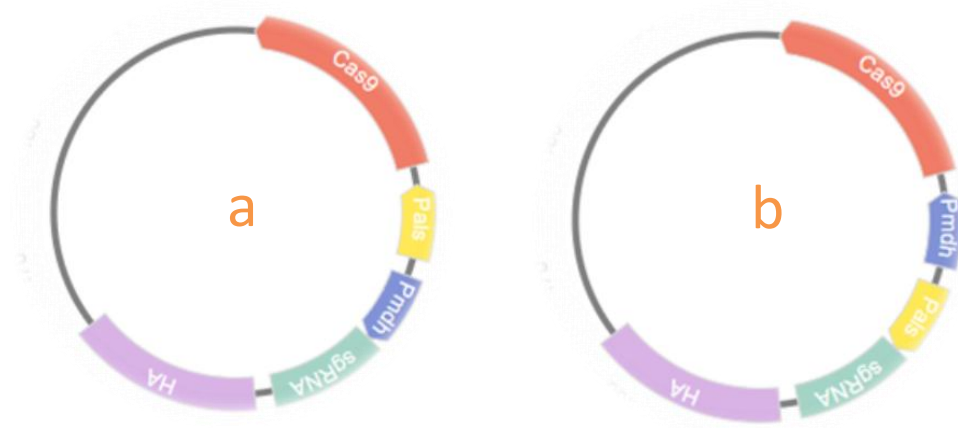


Figure 100: Plasmid design of pMTL90882_12P6 (a) and pMTL90882_12P11 (b) used for CRISPR_Cas9 genome editing of *M. parvus* (OBBP). With the exception of Cas9 and guide RNA promoters, both plasmids are identical.

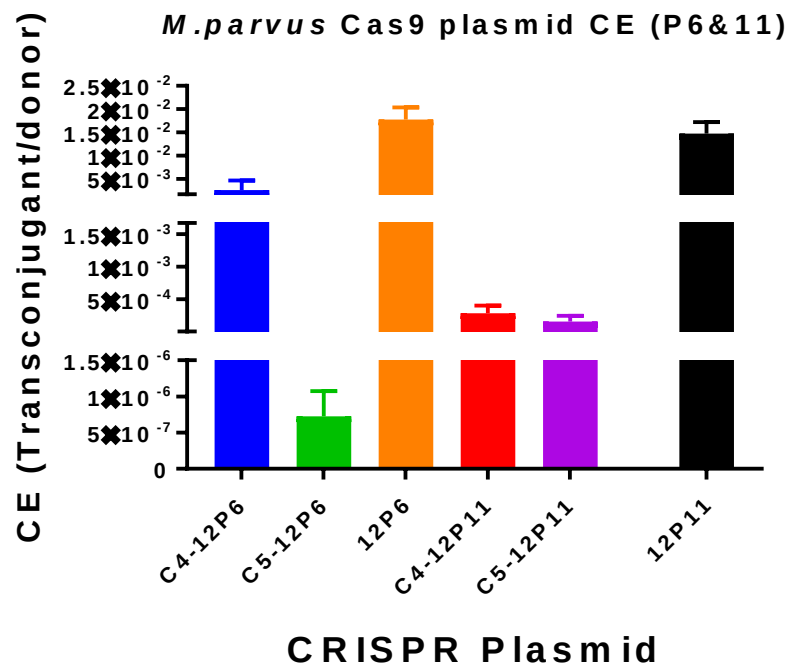


Figure 101: Conjugation efficiency of CRISPR-Cas9 plasmids used for *M. parvus* (OBBP) *phaC* gene knockout. Unpaired t test was used to compare the differences between control plasmids and test plasmid. There was significant difference between pMTL90882_C4-12P6 and pMTL90882_12P6 ($P < 0.0001$). The conjugation of efficiency of pMTL90882_C4-12P11 and pMTL90882_12P11 was also significantly different ($P < 0.0001$).

pMTL90882_C4-12P11, pMTL90882_C5-12P11 and pMTL90882_12P11

M. parvus (OBBP) was conjugated with another set of plasmids which only differed in the promoters used to transcribe Cas9 and guide RNA from the previous *M. parvus* (OBBP) plasmids used. Pmdh and Pals used to transcribe Cas9 and guide RNA in the first set of plasmids were switched; this time around Pmdh was used to transcribe Cas9 while Pals was used for guide RNA transcription (Figure 100b). The

conjugation efficiency of pMTL90882_C4-12P11 was 2.81×10^{-4} . There was no significant difference in conjugation efficiency when compared to pMTL90882_C5-12P11 (1.54×10^{-5}) which was the control plasmid carrying only Cas9 and guide RNA ($P = 0.1415$). This suggested the inactivity of Cas9 in pMTL90882_C5-12P11. The conjugation efficiency of pMTL90882_C4-12P611 was also compared to pMTL90882_12P11. pMTL90882_C4-12P11 conjugation efficiency was significantly lower than pMTL90882_12P11 (1.96×10^{-2}) with $P < 0.0001$ (Figure 101). This also suggested Cas9 double strand break did not occur in pMTL90882_12P11. As mentioned earlier this could be due to plasmid mutation thereby compromising the nuclease activity of Cas9. After screening 10 colonies of pMTL90882_12P11 conjugation, no Cas9 knockout was obtained as shown Figure 102b

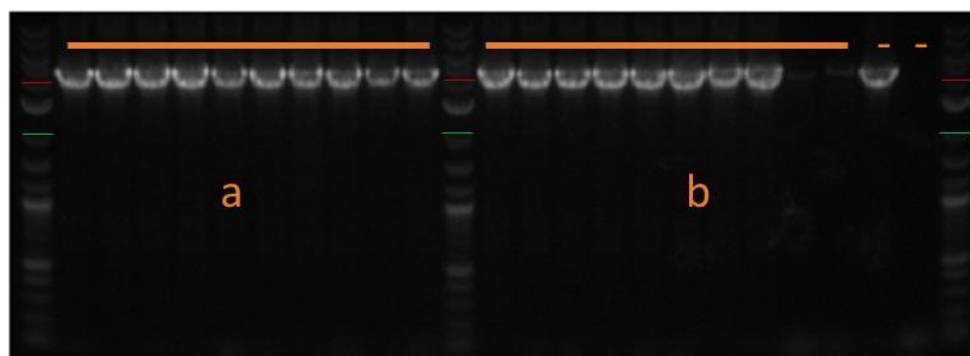


Figure 102: Screen for *phaC* knockouts in *M. parvus* (OBBP) after transforming with pMTL90882_12P6 (a) and pMTL90882_12P11 (b). *M. parvus* (OBBP) KOs were expected to have band size of 2324 bp (green streak) instead of 4208 bp of wild type shown by red streak on DNA ladder. The orange bar represents 10 colonies screened for each plasmid. The negative signs are wild type *M. parvus* (OBBP) colonies used as negative controls.

pMTL90882_C4-12P12, pMTL90882_C5-12P12 and pMTL90882_12P12

pMTL90882_C4-12P12, the control plasmid carrying Cas9 but without guide RNA and HAs was transformed into *M. parvus* (OBBP). This plasmid design was similar to pMTL90882_C4-12P6 except it has a different promoter for Cas9. Phik one of the weaker promoters tested was used for transcription of Cas9. The conjugation efficiency of this plasmid in *M. parvus* (OBBP) was expected to be relatively high as no Cas9 activity was expected due to the absence of guide RNA on the plasmid. The efficiency of conjugation was 1.66×10^{-4} . There was no significant difference in conjugation efficiency when compared to pMTL90882_C5-12P12 (2.7×10^{-3}) which

has only Cas9 and guide RNA but no HAs ($P = 0.2033$). The conjugation efficiency of pMTL90882_C4-12P12 was also compared with pMTL90882_12P12 (Figure 103a). The Cas9 and guide RNA of pMTL90882_12P12 were under the transcriptional control of Phik and P3 respectively. pMTL90882_C4-12P12 conjugation efficiency was significantly lower than pMTL90882_12P12 (1.77×10^{-2}) with $P < 0.0001$ (Figure 104). The significant difference suggests Cas9 did not generate double strand break. After screening 10 colonies of pMTL90882_12P12 conjugation, no Cas9 knockout was obtained as shown in (Figure 105a).



Figure 103: Plasmids pMTL90882_12P12 with Phik and P3 promoter (a), and pMTL90882_2.12P12 with Phik and P3 promoters, and in addition a different guide RNA (b) used for CRISPR-Cas9 knockout in *M. parvus* (OBBP).

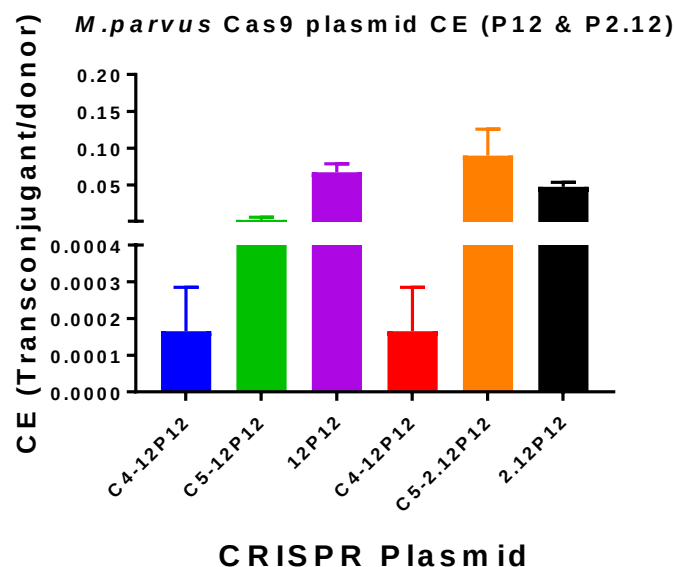


Figure 104: Conjugation efficiency of CRISPR-Cas9 plasmids used for *M. parvus* (OBBP) gene knockout. Unpaired t test was used to compare the differences between control plasmids and test plasmid. There was significant difference between pMTL90882_C4-12P12 and pMTL90882_12P12 ($P < 0.0001$) conjugation efficiency. The conjugation efficiency between pMTL90882_C4-2.12P12 and pMTL90882_2.12P12 was also significantly different ($P < 0.0001$).

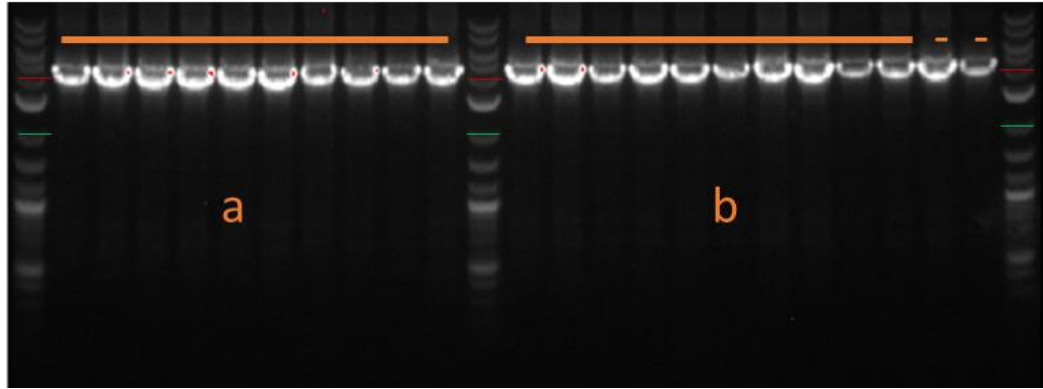


Figure 105: Screen for *phaC* knockouts in *M. parvus* (OB BP) after transforming with pMTL90882_12P12 (a) and pMTL90882_2.12P12 (b). *M. parvus* (OB BP) KOs were expected to have band size of 2324 bp (green streak) instead of 4208 bp of wild type shown by red streak on DNA ladder. The orange bar represents 10 colonies screened for each plasmid. The negative signs are wild type *M. parvus* (OB BP) colonies used as negative controls.

pMTL90882_C4-2.12P12, pMTL90882_C5-2.12P12 and pMTL90882_2.12P12

M. parvus (OB BP) was conjugated with another set of plasmids which had a different guide RNA orientation. Whereas the guide RNA in pMTL90882_C5-12P12 and pMTL90882_12P12 and all other plasmids tested were in 5-3 orientation (template strand), this guide RNA was in 3-5 orientation (non-template/coding strand) (Figure 103b). The conjugation efficiency of pMTL90882_C4-12P12 was 1.66×10^{-4} . There was significant difference in conjugation efficiency when compared to pMTL90882_C5-2.12P12 (9×10^{-2}) which was the control plasmid carrying only Cas9 and guide RNA ($P = 0.0024$). This suggested the inactivity of Cas9 in pMTL90882_C5-2.12P12. The conjugation efficiency of pMTL90882_C4-12P12 was also compared to pMTL90882_2.12P12. pMTL90882_C4-12P12 conjugation efficiency was significantly lower than pMTL90882_2.12P12 (4.75×10^{-2}) with $P < 0.0001$ (Figure 104). This also suggested Cas9 double strand break did not occur in pMTL90882_2.12P12. After screening 10 colonies of pMTL90882_2.12P12 conjugation, no Cas9 knockout was obtained as shown in Figure 105b.

pMTL90882-C512P11 + pMTL70E01_phaC

CRISPR-Cas9 conjugations carried out in this study involved the use of one plasmid systems. This section provides results obtained for two plasmid system conjugation where Cas9 and guide RNA were carried by one plasmid and HAs by a

second plasmid (Figure 106). This was hypothesised to increase the efficiency of homologous recombination thereby enhancing CRISPR-Cas9 gene knockout. The efficiency of conjugation obtained from pMTL90882_C5-12P11 + pMTL70E01_PhaC conjugation in *M. parvus* (OBBP) was 8.1×10^{-5} . Colonies were screened from agar plates but no *phaC* knockout was obtained (Figure 107).

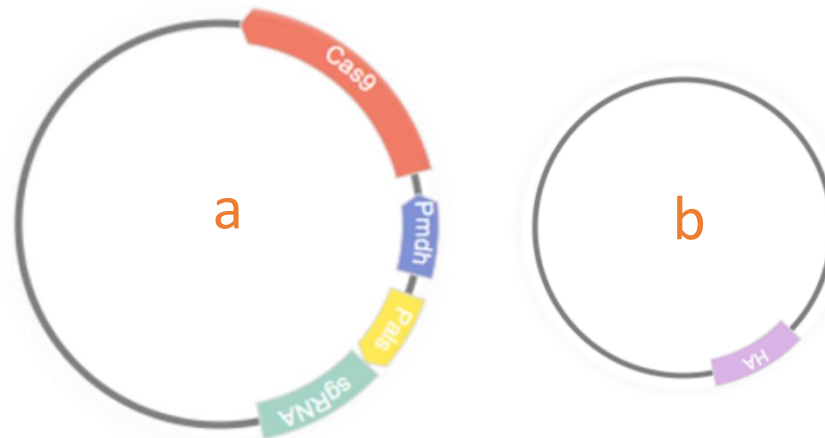


Figure 106: Plasmids pMTL90882_C5-12P11 with ColEI replicon, Cas9 and guide RNA but no homology arms (a), and pMTL71E01-Phac with pBBR1 replicon, erythromycin resistance gene and *phaC* homology arms (b) used for CRISPR-Cas9 knockout in *M. parvus* (OBBP).

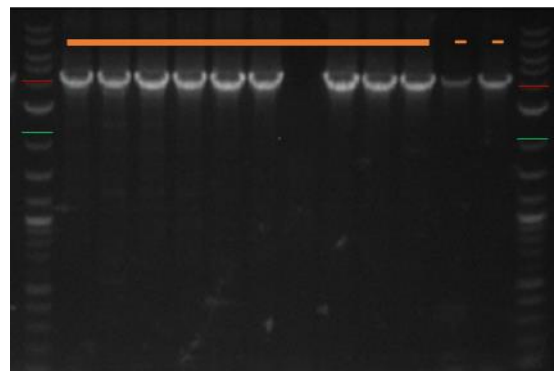


Figure 107: Screen for *phaC* knockouts in *M. parvus* (OBBP) after transforming with pMTL90882_C5-12P11 and pMTL_71E01-Phac. *M. parvus* (OBBP) KOs were expected to have band size of 2324 bp (green streak) instead of 4208 bp of wild type shown by red streak on DNA ladder. The orange bar represents 10 colonies screened. The negative signs are wild type *M. parvus* (OBBP) colonies used as negative controls.

pMTL90882_12P11 + Scr7

Plasmid pMTL90882_12P11 previously tested was conjugated in *M. parvus* (OBBP) in the presence of 100 μ M Scr7 pyrazine. Scr7 was reported to inhibit DNA ligase, consequently diminishing the ability of organisms to carry out double strand break

repair through NHEJ. This resulted in increased efficiency of homologous recombination [183, 184]. The conjugation efficiency obtained from this experiment was relatively low (1.86×10^{-6}) which was a good sign showing Cas9 DSB occurred, although after screening 10 colonies, no *phaC* knockout was obtained (Figure 108).

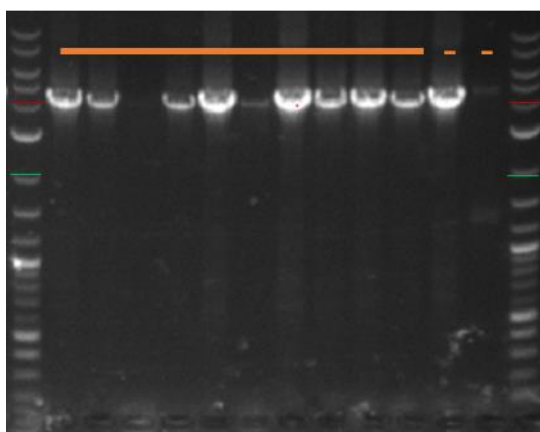


Figure 108: Screen for *phaC* knockouts in *M. parvus* (OBBP) after transforming with pMTL90882_12P11 in the presence of 100 μ M Scr7. *M. parvus* (OBBP) KOs were expected to have band size of 2324 bp (green streak) instead of 4208 bp of wild type shown by red streak on DNA ladder. The orange bar represents 10 colonies screened. The negative signs are wild type *M. parvus* (OBBP) colonies used as negative controls.

pMTL90882_C5-12L11 and pMTL90882_12L11

This was another strategy adopted to diminish the NHEJ genome repair ability of *M. parvus* (OBBP) which involves targeting *ligD* which is the ATP-dependent DNA Ligase gene thereby inhibiting NHEJ and increasing the efficiency of homologous recombination. Therefore this set of plasmids carry guide RNA for *ligD* and in the case of pMTL90882_12L11, *ligD* left and right homology arms. The efficiency of conjugation of pMTL90882_C5-12L11 and pMTL90882_12L11 were 2.1×10^{-5} and 1.44×10^{-5} respectively (Figure 109). This was significantly lower than pMTL90882_C4-12P11 (2.81×10^{-4}), the Cas9 plasmid without guide RNA and homology arms ($P = 0.049$) and ($P = 0.043$). This low conjugation efficiency was an early indication that Cas9 was active and DSB break had occurred. When 10 colonies were screened for *ligD* knockout, six out of the 10 gave the expected band size (Figure 110). Sequencing two of these PCR bands showed that *ligD* had

been knocked out. These colonies are currently being restreaked in order to lose the Cas9 plasmid.

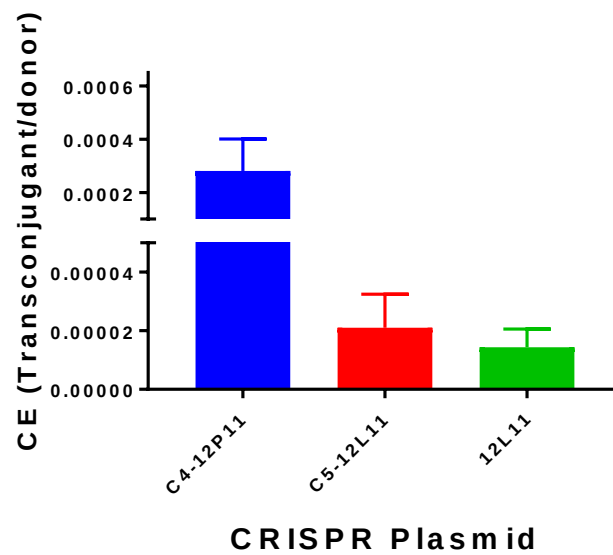


Figure 109: Conjugation efficiency of CRISPR-Cas9 plasmids used for *M. parvus* (OBBP) *ligD* gene knockout. Unpaired t test was used to compare the differences between control plasmids and test plasmid. There was significant Difference between pMTL90882_C4-12P11 and pMTL90882_C5-12L11 ($P < 0.049$) conjugation efficiency. The conjugation efficiency between pMTL90882_C4-12P11 and pMTL90882_12L11 was also significantly different ($P = 0.043$).

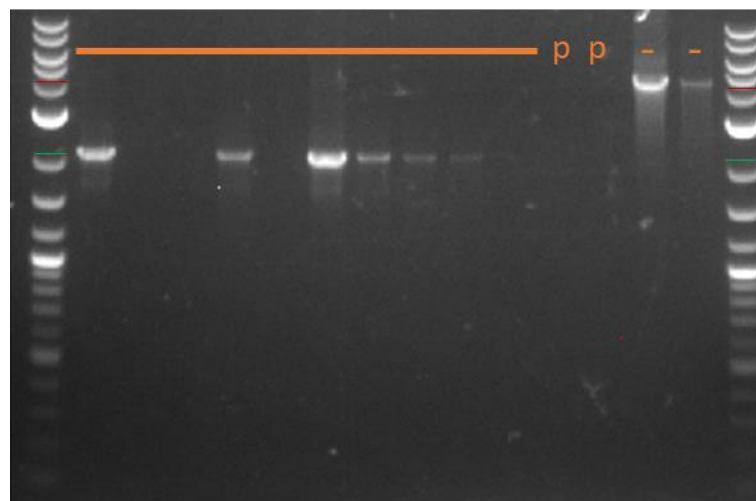


Figure 110: Screen for *ligD* knockouts in *M. parvus* (OBBP) after transforming with pMTL90882_12L11. *M. parvus* (OBBP) KOs were expected to have band size of 2134 bp (green streak) instead of 4582 bp of wild type shown by red streak on DNA ladder. The orange bar represents 10 colonies screened. The negative signs are wild type *M. parvus* (OBBP) colonies used as negative controls. Although the plasmid pMTL90882_12L11 does not have the primer region used for the screen, it was used as an additional negative control (P) to ensure it was not the plasmid that was amplified.

Isolate 12 is one of the few methanotrophs known to possess *ligD* in addition to *M. parvus* (OBBP). Therefore the same plasmid was conjugated in Isolate 12. Out of the colonies obtained, 10 were screened in the same manner as *M. parvus* (OBBP). Eight out of the 10 colonies were *ligD* knockouts, suggesting an 80% CRISPR-Cas9 gene knockout efficiency in Isolate 12 (Figure 111). Sequencing DNA from the bands obtained also confirmed *ligD* was deleted from the genome of Isolate 12.

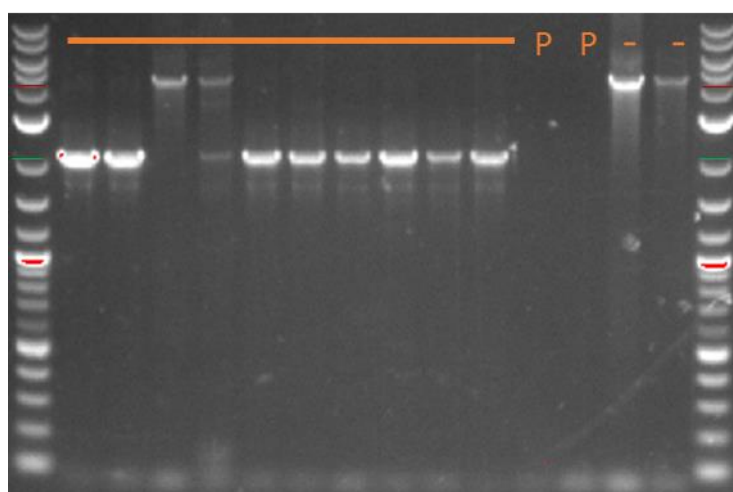


Figure 111: Screen for *ligD* knockouts in Isolate 12 after transforming with pMTL90882_12L11. Isolate 12 KOs were expected to have band size of 2134 bp (green streak) instead of 4582 bp of wild type shown by red streak on DNA ladder. The orange bar represents 10 colonies screened. The negative signs are wild type Isolate 12 colonies used as negative controls. Although the plasmid pMTL90882_12L11 does not have the primer region used for the screen, it was used as an additional negative control (P) to ensure it was not the plasmid that was amplified which gave the sizes of the homology arms.

6.5.5.1 *Investigating high conjugation efficiency of methanotrophs transformed with Cas9 plasmids*

The unexpected high conjugation efficiency of methanotrophs transformed with complete Cas9 plasmids which had guide RNA and homology arms was investigated. The first speculation was Cas9 mutation which rendered the plasmid benign hence the high survival of transconjugants. This led to an attempt to PCR amplify the plasmids from the transconjugants in order to sequence and find out if mutations to the plasmid actually occurred. The region amplified was from Cas9 through guide RNA to the terminator. No bands were obtained for any of the colonies. pMTL90882_12P6 plasmid used as positive control gave bands around

the region expected. Due to time factor, this PCR could not be repeated (Figure 112 and Figure 113)

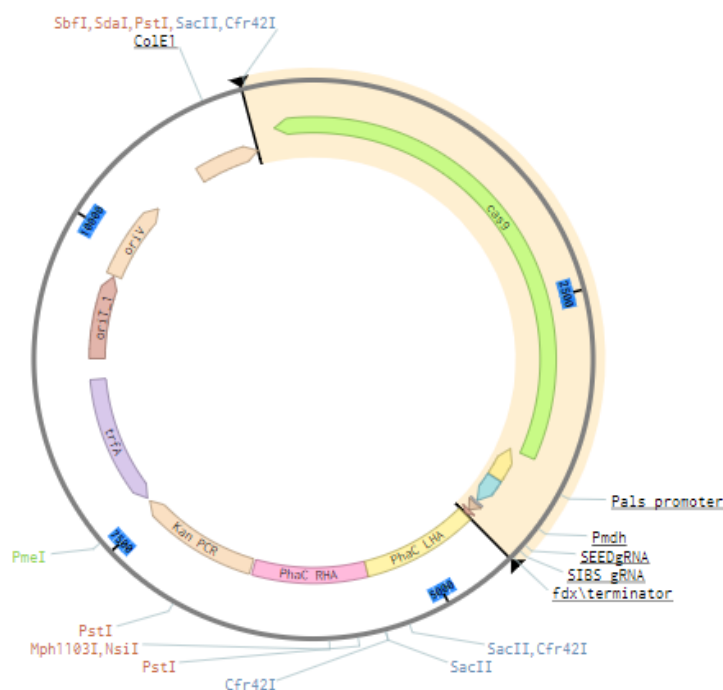


Figure 112: CRISPR-Cas9 plasmid region amplified to investigate mutation of Cas9 plasmid

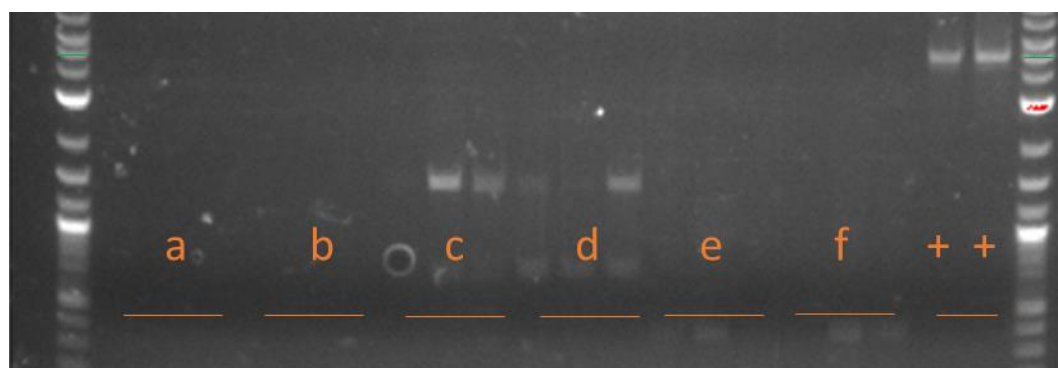


Figure 113: PCR of CRISPR-Cas9 transconjugants to amplify Cas9 to guide RNA region to investigate the sequence of Cas9 plasmid after transforming in methanotrophs using primer pair ColEI_F and Fdx_term_rev. PCR was carried on Isolate 3* carrying pMTL90882_3P6 (a) and pMTL90882_3P11 (b); Isolate 10 carrying pMTL90882_10E11 (c) pMTL90882_10E12 (d); and *M. parvus* (OBBP) carrying pMTL90882_12P6 (e) pMTL90882_12P11. Positive sign represent pMTL90882_12P6 plasmid used as positive control. Green streak on DNA ladder represents the point where PCR product bands were expected (4979 bp).

To summarise the plasmids used in this study after the first two set of experiments involving Cas9For3 and Cas9For6 a table was created (Table 47).

Table 47: Summary of Isolate 3* and *M. parvus* (OBPP) CRISPR-Cas9 plasmids, gene targets and conjugation efficiency data. Yellow colours in heat map represent high Conjugation Efficiency and green colours represent low Conjugation Efficiency

	Plasmid	Control	Cas9 promoter	Guide RNA promoter	Gene target	Conjugation Efficiency (CE)	CE Heat map
Isolate 3*	pMTL90882_C4-3P6	control 1	Pals	NA	<i>phaC</i>	1.2×10^{-2}	0.012
	pMTL90882_C5-3P6	control 2	Pals	Pmdh	<i>phaC</i>	1.25×10^{-5}	1.3E-05
	pMTL90882_3P6	Main plasmid	Pals	Pmdh	<i>phaC</i>	1.67×10^{-5}	1.7E-05
	pMTL90882_C4-3P11	control 1	Pmdh	NA	<i>phaC</i>	1.76×10^{-2}	0.0176
	pMTL90882_C5-3P11	control 2	Pmdh	Pals	<i>phaC</i>	4.54×10^{-6}	4.5E-06
	pMTL90882_3P11	Main plasmid	Pmdh	Pals	<i>phaC</i>	1.96×10^{-2}	0.0196
<i>M. parvus</i> (OBPP)	pMTL90882_C4-12P6	control 1	Pals	NA	<i>phaC</i>	2.6×10^{-3}	0.0026
	pMTL90882_C5-12P6	control 2	Pals	Pmdh	<i>phaC</i>	7.25×10^{-7}	7.3E-07
	pMTL90882_12P6	Main plasmid	Pals	Pmdh	<i>phaC</i>	1.77×10^{-2}	0.0177
	pMTL90882_C4-12P11	control 1	Pmdh	NA	<i>phaC</i>	2.81×10^{-4}	0.00028
	pMTL90882_C5-12P11	control 2	Pmdh	Pals	<i>phaC</i>	1.54×10^{-5}	1.5E-05
	pMTL90882_12P11	Main plasmid	Pmdh	Pals	<i>phaC</i>	1.96×10^{-2}	0.0196
	pMTL90882_C4-12P12	control 1	Phik	NA	<i>phaC</i>	1.66×10^{-4}	0.00017
	pMTL90882_C5-12P112	control 2	Phik	P3	<i>phaC</i>	2.7×10^{-3}	0.0027
	pMTL90882_12P112	Main plasmid	Phik	P3	<i>phaC</i>	1.77×10^{-2}	0.0177
	pMTL90882_C4-2.12P12	control 1	Phik	NA	<i>phaC</i>	1.66×10^{-4}	0.00017
	pMTL90882_C5-2.12P112	control 2	Phik	P3	<i>phaC</i>	9×10^{-2}	0.09
	pMTL90882_2.12P112	Main plasmid	Phik	P3	<i>phaC</i>	4.75×10^{-2}	0.0475
	pMTL90882_C4-12L11	control 1	Pmdh	NA	<i>ligD</i>	2.81×10^{-4}	0.00028
	pMTL90882_C5-12L11	control 2	Pmdh	Pals	<i>ligD</i>	2.1×10^{-5}	2.1E-05
	pMTL90882_12L11	Main plasmid	Pmdh	Pals	<i>ligD</i>	1.44×10^{-5}	1.4E-05

6.6 Discussion

CRISPR-Cas9 gene knockout was carried out on four methanotrophs, three of which were isolated in this study. Among them, one was a type I methanotroph from the genus *Methylocaldum* (Isolate 10) while the remaining three were type II methanotrophs from the *Methylocystis* genus (Isolate 3*, Isolate 12 and *M. parvus* (OBBP)). Among the criteria used to select these strains as candidates for CRISPR-Cas9 gene knockout include the availability of genome sequences, promoters that work, and their unique HR and NHEJ pathways. The absence of published work on CRISPR-Cas9 gene knockout in methanotrophs created a knowledge gap which necessitated a broad framework for implementing CRISPR-Cas9 as a gene knockout tool. Therefore different parameters were considered which included DSB repair pathways, promoter strengths for Cas9 and guide RNA, gene knockout targets, guide RNA seed orientation, plasmid delivery system and chemical supplementation to increase HR efficiency.

The promoter probe assay carried out did not take the separate activity of Ribosome Binding Sites (RBS) and promoter into account as both promoter and RBS were fused together. Therefore, the combination of promoters and RBS used were the same for all the assays carried out. Although this makes it difficult to determine the individual strength of promoters and RBS, it provides information about combined promoter-RBS activity sufficient to carry out the desired CRISPR-Cas9 plasmids construction. P3 promoter unanimously worked in all the tests carried out and had the highest strength. P3, P13 and PNpro1 are identical in sequences except for the -35 to -10 sequence which suggests that the essential DNA sequence required for promoters to operate is what P3 and PNpro1 have. The -35 and -10 regions may only determine the strength of the promoters but not the ability to function. PhiK, Pmdh and Pals are methanotroph promoters with very low activity in *E. coli*. This low activity could be background and was mistaken in the past for absence of activity before scales of the graphs plotted were readjusted to accommodate low fluorescent/OD readings. The absence of expression in *E. coli* makes it suitable for transcribing toxic genes during cloning.

However, confirmation of absence of activity of these promoters in *E. coli* need further experiments to increase the statistical power of the analyses carried out.

The initial CRISPR-Cas9 gene knockout experiments set up focused on *M. parvus* (OBBP). They involved the use of both nCas9 and Cas9; Poly-3-hydroxybutyrate gene (*phaC*) as knockout target and relatively stronger Cas9 promoters compared to guide RNA promoter. After screening colonies, no CRISPR-Cas9 gene knockouts were obtained. This led to broader experimental approaches to ensure success in obtaining knockouts. The reason why initial experiments were unsuccessful is not known. The one thing suspected to work was DSB induced by Cas9 as the efficiency of conjugation reduced when guide RNA was included in the plasmid compared to plasmids with only Cas9. This reinforced the hypothesis that homologous recombination efficiency required for gene knockout was low in *M. parvus* (OBBP). Hence other organisms were included in subsequent experiments.

Isolate 3* was the first methanotroph to test under the broad experimental framework designed after the unsuccessful attempt of the initial CRISPR-Cas9 gene knockout work. With regards to promoters used for Cas9 and guide RNA, only two plasmid series were successfully cloned. Plasmids with moderately strong promoter for Cas9 and weak promoter for guide RNA, and vice versa (3P6 and 3P11). The inability to clone the plasmid with very strong promoter for guide RNA (P3) could have been due to deleterious effects of the high expression levels of P3 promoter. When the two plasmid series were transformed in Isolate 3*, no CRISPR-Cas9 gene knockout of *phaC* was obtained. The first reason suspected was weak homologous recombination of Isolate 3* although there was no way to verify this. Secondly, conjugation efficiency data suggests only the plasmid series with moderately strong Cas9 promoter and weak guide RNA promoter (pMTL90882_3P6) induced Cas9 DSB due to the relatively low conjugation efficiency compared to the control plasmid (pMTL90882_C4-3P6). Therefore it was speculated that this series of plasmid was unsuitable for making CRISPR-Cas9 gene knockout. Finally it was speculated that *phaC* gene was essential in Isolate 3* and all the cells that had *phaC* knocked out did not survive. If this were to be true, the implication was all the cells that survived either had mutations in the seed guide

RNA region of *phaC* hence Cas9 did not induce DSBs, or the plasmid that transformed those individual cells was mutated and did not cause DSB. PCR amplification of the respective regions carried out to test these hypotheses did not result in DNA products of the expected size as shown for the latter hypothesis (Figure 113). PCR amplification experiments of the former hypothesis also did not yield bands although the result was not shown.

Among the sets of plasmids tested in Isolate 10, two plasmid series were used to carry out knockouts (pMTL90882_10E11 and pMTL90882_10E12). From the conjugation efficiency data, it was likely that cas9 induced double strand break as there was a 10 fold decrease in conjugation efficiency of plasmids carrying guide RNA and homology arms. There was no gene knock out obtained from both test plasmids which was not expected. This is because *RecBCD* is the *E. coli* homologous recombination pathway and has been shown to be efficient at using HDR for DSB repair [301]. Isolate 10 also has the *RecBCD* pathway for homologous recombination and was therefore expected to efficiently recombine HA arms and delete *efe* in the respective plasmids. One possibility may be that the *RecBCD* is not functional and *RecF* pathway which is another HDR pathway has lower repair efficiency. Attempts to PCR amplify the plasmid from the colonies that grew was also unsuccessful. Additionally the promoters used for Cas9 were shown to have very weak expression which could have an effect on the efficiency of homologous recombination. Finally, as mentioned earlier, an optimal Cas9:guide RNA promoter expression ratio may be required for successful and efficient HDR and this ratio was not attained.

The conjugation efficiency of *M. parvus* (OBBP) and Isolate 12 CRISPR-Cas9 test plasmids targeting *phaC* were much higher than expected. There are three reasons that can explain this outcome. Firstly there was a Cas9, guide RNA, promoter or terminator mutation in the plasmid used for conjugation hence Cas9 DSB was not induced. Secondly, the guide RNA was not effective in targeting *phaC*. This is unlikely as guide RNA on-target score on Benchling which predicts the effectiveness of a guide RNA was 71% which is relatively high. Additionally, results from conjugation with pMTL90882_C5-12P6 and pMTL90882_CasFor6 suggested

Cas9 DSB. Finally, the high conjugation efficiencies obtained was due to high efficiency of HDR which will result in a lot of knockouts. No knockout was obtained among all the colonies screened which ruled out this possibility. Low conjugation efficiency was seen in the two-plasmid system which can be attributed to pressure from growing on two antibiotics. This leaves the first possibility as the likely explanation for the high conjugation efficiency but efforts to PCR amplify the test plasmids from methanotrophs that grew failed. Hence this could not be verified. However, this line of enquiry will be investigated further.

When *ligD* was targeted for gene knockout the conjugation efficiency of CRISPR-Cas9 test plasmids for knockouts was significantly lower when compared to the control plasmid without guide RNA. This was the first hint suggesting Cas9-induced DSB occurred. Screening the colonies of *M. parvus* (OB BP) and Isolate 12 showed an 80% efficiency in gene knockout for Isolate 12. The knockout efficiency for *M. parvus* (OB BP) could not be calculated as not all the colonies screened resulted in PCR products. However, all six PCR products obtained were knockouts. This is the first time CRISPR-Cas9 gene knockout has been reported in methanotrophs. There may have been more than one reason why it was successful. The strongest argument is knocking out *ligD* deprived the use of NHEJ or alternative DSB repair pathways from working which left homologous recombination as the only pathway for repairing DSBs and avoiding a lethal event. The use of homologous recombination for DSB repair elicited gene knockout. Another reason why this would have worked was the ratio of Cas9:guide RNA promoter expression. This is the first time plasmid with Cas9, guide RNA and HAs under the transcriptional control of Pmdh and Pals respectively worked. This ratio could be key to efficient CRISPR-Cas9 gene knockout in *Methylocystis* species and will be reattempted in other gene targets. Finally, it is possible that the guide RNA used for targeting *ligD* was just optimal.

It was observed that there were a lot of 'escaper colonies', a term used to refer to colonies that escape the action of Cas9 [309]. This was observed even when *ligD* was used as target. It is difficult to identify the cause with the limited amount of data from this study but a strong link has been made between number of escaper

colonies and *mutL* [309]. *mutL* is a gene required for mismatch DNA repair and it was demonstrated that *mutL* mutant in *E. coli* had higher frequency of escaper colonies.

The results obtained however intriguing, raise more questions than answers. Will CRISPR-Cas9 gene knockout work or be as efficient if the promoters used for *ligD* plasmid were reversed between Cas9 and guide RNA? Will the same efficiency of gene knockout be observed if the promoters used for *ligD* were applied for a different target such as *phaC*. These are the principal questions that need to be answered first. Other questions that require investigation include investigating the effect of knocking out *ligD* variants such as *ykoV* in isolate 3*. Will this have any effect on CRISPR-Cas9 gene knockout efficiency? Isolate 10 does not have a *ligD* variant. Will knocking out *ligA* which is associated with A-EJ have similar impact if disruption of NHEJ by knocking out *ligD* was the reason behind the gene knockout success in this study? What about protein kinase which is essential for NHEJ? Will knocking it out also give the same results? If only *ligD* mutants can be edited by CRISPR-Cas9 do these mutants have a phenotype that can affect their use in day to day lab work? Will it be easier to knockout other genes using *ligD* mutants? Answering these questions will require carefully designed experiments and will no doubt add to the growing knowledge of CRISPR, non-homologous and homologous recombination.

6.7 Conclusion

ligD was successfully knocked out in Isolate 12 and *M. parvus* (OBBP) using CRISPR-Cas9 gene knockout system. The plasmid used for the knockout had a weaker promoter for Cas9 compared to guide RNA. It is the first time this has been demonstrated in methane-oxidising bacteria.

7 Chapter 7: Final Discussion

7.1 Isolation of methanotrophs

Isolation of pure methanotrophs was conducted over 18 months and resulted in nine isolates from different environments demonstrating the ubiquity of methanotrophs [15-19]. Regardless of the storage conditions (temperature and storage time before isolation), pure methanotrophs were isolated from environmental samples. The amount of starting environmental sample used for enrichment varied in this study. Samples as little as 10 μ L of environmental solutions were used to minimise carbon sources such as sugars for non-methanotrophs. This affected the time taken for enrichments to start growing. It is the opinion of this author that rather than start enrichment with very little sample volumes or mass, relatively higher amounts will likely lead to faster initial growth. Further along, the population of non-methanotrophic heterotrophs can be reduced by making dilutions of grown enrichment samples and streaking on agar plates to select bacterial colonies with methanotrophic properties. Among the properties of methanotrophic colonies that make them recognisable are their predominantly opaque nature and slow growth in the first week of incubation on agar plates. Most importantly, methanotrophs are identified by the presence of highly conserved *pmoA* and *mmoX* genes exclusive to them by PCR amplification using already published PCR primers [252]. One of the observations made during isolation was the propensity of methanotrophic colonies to grow alongside non-methanotrophs. The commensal relationship of methanotrophs has been studied and is well understood which explains the difficulty encountered when isolating pure strains [310-313]. Among the steps taken to solve this problem was removal of vitamins likely to encourage growth of non-methanotrophs [88]. Another step taken to increase the chances of isolating pure methanotrophs was making dilutions of liquid enrichment cultures and streaking the highest dilutions on agar plates. Colonies growing on plates were then transferred to liquid media for further dilution. This alternation was continued till pure methanotrophs were isolated and was used to purify Isolate 3*, 6, 7, and 12 [250]. In more peculiar

cases, alternating media forms was coupled with alternating growth temperature from 37 – 42°C which led to pure colonies of Isolate 10 [314]. The ability of *Methylococcus capsulatus* to grow at 45°C made Isolate 9 and 14 easier to purify as not many organisms were found growing on plates at that temperature. Methods used to confirm purity of methanotrophs included plating on media with rich carbon source such as LB and TSA media, phase contrast microscopy and visual observation of morphology on plates. Other extensive methods have been reported [31].

After confirming purity, experiments were carried out to determine the characteristics of pure isolates. The morphology of isolates was resolved with visual observation on agar plates, Phase Contrast Microscopy images and Transmission Electron Microscopy (TEM) images. Visually, all isolates appeared opaque and concave on agar plates with colour ranging from cream, pink to brown. The pink colouration in most cases may have resulted from synthesis of carotenoids and this is supported by the presence of carotenoid-synthesizing genes in these isolates. These genes have been reported in other methanotrophs [33]. Unlike Isolate 3* and 6 where pink colouration was acquired and maintained at later growth stages, Isolate 7 pink colouration appeared at the beginning of growth. Brown colony colour was observed in Isolate 10 (*Methylocaldum* species) only which is attributed to melanin known to be synthesized by other methanotrophs [270]. Microscopy showed all the Isolates were diplococci although Isolate 9, 10 and 14 appeared mixed with single coccus cells both under Phase Contrast and TEM. TEM was also very instrumental in understanding the inclusion bodies of Isolates. The nature of methane oxidation site which is the Intracytoplasmic Membrane (ICM) was in agreement with prediction based on whether an isolate was type I, type II or type X as described by Whittenbury *et al* [2]. TEM also showed PHB storage granules which was expected in type II methanotrophs known for PHB accumulation under nitrogen limiting growth conditions [269].

Genome engineering, molecular biology applications, forward and reverse genetics constituted a significant part of this study. As a prerequisite, amenability to DNA

transfer through conjugation was investigated. Conjugation was demonstrated in Isolate 3*, 6, 10, 12 and 14. Isolate 3* and 12 which are type II methanotrophs were shown to have higher conjugation efficiencies compared to Isolate 10 which is type I (Figure 46). The difference between plasmid replicons also had significant effect on conjugation efficiencies. For example plasmids with ColE1 replicons had higher efficiencies than pBBR1 based plasmids. This was expected based on the copy number of these plasmids and ColE1 was reported to be higher than pBBR1 based plasmids [271]. For everyday molecular biology tasks, high conjugation efficiency is not essential. However in applications such as building transposon libraries high conjugation efficiency reduces the time required to build a library of high number of transposon mutants. An important step in plasmid conjugation is mating of donor and recipient cells. Results from this study showed this time could be reduced to as low as 6 hours with even higher efficiency compared to 48 hours practiced in reported studies [272, 286]. Electroporation was also attempted due to certain advantages such as transfer of linear DNA molecules and transfer of plasmids without mobilisation elements such as oriT (origin of transfer). However no success was achieved in electroporation of all isolates attempted. These could be due to physical barriers in the cell membranes that prevent electroporation based DNA transfer. However, electroporation has been reported in both type I and type II methanotrophs and future work will investigate ways of adopting this mode of DNA transfer in isolated strains [315, 316].

Phylogenetic tree was used to determine the relationship between isolated strains as well as between isolated strains and other organisms. As expected the relationship between methanotrophs of the same type (type I or type II) was closer compared to methanotrophs of different types. What was unexpected was the closer relationship between type I methanotrophs and other organisms compared to type I and type II methanotrophs. It is beyond the scope of this study to investigate whether type I and type II methanotrophs evolved separately but nonetheless an important consideration.

There was no striking pattern observed with regards to antibiotic resistance of isolates tested except that they were all resistant to nalidixic acid at

concentrations *E. coli* found sensitive (25 µg/mL). Resistance to nalidixic acid was an advantage exploited when conjugation was carried out between *E. coli* and methanotrophs. The methods used to investigate antibiotic resistance employed the use of antibiotic resistant strips which provided values lower than observed when methanotrophs were grown on agar plates with antibiotics. For example the strips suggested all isolates to be sensitive to kanamycin at concentrations above 1 µg/mL. However, wild type colonies of Isolate 3*, 6 and 12 were found to grow at kanamycin concentrations as low as 30 µg/mL on agar plates, although the growth was significantly lower than when grown in agar plates without antibiotics.

Some organisms use CH₄ as their sole source of carbon and energy and they are referred to as obligate methanotrophs. Organisms that use other C1 based compounds such as methanol, and compounds without carbon-carbon bonds such as dimethylamine are known as methylotrophs. Finally methanotrophs and methylotrophs that can grow on compounds with carbon-carbon bonds like acetate are referred to as facultative methanotrophs and facultative methylotrophs respectively. In essence, all methanotrophs are methylotrophs but not all methylotrophs are methanotrophs. In this study Isolate 01, 03, 7, 9, 10 and 14 showed characteristics of obligate methanotrophs. Isolate 12 was able to grow on methanol in addition to growth on CH₄ therefore is not an obligate methanotroph. Isolate 3* was able to grow on acetate making it a facultative methanotroph. The broad spectrum of growth characteristics provides flexibility for various applications. For example Isolate 01, 03, 7, 10 and 14 can be used for tasks requiring strict utilization of CH₄ such as climate change mitigation; Isolate 12 can be used in applications where methanol is preferred to CH₄ such as in growth monitoring with the Biolecta; and finally Isolate 3* will be preferred in applications requiring the use of non-volatile compound like methanol or gases such as CH₄.

The whole genome sequence of methanotrophs obtained was useful in differentiating isolates such as 3* and 6 that had identical 16S rRNA sequences. It was also used in identifying gene knockout targets as well as designing components for genome editing using CRISPR-Cas9. The identification of transposon insertion sites in Isolate 12 would have been impossible without

genome sequence data which demonstrates its importance. The difficulty experienced with implementing electroporation in methanotrophs may be solved by exploiting the RM systems of organisms like isolate 6, 12 and 14 that have PacBio sequences.

Three chosen chemical products in this study that methanotrophs can potentially produce are 3HP, isobutanol and methanol. These products have been successfully synthesized by other bacteria either by native pathways or through expression of heterologous genes [260, 317, 318]. What makes it different using methanotrophs is the potential of using a greenhouse gas (CH_4) which is a cheap source of energy that could otherwise be released into the environment for example in landfill or oil fields where CH_4 is flared. To produce these chemicals, methanotrophs have to be able to tolerate them at desired concentrations. Therefore tolerance of isolates to various compounds was investigated with Isolate 12 being the focus due to plans to use it for making a transposon mutant library. The stress caused by these chemicals was thought to have genetic signatures which this study wanted to explore with a transposon library and TraDIS. The sensitivity of Isolate 12 to 3HP was not surprising as laboratory communication in the SBRC Nottingham revealed sensitivity of another organism *C. necator* H16 to 3HP. Isobutanol was also shown to be toxic in *E. coli* [319]. Methanol on the other hand is an intermediate during CH_4 metabolism in methanotrophs and was not expected to be toxic. The results obtained in this study agreed with what was expected.

The components (vitamins, Fe-EDTA and $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$) added to NMS media can potentially increase cost of production if done on a large scale. Additionally, certain components such as vitamins are likely to increase contamination risk of methanotrophic cultures. With Isolate 12 as the focus, the effect of the various components were investigated and the results showed insignificant consequences of omitting these components in NMS media as there was no significant difference of Isolate 12 observed within a wide range of concentration of these components in NMS media. In future experiments, addition of these components to NMS media will be minimized or completely removed especially when working with Isolate 12.

For methanotrophs to be used for viable industrial applications, an unwanted or very cheap source of CH₄ must be available. As such it is important to investigate if methanotrophs can grow on different sources of CH₄. One source of CH₄ that is readily available and can be generated in rural, semi-urban and urban areas is biogas. The ability of all isolates to grow on two different biogas sources was demonstrated. This is not the first time growth of methanotrophs on biogas was reported. Biogas was used by thermotolerant methanotrophic consortia for methanol biosynthesis [317]. *Methylocystis hirsuta* was used to accumulate PHB using artificial biogas [320]. However this is the first report of a wide array of methanotrophic species that can grow on two different biogas sources. More importantly accumulation of PHB on both biogas sources was shown to increase by 10 fold compared to growth on pure CH₄. Biogas is a relatively desired product as it is used for electricity generation. A cheaper CH₄ source will likely be where CH₄ composition is too low to be used for electricity generation such as in landfills. Future studies plan to investigate the growth of methanotrophs in such CH₄ sources.

Investigating different methanotrophic characteristics provided a basis for identifying the novelty of some isolates. For example the presence of fimbriae in Isolate 3* presented a significant morphological distinction compared to its closest related species (*Methylocystis rosea*) which lacked fimbriae [1]. Isolate 10 was also considered novel based on differences with its closest relative (*Methylocaldum marinum*) which include inability to grow on methanol, growth temperature, salt requirement, growth of colonies on plate and absence of sMMO [26].

7.2 Random transposon mutagenesis in methanotrophs

Transposon mutagenesis has been used as an important tool in molecular biology and understanding of gene function. The scope of this study was to exploit the use of transposons as tools for forward genetics study in methanotrophs, in which transposons can be used to determine the genetic basis responsible for a particular phenotype. For example, the inability of a methanotroph such as *M. parvus* (OBBP) to accumulate PHB when *phaC* gene is knocked out [306]. Another

example is the inability of a cell to grow when an essential gene in its chromosome is knocked out. This forms the basis of NGS sequencing technologies like TraDIS which involves sequencing thousands of transposon mutants to find out genes with multiple transposon insertions (non-essential genes), and genes which have very few or no insertions at all (essential genes). The idea being genes that have insertions and have therefore being inactivated are not essential to the cell hence allowing the cell to survive and multiply to such high cell numbers that its genome can be detected in a pool of other mutants' genome. On the other hand, inactivation of essential genes leads to cell death which significantly reduces the population of the subject cell in a mutant pool such that it cannot be detected by sequencing. However to have a large mutant pool, there is need for controlled mutations such that you have one cell having one mutation different from the mutation in other cells found in the same library of mutants. Random transposon mutagenesis with plasmid loss provides a very efficient way of carrying this out.

In this study, the ultimate aim was to determine essential genes in methanotrophs using TraDIS which can provide information on which genes can be deleted and which deletions are lethal. However, TraDIS has never been carried out in methanotrophs so the core part of this project involved implementing a system known to work in *C. necator* H16 (SBRC Nottingham) and other bacteria such as *Salmonella typhi*, *E. coli* and *Clostridium difficile* [35, 217, 321]. In three out of the four studies referenced Tn5 was the choice of transposon used to carry out mutagenesis. Tn5 transposon insertion is random which makes it desirable. Although mariner transposon is also random, it has bias of A-T DNA sites in the genome which is suitable for organisms like *C. difficile* which have high A-T content in the genome [322].

After determining which transposon to use it was important to select the right methanotroph species on which to carry out TraDIS. One important factor for selecting species was the conjugation efficiency. It was shown in this study that type II methanotrophs tested had higher conjugation efficiencies. Hence type II methanotrophs were the preferred candidates. Among the type II methanotrophs,

PacBio sequence was available for Isolate 12 in addition to desired characteristics such as high PHB accumulation when grown with biogas hence it was selected.

The Tn5 derivative mini-Tn5Kan_R6K plasmid used for transposon mutagenesis in this study was synthesized by the author. One important part of the plasmid was the use of non-replicative replicon known as R6K which has been used in other studies as a replicon for suicide vectors [35, 281]. In this study R6K was tested in Isolate 12 and shown to be indeed a non-replicative replicon ideal for use in suicide plasmids. The transposition efficiency obtained by the Tn5 derivative was also determined and found to be enough to make a transposon library within a reasonable amount of time. These are all new findings in the field methanotrophs.

Although Tn5 transposition is random, it was important to show evidence for this in methanotrophs. This is the first study showing random Tn5 mutagenesis in type II methanotrophs with 36 random insertion sites identified. A lot of effort was committed into creating one million transposon mutant library. Due to time factor, this was scaled down to about 600000 mutants. The mutant library size used in the past varied. Whereas some studies used up to a 1.1 million mutants [35], others used as low as 0.5 million mutants [321]. An unexpected outcome of the library of transposon mutants created in this study was contamination observed. Steps taken to minimize contamination in the future include segregation of transposon mutant batches. Therefore instead of storing them in the same screw cap tube, each batch will be stored in a different screw cap tube and revived independently before pooling together. Experiments and preparations such as plate drying will also be carried out in sterile laminar flow cupboards such as ThermoElectron Corporation Hersafe KS 12. Finally, In addition to testing methanotroph growth cultures for contamination, bacterial mating resuspension were also tested for contamination. These challenges of contamination were likely not encountered during studies involving organisms like *E. coli* due to their fast growth. Unfortunately, methanotroph colonies grow after two weeks which is enough time for even insignificant contamination to grow and dominate. The challenges of contamination were not reported in past studies which was why it was not fully anticipated in this study.

Regardless of the challenges encountered, this study has made profound contributions by establishing transposon mutagenesis tools and paving a road map for TraDIS applications in methanotrophs.

7.3 CRISPR-Cas genome editing in methanotrophs

A series of promoters were tested for use in CRISPR-Cas9 genome editing. These promoters were obtained from *M. parvus* (OBBP), *M. capsulatus* (Bath) and finally some were synthetic promoters from Naglis Malys in SBRC Nottingham. All the synthetic promoters tested (NPR01, P13 and P3) were active and their DNA sequence differed only in the -35 and -10 regions suggesting it could be the point determining promoter activity. These three promoters had the same RBS sequences. All the other promoters had different RBS regions which made it difficult to speculate their actual strength. A study involving the methanotroph *Methylobacterium buryatense* 5GB1 showed the effect of promoters and RBS and concluded there was a significant effect of promoter-RBS combination on gene expression [323]. Future studies will investigate individual RBS and promoter effect.

Past studies have shown the effect of copper on gene expression in methanotrophs [324]. It was hypothesized that the effect of copper on gene expression could be at the transcriptional level and this was investigated by measuring reporter protein expression using different promoters under copper-free and copper conditions in the form of $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$. A significant difference was observed in favour of some promoters in *M. parvus* (OBBP) grown in the presence of $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$. RNAseq can be carried out to ascertain these results. More importantly, nine promoters and promoter-RBS combinations were identified to work in *M. parvus* (OBBP) and six of them in Isolate 3*, 6, 10 and 12 at different levels of expression. This is one of the most extensive promoter assays conducted which involved different methanotroph types and numerous promoters and will be useful to the methanotroph community when published. Other published data on promoters for methanotrophs focused on *M. buryatense* which is a type I methanotrophs [286, 323].

CRISPR-Cas genome editing has become very popular and it is used in genome editing of eukaryotes, prokaryotes and archaea [167-178, 180]. As a result it was important to try and establish it in methanotrophs as it had never been reported. More importantly, genome editing in methanotrophs was carried out using sucrose counter selection after homologous recombination which required screening of single and double crossovers, a time consuming process especially for methanotrophs that are slow growers [286]. CRISPR-Cas9 genome editing is less time consuming and as such potentially more efficient. The predominant Cas9 target used in this study was *phaC* gene required for PHB synthesis which was tested in all the type II methanotrophs used. Other targets included *ege* (Isolate 10) and *ligD* (Isolate 12 and *M. parvus* (OBBP)). From preliminary experiments, it was clear that genome editing was not taking place with the set of promoters used (strong promoter for Cas9 and weak promoter for guide RNA) and *phaC* gene as target. However studies showed successful CRISPR-Cas9 genome editing when a stronger promoter (U6) was used for guide RNA [325]. The SBRC Nottingham CRISPR-nCas9 plasmid (unpublished) was also successfully used to carry out genome editing in Clostridia species and it used a stronger promoter for guide RNA. As such this approach was taken and plasmids were made which had weak Cas9 promoters and strong guide RNA promoters. It is suspected that the plasmids with stronger guide RNA promoters such as pMTL90882_3P11 and pMTL90882_12P11 mutated and as such were unable to induce double strand break. However, the plasmid targeting *ligD* pMTL90882_12L11 remained intact and induced double strand break evident from the low conjugation efficiency when it was conjugated. Studies had shown increased homologous recombination efficiency when NHEJ machinery in cells were inhibited. These inhibitions can be as a result of using DNA ligase (LigD) inhibitors such as the pyrazine Scr7 [183, 184]. Additionally homologous recombination efficiency can be increased by deleting the protein kinase gene (Ku) also known to be involved in NHEJ [326, 327]. This information backed the authors idea of using *ligD* as a target for CRISPR-Cas9 genome editing in methanotrophs. An 80% efficiency of genome editing was achieved in Isolate 12 using a combination of strong promoter for guide RNA and *ligD* as the target gene. *ligD* gene knockout was also achieved in *M. parvus* (OBBP).

The next step of this project will be to confirm whether gene knockout success was because *ligD* was used as target or because a stronger guide RNA promoter was used, or both. If knocking out *ligD* was the reason for the success it validates reports which suggest diminishing NHEJ increases homologous recombination efficiency. The *ligD* mutants will be used to target other genes to find out whether *ligD* mutants will maintain the high homologous recombination efficiency. The use of a strong promoter for guide RNA using *phaC* target will also be explored.

It is important to emphasize the role of controls in these experiments as they were instrumental in indicating which plasmids worked and which ones did not. It was by experimental design that plasmids successful in introducing double strand breaks had significantly lower conjugation efficiencies compared to their control plasmids which did not have guide RNAs used for directing the nuclease activities of Cas9. For example there was significantly lower conjugation efficiency in the plasmid targeting *ligD* (pMTL90882_12L11) which had guide RNA compared to the control plasmid pMTL90882_C4-12L11 which did not have guide RNA. pMTL90882_12L11 was successful in inducing double strand break and causing gene knockout. On the other hand the plasmid targeting *phaC* pMTL90882_12P11 was significantly higher than its control plasmid which did not have guide RNA. This suggested cas9 double strand break did not occur in the pMTL90882_12P11 and consequently there was no success in gene knockout.

It is difficult to confirm whether gene knockout success was as a result of *ligD* being the gene target or because a stronger guide RNA promoter was used compared to Cas9 promoter. If *ligD* being the target was the reason for success, it is not clear whether it had to do with good guide RNA or it was solely because of the absence of NHEJ due to *ligD* knockout. Future experiments have been planned to answer these questions. Another important experiment planned is to see if knocking out other genes in Isolate 12 and *M. parvus* (OB BP) *ligD*⁻ mutants can be carried out with high efficiencies. Regardless of the uncertainties, making *ligD*⁻ mutants is an important achievement as CRSIPR-Cas9 genome editing has not been reported in methane-oxidising bacteria.

8 Conclusion

In conclusion, this study resulted in the isolation of nine methanotrophs. Two of these, Isolate 7 (*Methylomonas* species) and Isolate 10 (*Methylocaldum* species) are type I methanotrophs; five species, Isolate 01, 03, 3*, 6 and 12 are *Methylocystis* species which are type II methanotrophs; and two species, Isolate 9 and 14 (*Methylococcus capsulatus*) are type X methanotrophs.

The morphology of these isolates was resolved using Phase Contrast Microscopy and for some (Isolate 3*, 6, 10, 12 and 14), Transmission Electron Microscopy. The genomes of five of these isolates (3*, 6, 10, 12 and 14) were sequenced with Illumina while three isolates (6, 12 and 14) in addition to Illumina were sequenced with PacBio. The antibiotic resistance profile of these isolates was determined which showed uniformity to nalidixic acid resistance at concentration up to 25 µg/mL. The phylogenetic relationship of these isolates was also determined. Conjugation efficiency of Isolate 3*, 10 and 12 were determined as well as *M. parvus* (OBBP) which showed type II methanotrophs to have higher conjugation efficiency. Isolate 3* was shown to grow on acetate in addition to CH₄ whereas Isolate 12 was shown to grow on methanol. All the isolates were able to grow on two different biogas sources with Isolate 12 accumulating about 10 times more PHB when grown in biogas compared to growth on CH₄. All the type II methanotrophs tested accumulated PHB whereas all the type I and type X did not. Isolate 3* and 12 showed sensitivity to 3HP at 1 mM concentration when grown in NMS media. Sensitivity to Isobutanol was also observed at 0.5 mM concentrations. Finally Isolate 12 was able to grow when either vitamins, Fe-EDTA or CuSO₄·7H₂O was removed from the culture media.

Characterisation of methanotrophs provided a basis for considering Isolate 3* as a novel species based on the presence of fimbriae which was not observed in its closest related species (*Methylocystis rosea*). Isolate 10 was also considered novel based on differences with its closest relative (*Methylocaldum marinum*) which include inability to grow on methanol, growth temperature, salt requirement, growth of colonies on plate and absence of sMMO.

This study was also successful in establishing random transposon mutagenesis in Isolate 12, a *Methylocystis* species, using a suicide plasmid (mini-Tn5Kan_R6K) with transposition efficiency of 4.43×10^{-4} after mating for six hours. The plasmid was able to induce transposition without replicating in recipient cell (Isolate 12). A transposon library of Isolate 12 was also made which was stored for over three months at -80°C and successfully revived although it was not used for TraDIS due to contamination.

Finally, this study established for the first time, successful genome editing in methanotrophs with CRISPR-Cas9 using *ligD* as target for guide RNA. The plasmid used had Cas9 under the transcriptional control of a weak promoter (Pmdh) while guide RNA was under the transcriptional control of a stronger promoter (Pals).

9 References

1. Wartainen, I., et al., *Methylocystis rosea* sp. nov., a novel methanotrophic bacterium from Arctic wetland soil, Svalbard, Norway (78 degrees N). *Int J Syst Evol Microbiol*, 2006. **56**(Pt 3): p. 541-7.
2. Whittenbury, R., K. Phillips, and J. Wilkinson, *Enrichment, isolation and some properties of methane-utilizing bacteria*. *Microbiology*, 1970. **61**(2): p. 205-218.
3. Anthony, C., *The biochemistry of methylotrophs*. Vol. 17. 1982: Academic press London.
4. Mutalik, V.K., et al., *Precise and reliable gene expression via standard transcription and translation initiation elements*. *Nature Methods*, 2013. **10**: p. 354.
5. Wartainen, I., et al., *Methylocystis rosea* sp. nov., a novel methanotrophic bacterium from Arctic wetland soil, Svalbard, Norway (78° N). *International Journal of Systematic and Evolutionary Microbiology*, 2006. **56**(3): p. 541-547.
6. Semrau, J.D., *Bioremediation via Methanotrophy: Overview of Recent Findings and Suggestions for Future Research*. *Frontiers in Microbiology*, 2011. **2**: p. 209.
7. Reeburgh, W., S. Whalen, and M. Alperin, *The role of methylotrophy in the global methane budget, Microbial Growth on C 1 Compounds*. JC Murrell, DP Kelly: p. 1-14.
8. Agency, U.S.E.P., *Carbon Dioxide Emissions* 2018.
9. Basis, I.C.C.T.P.S., *Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. 2007.
10. Agency, I.E., *World Energy Outlook: Trends by Sector - Transport*. 2015.
11. Ribeiro, S.K. and R. Balassiano, *CO2 emissions from passenger transport in Rio de Janeiro*. *Transport policy*, 1997. **4**(2): p. 135-139.
12. Vosloo, A.C., *Fischer-Tropsch: a futuristic view*. *Fuel processing technology*, 2001. **71**(1-3): p. 149-155.
13. Steynberg, A., et al., *Fischer-Tropsch reactors*, in *Studies in Surface Science and Catalysis*. 2004, Elsevier. p. 64-195.
14. Dry, M.E., *The fischer-tropsch process: 1950–2000*. *Catalysis today*, 2002. **71**(3-4): p. 227-241.
15. Tofalo, R., et al., *Biogeographical characterisation of Saccharomyces cerevisiae wine yeast by molecular methods*. *Frontiers in Microbiology*, 2013. **4**.
16. Johnson, I.S., *Human insulin from recombinant DNA technology*. *Science*, 1983. **219**(4585): p. 632-637.
17. Meulepas, R.J.W., et al., *Enrichment of anaerobic methanotrophs in sulfate-reducing membrane bioreactors*. *Biotechnology and Bioengineering*, 2009. **104**(3): p. 458-470.

18. Bárcena, T.G., J.C. Yde, and K.W. Finster, *Methane flux and high-affinity methanotrophic diversity along the chronosequence of a receding glacier in Greenland*. *Annals of Glaciology*, 2010. **51**(56): p. 23-31.
19. Lindner, A.S., et al., *Methylocystis hirsuta* sp. nov., a novel methanotroph isolated from a groundwater aquifer. *International journal of systematic and evolutionary microbiology*, 2007. **57**(8): p. 1891-1900.
20. Durisch-Kaiser, E., et al., *Evidence of intense archaeal and bacterial methanotrophic activity in the Black Sea water column*. *Applied and environmental microbiology*, 2005. **71**(12): p. 8099-8106.
21. Dubey, S., *Microbial ecology of methane emission in rice agroecosystem: a review*. *Applied ecology and environmental research*, 2005. **3**(2): p. 1-27.
22. Tsubota, J., et al., *Methylothermus thermalis* gen. nov., sp. nov., a novel moderately thermophilic obligate methanotroph from a hot spring in Japan. *International journal of systematic and evolutionary microbiology*, 2005. **55**(5): p. 1877-1884.
23. Lee, E.H., et al., *Characterization of methane oxidation by a methanotroph isolated from a landfill cover soil, South Korea*. *Journal of microbiology and biotechnology*, 2011. **21**(7): p. 753-756.
24. Khmelenina, V.N., et al., *Isolation and characterization of halotolerant alkaliphilic methanotrophic bacteria from Tuva soda lakes*. *Current Microbiology*, 1997. **35**(5): p. 257-261.
25. Dedysh, S.N., et al., *Isolation of acidophilic methane-oxidizing bacteria from northern peat wetlands*. *Science*, 1998. **282**(5387): p. 281-284.
26. Takeuchi, M., et al., *Methylocaldum marinum* sp. nov., a thermotolerant, methane-oxidizing bacterium isolated from marine sediments, and emended description of the genus *Methylocaldum*. *Int J Syst Evol Microbiol*, 2014. **64**(Pt 9): p. 3240-6.
27. Pandey, V.C., et al., *Methanotrophs: promising bacteria for environmental remediation*. *International Journal of Environmental Science and Technology*, 2014. **11**(1): p. 241-250.
28. Theisen, A.R., et al., *Regulation of methane oxidation in the facultative methanotroph *Methylocella silvestris* BL2*. *Molecular microbiology*, 2005. **58**(3): p. 682-692.
29. Murrell, J.C., I.R. McDonald, and B. Gilbert, *Regulation of expression of methane monooxygenases by copper ions*. *Trends in Microbiology*, 2000. **8**(5): p. 221-225.
30. Macalady, J.L., et al., *Population dynamics of type I and II methanotrophic bacteria in rice soils*. *Environmental Microbiology*, 2002. **4**(3): p. 148-157.
31. Dedysh, S.N., C. Knief, and P.F. Dunfield, *Methylocella* Species Are Facultatively Methanotrophic. *Journal of Bacteriology*, 2005. **187**(13): p. 4665-4670.

32. Crombie, A. and J.C. Murrell, *Development of a system for genetic manipulation of the facultative methanotroph Methylocella silvestris BL2*. Methods Enzymol, 2011. **495**: p. 119-33.
33. Sharpe, P.L., et al., *Use of transposon promoter-probe vectors in the metabolic engineering of the obligate methanotroph Methylomonas sp. strain 16a for enhanced C40 carotenoid synthesis*. Appl Environ Microbiol, 2007. **73**(6): p. 1721-8.
34. Henard, C.A., et al., *Bioconversion of methane to lactate by an obligate methanotrophic bacterium*. Scientific Reports, 2016. **6**: p. 21585.
35. Langridge, G.C., et al., *Simultaneous assay of every Salmonella Typhi gene using one million transposon mutants*. Genome Research, 2009. **19**(12): p. 2308-2316.
36. Söhngen, N., *Über bakterien, welche methan als kohlenstoffnahrung und energiequelle gebrauchen*. Zentrabl Bakteriöl Parasitenk Infektionskr, 1906. **15**: p. 513-517.
37. Brown, L., R. Strawinski, and C. McCleskey, *The isolation and characterization of Methanomonas methanooxidans Brown and Strawinski*. Canadian journal of microbiology, 1964. **10**(5): p. 791-799.
38. Davis, J., V. Coty, and J. Stanley, *Atmospheric nitrogen fixation by methane-oxidizing bacteria*. Journal of bacteriology, 1964. **88**(2): p. 468-472.
39. Foster, J. and R.H. Davis, *A methane-dependent coccus, with notes on classification and nomenclature of obligate, methane-utilizing bacteria*. Journal of bacteriology, 1966. **91**(5): p. 1924-1931.
40. Patt, T. and R. Hanson, *Intracytoplasmic membrane, phospholipid, and sterol content of Methylobacterium organophilum cells grown under different conditions*. Journal of bacteriology, 1978. **134**(2): p. 636-644.
41. Lynch, M.J., A.E. Wopat, and M.L. O'Connor, *Characterization of two new facultative methanotrophs*. Applied and environmental microbiology, 1980. **40**(2): p. 400-407.
42. Lidstrom-Oconnor, M.E., G.L. Fulton, and A.E. Wopat, *"Methylobacterium ethanolicum": a Syntrophic Association of Two Methylophilic Bacteria*. Microbiology, 1983. **129**(10): p. 3139-3148.
43. Stanley, S.H., et al., *Copper stress underlies the fundamental change in intracellular location of methane mono-oxygenase in methane-oxidizing organisms: Studies in batch and continuous cultures*. Biotechnology Letters, 1983. **5**(7): p. 487-492.
44. Ward, N., et al., *Genomic Insights into Methanotrophy: The Complete Genome Sequence of Methylococcus capsulatus (Bath)*. PLoS Biology, 2004. **2**(10): p. e303.
45. Hanson, R.S. and T.E. Hanson, *Methanotrophic bacteria*. Microbiological reviews, 1996. **60**(2): p. 439-471.
46. Semrau, J.D., A.A. DiSpirito, and S. Yoon, *Methanotrophs and copper*. FEMS Microbiology Reviews, 2010. **34**(4): p. 496-531.

47. Vigliotta, G., et al., *Clonothrix fusca* Roze 1896, a Filamentous, Sheathed, Methanotrophic γ -Proteobacterium. Applied and Environmental Microbiology, 2007. **73**(11): p. 3556-3565.
48. Stewart, E.J., *Growing Unculturable Bacteria*. Journal of Bacteriology, 2012. **194**(16): p. 4151-4160.
49. Burlage, R.S., et al., *Techniques in microbial ecology*. 1998: Oxford University Press on Demand.
50. Bowman, J.P., et al., *Revised taxonomy of the methanotrophs: description of Methylobacter gen. nov., emendation of Methylococcus, validation of Methylosinus and Methylocystis species, and a proposal that the family Methylococcaceae includes only the group I methanotrophs*. International Journal of Systematic and Evolutionary Microbiology, 1993. **43**(4): p. 735-753.
51. Strong, P.J., S. Xie, and W.P. Clarke, *Methane as a resource: can the methanotrophs add value?* Environmental science & technology, 2015. **49**(7): p. 4001-4018.
52. Malashenko, Y., et al., *Thermophilic and thermotolerant methane-assimilating bacteria*. Microbiology, 1975. **44**(5): p. 773-779.
53. Bowman, J., *The methanotrophs—the families Methylococcaceae and Methylocystaceae*, in *The prokaryotes*. 2006, Springer. p. 266-289.
54. Svenning, M.M., et al., *Isolation of methane oxidising bacteria from soil by use of a soil substrate membrane system*. FEMS Microbiology Ecology, 2003. **44**(3): p. 347-354.
55. Putzer, K., et al., *Separation of methanotrophic bacteria by using Percoll and its application to isolation of mixed and pure cultures*. Applied and environmental microbiology, 1991. **57**(12): p. 3656-3659.
56. Dalton, H., *Oxidation of hydrocarbons by methane monooxygenases from a variety of microbes*, in *Advances in Applied Microbiology*. 1980, Elsevier. p. 71-87.
57. Fox, B., et al., *Methane monooxygenase from Methylosinus trichosporium OB3b. Purification and properties of a three-component system with high specific activity from a type II methanotroph*. Journal of Biological Chemistry, 1989. **264**(17): p. 10023-10033.
58. Knapp, C.W., et al., *Methane monooxygenase gene expression mediated by methanobactin in the presence of mineral copper sources*. Proceedings of the National Academy of Sciences, 2007. **104**(29): p. 12040-12045.
59. Nies, D.H., *Microbial heavy-metal resistance*. Applied microbiology and biotechnology, 1999. **51**(6): p. 730-750.
60. Hakemian, A.S. and A.C. Rosenzweig, *The biochemistry of methane oxidation*. Annu. Rev. Biochem., 2007. **76**: p. 223-241.
61. Lieberman, R.L. and A.C. Rosenzweig, *Crystal structure of a membrane-bound metalloenzyme that catalyses the biological oxidation of methane*. Nature, 2005. **434**: p. 177.

62. Semrau, J.D., *Bioremediation via Methanotrophy: Overview of Recent Findings and Suggestions for Future Research*. Frontiers in microbiology, 2011. **2**: p. 209-209.
63. Burrows, K.J., et al., *Substrate specificities of the soluble and particulate methane mono-oxygenases of Methylosinus trichosporium OB3b*. Microbiology, 1984. **130**(12): p. 3327-3333.
64. DiSpirito, A.A., et al., *Trichloroethylene oxidation by the membrane-associated methane monooxygenase in type I, type II and type X methanotrophs*. Biodegradation, 1991. **2**(3): p. 151-164.
65. Stolyar, S., et al., *Role of multiple gene copies in particulate methane monooxygenase activity in the methane-oxidizing bacterium Methylococcus capsulatus Bath*. Microbiology, 1999. **145**(5): p. 1235-1244.
66. Jahng, D. and T. Wood, *Metal ions and chloramphenicol inhibition of soluble methane monooxygenase from Methylosinus trichosporium OB3b*. Applied Microbiology and Biotechnology, 1996. **45**(6): p. 744-749.
67. Sullivan, J.P., D. Dickinson, and H.A. Chase, *Methanotrophs, Methylosinus trichosporium OB3b, sMMO, and their application to bioremediation*. Critical reviews in microbiology, 1998. **24**(4): p. 335-373.
68. Rosenzweig, A.C., et al., *Crystal structures of the methane monooxygenase hydroxylase from Methylococcus capsulatus (Bath): implications for substrate gating and component interactions*. Proteins: Structure, Function, and Bioinformatics, 1997. **29**(2): p. 141-152.
69. Green, J. and H. Dalton, *Protein B of soluble methane monooxygenase from Methylococcus capsulatus (Bath). A novel regulatory protein of enzyme activity*. Journal of Biological Chemistry, 1985. **260**(29): p. 15795-15801.
70. LUND, J., M.P. WOODLAND, and H. DALTON, *Electron transfer reactions in the soluble methane monooxygenase of Methylococcus capsulatus (Bath)*. European journal of biochemistry, 1985. **147**(2): p. 297-305.
71. West, C.A., et al., *Functional expression in Escherichia coli of proteins B and C from soluble methane monooxygenase of Methylococcus capsulatus (Bath)*. J Gen Microbiol, 1992. **138**(7): p. 1301-7.
72. Lock, M., et al., *Mutagenesis and expression of methane monooxygenase to alter regioselectivity with aromatic substrates*. FEMS Microbiology Letters, 2017. **364**(13): p. fnx137.
73. S. Lloyd, J., et al., *Heterologous expression of soluble methane monooxygenase genes in methanotrophs containing only particulate methane monooxygenase*. Vol. 171. 1999. 364-370.
74. Murrell, J.C., B. Gilbert, and I.R. McDonald, *Molecular biology and regulation of methane monooxygenase*. Archives of microbiology, 2000. **173**(5-6): p. 325-332.
75. Costello, A.M. and M.E. Lidstrom, *Molecular Characterization of Functional and Phylogenetic Genes from Natural Populations of*

- Methanotrophs in Lake Sediments*. Applied and Environmental Microbiology, 1999. **65**(11): p. 5066-5074.
76. Baesman, S.M., et al., *Methane Oxidation and Molecular Characterization of Methanotrophs from a Former Mercury Mine Impoundment*. Microorganisms, 2015. **3**(2): p. 290-309.
 77. Dianou, D. and K. Adachi, *Characterization of methanotrophic bacteria isolated from a subtropical paddy field*. FEMS Microbiology Letters, 1999. **173**(1): p. 163-173.
 78. Takeda, K., et al., *Characterization of cultivable methanotrophs from paddy soils and rice roots*. Soil Science and Plant Nutrition, 2008. **54**(6): p. 876-885.
 79. Kip, N., et al., *Detection, Isolation, and Characterization of Acidophilic Methanotrophs from Sphagnum Mosses*. Applied and Environmental Microbiology, 2011. **77**(16): p. 5643-5654.
 80. Pieja, A.J., K.H. Rostkowski, and C.S. Criddle, *Distribution and Selection of Poly-3-Hydroxybutyrate Production Capacity in Methanotrophic Proteobacteria*. Microbial Ecology, 2011. **62**(3): p. 564-573.
 81. Calhoun, A. and G.M. King, *Characterization of Root-Associated Methanotrophs from Three Freshwater Macrophytes: Pontederia cordata, Sparganium eurycarpum, and Sagittaria latifolia*. Applied and Environmental Microbiology, 1998. **64**(3): p. 1099-1105.
 82. Graham, D.W., et al., *Applications of a colorimetric plate assay for soluble methane monooxygenase activity*. Applied and Environmental Microbiology, 1992. **58**(7): p. 2231-2236.
 83. Han, X., et al., *Mitigating methane emission from paddy soil with rice-straw biochar amendment under projected climate change*. Scientific Reports, 2016. **6**: p. 24731.
 84. Im, J. and J.D. Semrau, *Pollutant degradation by a Methylocystis strain SB2 grown on ethanol: bioremediation via facultative methanotrophy*. FEMS Microbiol Lett, 2011. **318**(2): p. 137-42.
 85. Baani, M. and W. Liesack, *Two isozymes of particulate methane monooxygenase with different methane oxidation kinetics are found in Methylocystis sp. strain SC2*. Proc Natl Acad Sci U S A, 2008. **105**(29): p. 10203-8.
 86. Marx, C.J. and M.E. Lidstrom, *Broad-host-range cre-lox system for antibiotic marker recycling in gram-negative bacteria*. BioTechniques, 2002. **33**(5): p. 1062-1067.
 87. Toukdarian, A.E. and M.E. Lidstrom, *Molecular construction and characterization of nif mutants of the obligate methanotroph Methylosinus sp. strain 6*. Journal of Bacteriology, 1984. **157**(3): p. 979-983.
 88. Commons, M., *Genetics*. 2018.
 89. Kalyuzhnaya, M.G., A.W. Puri, and M.E. Lidstrom, *Metabolic engineering in methanotrophic bacteria*. Metab Eng, 2015. **29**: p. 142-152.
 90. News, U.o.W., *Energy Dept. funds UW project to turn wasted natural gas into diesel*. 2012.
 91. Coleman, W.J., et al., *Biological Conversion of Multi-Carbon Compounds from Methane*. 2014, Google Patents.

92. Swana, J., et al., *An analysis of net energy production and feedstock availability for biobutanol and bioethanol*. Bioresour Technol, 2011. **102**: p. 2112 - 2117.
93. Periana, R.A., et al., *A Mercury-Catalyzed, High-Yield System for the Oxidation of Methane to Methanol*. Science, 1993. **259**(5093): p. 340-343.
94. Duan, C., M. Luo, and X. Xing, *High-rate conversion of methane to methanol by Methylosinus trichosporium OB3b*. Bioresource Technology, 2011. **102**(15): p. 7349-7353.
95. Patel, S.K.S., et al., *Methanol production from simulated biogas mixtures by co-immobilized Methylobacterium methanica and Methylocella tundrata*. Bioresource Technology, 2018. **263**: p. 25-32.
96. Ivanova, E.G., et al., *Production of vitamin B12 in aerobic methylotrophic bacteria*. Microbiology, 2006. **75**(4): p. 494-496.
97. Sharpe, P.L., et al., *Use of Transposon Promoter-Probe Vectors in the Metabolic Engineering of the Obligate Methanotroph Methylobacterium sp. Strain 16a for Enhanced C40 Carotenoid Synthesis*. Applied and Environmental Microbiology, 2007. **73**(6): p. 1721-1728.
98. Asenjo, J.A. and J.S. Suk, *Microbial Conversion of Methane into poly-β-hydroxybutyrate (PHB): Growth and intracellular product accumulation in a type II methanotroph*. Journal of Fermentation Technology, 1986. **64**(4): p. 271-278.
99. Mousavioun, P., P.J. Halley, and W.O.S. Doherty, *Thermophysical properties and rheology of PHB/lignin blends*. Industrial Crops and Products, 2013. **50**: p. 270-275.
100. Cal, A.J., et al., *Methanotrophic production of polyhydroxybutyrate-co-hydroxyvalerate with high hydroxyvalerate content*. International Journal of Biological Macromolecules, 2016. **87**: p. 302-307.
101. Wendlandt, K.D., et al., *Producing poly-3-hydroxybutyrate with a high molecular mass from methane*. Journal of Biotechnology, 2001. **86**(2): p. 127-133.
102. López, J.C., et al., *Biogas-based polyhydroxyalkanoates production by Methylocystis hirsuta: A step further in anaerobic digestion biorefineries*. Chemical Engineering Journal, 2018. **333**: p. 529-536.
103. Cal, A.J., et al., *Methanotrophic production of polyhydroxybutyrate-co-hydroxyvalerate with high hydroxyvalerate content*. Int J Biol Macromol, 2016. **87**: p. 302-7.
104. Materials, M., 2018.
105. Bothe, H., et al., *Heterotrophic bacteria growing in association with Methylococcus capsulatus (Bath) in a single cell protein production process*. Applied Microbiology and Biotechnology, 2002. **59**(1): p. 33-39.
106. Calysta, *CalystaCommercialization*. 2018.
107. Calysta, *Loop Reactor*. 2018.
108. Strong, P.J., et al., *The Opportunity for High-Performance Biomaterials from Methane*. Microorganisms, 2016. **4**(1): p. 11.

109. Carlson, D.F., et al., *Efficient TALEN-mediated gene knockout in livestock*. Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(43): p. 17382-17387.
110. Miller, J.C., et al., *An improved zinc-finger nuclease architecture for highly specific genome editing*. Nature Biotechnology, 2007. **25**: p. 778.
111. Deveau, H., J.E. Garneau, and S. Moineau, *CRISPR/Cas System and Its Role in Phage-Bacteria Interactions*. Annual Review of Microbiology, 2010. **64**(1): p. 475-493.
112. Sapranaukas, R., et al., *The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli*. Nucleic Acids Research, 2011. **39**(21): p. 9275-9282.
113. Barrangou, R., et al., *CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes*. Science, 2007. **315**(5819): p. 1709-1712.
114. Garneau, J.E., et al., *The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA*. Nature, 2010. **468**: p. 67.
115. Rouet, P., F. Smih, and M. Jasin, *Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells*. Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(13): p. 6064-6068.
116. Ran, F.A., et al., *Genome engineering using the CRISPR-Cas9 system*. Nature protocols, 2013. **8**(11): p. 2281-2308.
117. Ishino, Y., et al., *Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product*. Journal of Bacteriology, 1987. **169**(12): p. 5429-5433.
118. Hermans, P.W., et al., *Insertion element IS987 from Mycobacterium bovis BCG is located in a hot-spot integration region for insertion elements in Mycobacterium tuberculosis complex strains*. Infection and Immunity, 1991. **59**(8): p. 2695-2705.
119. Mojica, F.J.M., G. Juez, and F. Rodriguez - Valera, *Transcription at different salinities of Haloferax mediterranei sequences adjacent to partially modified PstI sites*. Molecular Microbiology, 1993. **9**(3): p. 613-621.
120. Mojica, F.J.M., et al., *Long stretches of short tandem repeats are present in the largest replicons of the Archaea Haloferax mediterranei and Haloferax volcanii and could be involved in replicon partitioning*. Molecular Microbiology, 1995. **17**(1): p. 85-93.
121. Mojica, F.J.M., et al., *Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements*. Journal of Molecular Evolution, 2005. **60**(2): p. 174-182.
122. Barrangou, R., et al., *CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes*. Science, 2007. **315**(5819): p. 1709.

123. Mojica Francisco, J.M., et al., *Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria*. Molecular Microbiology, 2002. **36**(1): p. 244-246.
124. Jansen, R., et al., *Identification of genes that are associated with DNA repeats in prokaryotes*. Molecular Microbiology, 2002. **43**(6): p. 1565-1575.
125. Jinek, M., et al., *A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity*. Science, 2012. **337**(A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity6096): p. 816-821.
126. Tang, T.-H., et al., *Identification of 86 candidates for small non-messenger RNAs from the archaeon *Archaeoglobus fulgidus**. Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(11): p. 7536-7541.
127. Deltcheva, E., et al., *CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III*. Nature, 2011. **471**: p. 602.
128. Zetsche, B., et al., *Cpf1 is a single RNA-guided endonuclease of a Class 2 CRISPR-Cas system*. Cell, 2015. **163**(3): p. 759-771.
129. Deveau, H., et al., *Phage Response to CRISPR-Encoded Resistance in *Streptococcus thermophilus**. Journal of Bacteriology, 2008. **190**(4): p. 1390-1400.
130. Makarova, K.S. and E.V. Koonin, *Annotation and Classification of CRISPR-Cas Systems*, in *CRISPR: Methods and Protocols*, M. Lundgren, E. Charpentier, and P.C. Fineran, Editors. 2015, Springer New York: New York, NY. p. 47-75.
131. Anders, C., et al., *Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease*. Nature, 2014. **513**(7519): p. 569-573.
132. Mojica, F.J.M., et al., *Short motif sequences determine the targets of the prokaryotic CRISPR defence system*. Microbiology, 2009. **155**(3): p. 733-740.
133. Ruud., J., et al., *Identification of genes that are associated with DNA repeats in prokaryotes*. Molecular Microbiology, 2002. **43**(6): p. 1565-1575.
134. Haft, D.H., et al., *A Guild of 45 CRISPR-Associated (Cas) Protein Families and Multiple CRISPR/Cas Subtypes Exist in Prokaryotic Genomes*. PLoS Computational Biology, 2005. **1**(6): p. e60.
135. Wiedenheft, B., S.H. Sternberg, and J.A. Doudna, *RNA-guided genetic silencing systems in bacteria and archaea*. Nature, 2012. **482**: p. 331.
136. Rodolphe, B., *CRISPR - Cas systems and RNA - guided interference*. Wiley Interdisciplinary Reviews: RNA, 2013. **4**(3): p. 267-278.
137. Westra, E.R., et al., *The CRISPRs, They Are A-Changin': How Prokaryotes Generate Adaptive Immunity*. Annual Review of Genetics, 2012. **46**(1): p. 311-339.
138. Koonin, E.V., K.S. Makarova, and F. Zhang, *Diversity, classification and evolution of CRISPR-Cas systems*. Current opinion in microbiology, 2017. **37**: p. 67-78.

139. Shmakov, S., et al., *Diversity and evolution of class 2 CRISPR–Cas systems*. Nature reviews. Microbiology, 2017. **15**(3): p. 169-182.
140. Makarova, K.S., et al., *An updated evolutionary classification of CRISPR–Cas systems*. Nature Reviews Microbiology, 2015. **13**: p. 722.
141. Nuñez, J.K., et al., *Cas1–Cas2 complex formation mediates spacer acquisition during CRISPR–Cas adaptive immunity*. Nature Structural & Molecular Biology, 2014. **21**: p. 528.
142. Yosef, I., M.G. Goren, and U. Qimron, *Proteins and DNA elements essential for the CRISPR adaptation process in Escherichia coli*. Nucleic Acids Research, 2012. **40**(12): p. 5569-5576.
143. Hooton, S.P.T. and I.F. Connerton, *Campylobacter jejuni acquire new host-derived CRISPR spacers when in association with bacteriophages harboring a CRISPR-like Cas4 protein*. Frontiers in Microbiology, 2014. **5**: p. 744.
144. Wei, Y., R.M. Terns, and M.P. Terns, *Cas9 function and host genome sampling in Type II-A CRISPR–Cas adaptation*. Genes & Development, 2015. **29**(4): p. 356-361.
145. Makarova, K.S., et al., *An updated evolutionary classification of CRISPR–Cas systems*. Nature reviews. Microbiology, 2015. **13**(11): p. 722-736.
146. Wang, R., et al., *Interaction of the Cas6 Riboendonuclease with CRISPR RNAs: Recognition and Cleavage*. Structure, 2011. **19**(2): p. 257-264.
147. Huo, Y., et al., *Structures of CRISPR Cas3 offer mechanistic insights into Cascade-activated DNA unwinding and degradation*. Nature Structural & Molecular Biology, 2014. **21**: p. 771.
148. Beloglazova, N., et al., *Structure and activity of the Cas3 HD nuclease MJ0384, an effector enzyme of the CRISPR interference*. The EMBO Journal, 2011. **30**(22): p. 4616-4627.
149. Jinek, M., et al., *A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity*. Science, 2012. **337**(6096): p. 816-821.
150. Brendel, J., et al., *A Complex of Cas Proteins 5, 6, and 7 Is Required for the Biogenesis and Stability of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-derived RNAs (crRNAs) in Haloferax volcanii*. Journal of Biological Chemistry, 2014. **289**(10): p. 7164-7177.
151. Samai, P., et al., *Co-transcriptional DNA and RNA Cleavage during Type III CRISPR-Cas Immunity*. Cell, 2015. **161**(5): p. 1164-1174.
152. Ramia, Nancy F., et al., *Essential Structural and Functional Roles of the Cmr4 Subunit in RNA Cleavage by the Cmr CRISPR-Cas Complex*. Cell Reports, 2014. **9**(5): p. 1610-1617.
153. Bengtsson, N.E., et al., *Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy*. Nature Communications, 2017. **8**: p. 14454.

154. Hu, W., et al., *RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection*. Proceedings of the National Academy of Sciences of the United States of America, 2014. **111**(31): p. 11461-11466.
155. Kaminski, R., et al., *Elimination of HIV-1 Genomes from Human T-lymphoid Cells by CRISPR/Cas9 Gene Editing*. Scientific Reports, 2016. **6**: p. 22555.
156. Ghorbal, M., et al., *Genome editing in the human malaria parasite Plasmodium falciparum using the CRISPR-Cas9 system*. Nature Biotechnology, 2014. **32**: p. 819.
157. Gantz, V.M., et al., *Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi**. Proceedings of the National Academy of Sciences, 2015. **112**(49): p. E6736-E6743.
158. Xue, W., et al., *CRISPR-mediated direct mutation of cancer genes in the mouse liver*. Nature, 2014. **514**: p. 380.
159. Chen, S., et al., *Genome-wide CRISPR Screen in a Mouse Model of Tumor Growth and Metastasis*. Cell, 2015. **160**(6): p. 1246-1260.
160. Hu, Z., et al., *Disruption of HPV16-E7 by CRISPR/Cas System Induces Apoptosis and Growth Inhibition in HPV16 Positive Human Cervical Cancer Cells*. BioMed Research International, 2014. **2014**: p. 612823.
161. Kochenderfer, J.N., et al., *Chemotherapy-Refractory Diffuse Large B-Cell Lymphoma and Indolent B-Cell Malignancies Can Be Effectively Treated With Autologous T Cells Expressing an Anti-CD19 Chimeric Antigen Receptor*. Journal of Clinical Oncology, 2015. **33**(6): p. 540-549.
162. Rodríguez-Leal, D., et al., *Engineering Quantitative Trait Variation for Crop Improvement by Genome Editing*. Cell, 2017. **171**(2): p. 470-480.e8.
163. Oishi, I., et al., *Targeted mutagenesis in chicken using CRISPR/Cas9 system*. Scientific Reports, 2016. **6**: p. 23980.
164. Ma, H., et al., *Correction of a pathogenic gene mutation in human embryos*. Nature, 2017. **548**: p. 413.
165. Gratz, S.J., et al., *CRISPR-Cas9 genome editing in Drosophila*. Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.], 2015. **111**: p. 31.2.1-31.2.20.
166. Vejnar, C.E., et al., *Optimized CRISPR–Cas9 System for Genome Editing in Zebrafish*. Cold Spring Harbor Protocols, 2016. **2016**(10): p. pdb.prot086850.
167. Dickinson, D.J. and B. Goldstein, *CRISPR-Based Methods for *Caenorhabditis elegans* Genome Engineering*. Genetics, 2016. **202**(3): p. 885.
168. Yan, M.-Y., et al., *CRISPR-Cas12a-Assisted Recombineering in Bacteria*. Applied and Environmental Microbiology, 2017. **83**(17): p. e00947-17.
169. Jiang, W., et al., *RNA-guided editing of bacterial genomes using CRISPR-Cas systems*. Nature Biotechnology, 2013. **31**: p. 233.

170. McAllister, K.N., et al., *Using CRISPR-Cas9-mediated genome editing to generate C. difficile mutants defective in selenoproteins synthesis*. Scientific Reports, 2017. **7**(1): p. 14672.
171. Liu, J., et al., *Development of a CRISPR/Cas9 genome editing toolbox for Corynebacterium glutamicum*. Microbial Cell Factories, 2017. **16**(1): p. 205.
172. Wang, Y., et al., *Bacterial Genome Editing with CRISPR-Cas9: Deletion, Integration, Single Nucleotide Modification, and Desirable "Clean" Mutant Selection in Clostridium beijerinckii as an Example*. ACS Synthetic Biology, 2016. **5**(7): p. 721-732.
173. Song, X., et al., *CRISPR-Cas9(D10A) Nickase-Assisted Genome Editing in Lactobacillus casei*. Applied and Environmental Microbiology, 2017. **83**(22): p. e01259-17.
174. Rock, J.M., et al., *Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform*. Nature microbiology, 2017. **2**: p. 16274-16274.
175. Mougiakos, I., et al., *Characterizing a thermostable Cas9 for bacterial genome editing and silencing*. Nature Communications, 2017. **8**(1): p. 1647.
176. Tan, S.Z., C.R. Reisch, and K.L.J. Prather, *A Robust CRISPR Interference Gene Repression System in Pseudomonas*. Journal of Bacteriology, 2018. **200**(7).
177. Chen, W., et al., *Rapid and Efficient Genome Editing in Staphylococcus aureus by Using an Engineered CRISPR/Cas9 System*. Journal of the American Chemical Society, 2017. **139**(10): p. 3790-3795.
178. Bernheim, A., et al., *Inhibition of NHEJ repair by type II-A CRISPR-Cas systems in bacteria*. Nature Communications, 2017. **8**(1): p. 2094.
179. Stachler, A.-E., et al., *High tolerance to self-targeting of the genome by the endogenous CRISPR-Cas system in an archaeon*. Nucleic Acids Research, 2017. **45**(9): p. 5208-5216.
180. Nayak, D.D. and W.W. Metcalf, *Cas9-mediated genome editing in the methanogenic archaeon Methanosarcina acetivorans*. Proceedings of the National Academy of Sciences, 2017. **114**(11): p. 2976-2981.
181. Gasiunas, G., et al., *Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria*. Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(39): p. E2579-E2586.
182. Renaud, J.-B., et al., *Improved Genome Editing Efficiency and Flexibility Using Modified Oligonucleotides with TALEN and CRISPR-Cas9 Nucleases*. Cell Reports, 2016. **14**(9): p. 2263-2272.
183. Maruyama, T., et al., *Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining*. Nat Biotechnol, 2015. **33**(5): p. 538-42.
184. Li, G., et al., *Small molecules enhance CRISPR/Cas9-mediated homology-directed genome editing in primary cells*. Scientific Reports, 2017. **7**(1): p. 8943.

185. Zhang, J.-P., et al., *Efficient precise knockin with a double cut HDR donor after CRISPR/Cas9-mediated double-stranded DNA cleavage*. *Genome Biology*, 2017. **18**(1): p. 35.
186. Clarke, R., et al., *Enhanced Bacterial Immunity and Mammalian Genome Editing via RNA-Polymerase-Mediated Dislodging of Cas9 from Double-Strand DNA Breaks*. *Molecular Cell*, 2018. **71**(1): p. 42-55.e8.
187. SanMiguel, P., et al., *Nested Retrotransposons in the Intergenic Regions of the Maize Genome*. *Science*, 1996. **274**(5288): p. 765-768.
188. Creighton, H.B. and B. McClintock, *The Correlation of Cytological and Genetical Crossing-Over in Zea Mays. A Corroboration*. *Proceedings of the National Academy of Sciences of the United States of America*, 1935. **21**(3): p. 148-150.
189. McClintock, B., *The Origin and Behavior of Mutable Loci in Maize*. *Proceedings of the National Academy of Sciences of the United States of America*, 1950. **36**(6): p. 344-355.
190. Jordan, E., H. Saedler, and P. Starlinger, *OO and strong-polar mutations in the gal operon are insertions*. Vol. 102. 1968. 353-363.
191. Rubin, G.M., M.G. Kidwell, and P.M. Bingham, *The molecular basis of P-M hybrid dysgenesis: The nature of induced mutations*. *Cell*, 1982. **29**(3): p. 987-994.
192. Kazazian Jr, H.H., et al., *Haemophilia A resulting from de novo insertion of L1 sequences represents a novel mechanism for mutation in man*. *Nature*, 1988. **332**: p. 164.
193. Lerman, D.N. and M.E. Feder, *Naturally Occurring Transposable Elements Disrupt hsp70 Promoter Function in Drosophila melanogaster*. *Molecular Biology and Evolution*, 2005. **22**(3): p. 776-783.
194. Clegg, M.T. and M.L. Durbin, *Tracing floral adaptations from ecology to molecules*. *Nature Reviews Genetics*, 2003. **4**: p. 206.
195. Kidwell, M.G. and D.R. Lisch, *PERSPECTIVE: TRANSPOSABLE ELEMENTS, PARASITIC DNA, AND GENOME EVOLUTION*. *Evolution*, 2001. **55**(1): p. 1-24.
196. Alekshun, M.N. and S.B. Levy, *Molecular Mechanisms of Antibacterial Multidrug Resistance*. *Cell*, 2007. **128**(6): p. 1037-1050.
197. Burns, K.H., *Transposable elements in cancer*. *Nature Reviews Cancer*, 2017. **17**: p. 415.
198. Collier, L.S., et al., *Cancer gene discovery in solid tumours using transposon-based somatic mutagenesis in the mouse*. *Nature*, 2005. **436**: p. 272.
199. Lidholm, D.A., A.R. Lohe, and D.L. Hartl, *The Transposable Element Mariner Mediates Germline Transformation in Drosophila Melanogaster*. *Genetics*, 1993. **134**(3): p. 859-868.
200. Rubin, G. and A. Spradling, *Genetic transformation of Drosophila with transposable element vectors*. *Science*, 1982. **218**(4570): p. 348-353.

201. Meissner, R., et al., *A high throughput system for transposon tagging and promoter trapping in tomato*. The Plant Journal, 2000. **22**(3): p. 265-274.
202. Barquist, L., et al., *A comparison of dense transposon insertion libraries in the Salmonella serovars Typhi and Typhimurium*. Nucleic Acids Research, 2013. **41**(8): p. 4549-4564.
203. Dziva, F., et al., *Sequencing and Functional Annotation of Avian Pathogenic Escherichia coli Serogroup O78 Strains Reveal the Evolution of E. coli Lineages Pathogenic for Poultry via Distinct Mechanisms*. Infection and Immunity, 2013. **81**(3): p. 838-849.
204. Luft, F.C., *Sleeping Beauty jumps to new heights*. Journal of Molecular Medicine, 2010. **88**(7): p. 641-643.
205. Goodier, J.L. and H.H. Kazazian, Jr., *Retrotransposons Revisited: The Restraint and Rehabilitation of Parasites*. Cell, 2008. **135**(1): p. 23-35.
206. Beauregard, A., M.J. Curcio, and M. Belfort, *The take and give between retrotransposable elements and their hosts*. Annual review of genetics, 2008. **42**: p. 587-617.
207. Muñoz-López, M. and J.L. García-Pérez, *DNA Transposons: Nature and Applications in Genomics*. Current Genomics, 2010. **11**(2): p. 115-128.
208. Krystine Garcia, T.J., Alexander Meyers. Available from: <https://www.google.co.uk/search?q=Krystine+Garcia%2C+Tao+Jing%2C+Alexander+Meyers&oq=Krystine+Garcia%2C+Tao+Jing%2C+Alexander+Meyers&aqs=chrome..69i57.680j0j7&sourceid=chrome&ie=UTF-8>
209. Arkhipova, I.R. and M. Meselson, *Diverse DNA transposons in rotifers of the class Bdelloidea*. Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(33): p. 11781-11786.
210. Robertson, H.M., *The mariner transposable element is widespread in insects*. Nature, 1993. **362**: p. 241.
211. Plasterk, R.H.A., Z. Izsvák, and Z. Ivics, *Resident aliens: the Tc1/mariner superfamily of transposable elements*. Trends in Genetics, 1999. **15**(8): p. 326-332.
212. Radice, A.D., et al., *Widespread occurrence of the Tc1 transposon family: Tc1-like transposons from teleost fish*. Molecular and General Genetics MGG, 1994. **244**(6): p. 606-612.
213. Lampe, D.J., M.E. Churchill, and H.M. Robertson, *A purified mariner transposase is sufficient to mediate transposition in vitro*. The EMBO Journal, 1996. **15**(19): p. 5470-5479.
214. Morisato, D. and N. Kleckner, *Tn10 transposition and circle formation in vitro*. Cell, 1987. **51**(1): p. 101-111.
215. Kristich, C.J., et al., *Development and Use of an Efficient System for Random mariner Transposon Mutagenesis To Identify Novel Genetic Determinants of Biofilm Formation in the Core Enterococcus faecalis Genome*. Applied and Environmental Microbiology, 2008. **74**(11): p. 3377-3386.

216. Liu, H., et al., *Use of a Mariner-Based Transposon Mutagenesis System To Isolate Clostridium perfringens Mutants Deficient in Gliding Motility*. Journal of Bacteriology, 2013. **195**(3): p. 629-636.
217. Dembek, M., et al., *High-Throughput Analysis of Gene Essentiality and Sporulation in Clostridium difficile*. mBio, 2015. **6**(2): p. e02383-14.
218. Reznikoff, W.S., *The Tn5 transposon*. Annu Rev Microbiol, 1993. **47**: p. 945-63.
219. Berg, D.E., et al., *Transposition of R factor genes to bacteriophage lambda*. Proceedings of the National Academy of Sciences of the United States of America, 1975. **72**(9): p. 3628-3632.
220. Wiegand, T.W. and W.S. Reznikoff, *Characterization of two hypertransposing Tn5 mutants*. J Bacteriol, 1992. **174**(4): p. 1229-39.
221. Ahmed, A. and L. Podemski, *The revised nucleotide sequence of Tn5*. Gene, 1995. **154**(1): p. 129-30.
222. Makris, J.C., P.L. Nordmann, and W.S. Reznikoff, *Mutational analysis of insertion sequence 50 (IS50) and transposon 5 (Tn5) ends*. Proc Natl Acad Sci U S A, 1988. **85**(7): p. 2224-8.
223. Johnson, R.C. and W.S. Reznikoff, *Role of the IS50 R proteins in the promotion and control of Tn5 transposition*. J Mol Biol, 1984. **177**(4): p. 645-61.
224. Krebs, M.P. and W.S. Reznikoff, *Transcriptional and translational initiation sites of IS50. Control of transposase and inhibitor expression*. J Mol Biol, 1986. **192**(4): p. 781-91.
225. Reznikoff, W.S., *Transposon Tn5*. Annu Rev Genet, 2008. **42**: p. 269-86.
226. Schulz, V.P. and W.S. Reznikoff, *Translation initiation of IS50R read-through transcripts*. Journal of Molecular Biology, 1991. **221**(1): p. 65-80.
227. Yin, J.C.P., M.P. Krebs, and W.S. Reznikoff, *Effect of dam methylation on Tn5 transposition*. Journal of Molecular Biology, 1988. **199**(1): p. 35-45.
228. Yin, J.C. and W.S. Reznikoff, *p2 and inhibition of Tn5 transposition*. Journal of Bacteriology, 1988. **170**(7): p. 3008-3015.
229. Ason, B., et al., *Targeting Tn5 Transposase Identifies Human Immunodeficiency Virus Type 1 Inhibitors*. Antimicrobial Agents and Chemotherapy, 2005. **49**(5): p. 2035-2043.
230. Craig, N.L., *Tn7: a target site-specific transposon*. Molecular Microbiology, 1991. **5**(11): p. 2569-2573.
231. van Opijnen, T., K.L. Bodi, and A. Camilli, *Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms*. Nat Methods, 2009. **6**(10): p. 767-72.
232. Khatiwara, A., et al., *Genome scanning for conditionally essential genes in Salmonella enterica Serotype Typhimurium*. Appl Environ Microbiol, 2012. **78**(9): p. 3098-107.
233. Gawronski, J.D., et al., *Tracking insertion mutants within libraries by deep sequencing and a genome-wide screen for Haemophilus genes required in the lung*. Proc Natl Acad Sci U S A, 2009. **106**(38): p. 16422-7.

234. Goodman, A.L., et al., *Identifying genetic determinants needed to establish a human gut symbiont in its habitat*. Cell Host Microbe, 2009. **6**(3): p. 279-89.
235. van Opijnen, T. and A. Camilli, *Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms*. Nature Reviews Microbiology, 2013. **11**: p. 435.
236. Andrews-Polymenis, H.L., C.A. Santiviago, and M. McClelland, *Novel genetic tools for studying food-borne Salmonella*. Curr Opin Biotechnol, 2009. **20**(2): p. 149-57.
237. Barquist, L., et al., *The TraDIS toolkit: sequencing and analysis for dense transposon mutant libraries*. Bioinformatics, 2016. **32**(7): p. 1109-1111.
238. Martin, H. and J.C. Murrell, *Methane monooxygenase mutants of Methylosinus trichosporium constructed by marker-exchange mutagenesis*. FEMS Microbiology Letters, 1995. **127**(3): p. 243-248.
239. Hanahan, D., *Studies on transformation of Escherichia-coli with plasmids*. Journal of Molecular Biology, 1983. **166**(4): p. 557-580.
240. NEB, *NEB® 5-alpha Competent E. coli (High Efficiency)*. 2018.
241. Simon, R., U. Priefer, and A. Puhler, *A broad host range mobilization system for invivo genetic-engineering - transposon mutagenesis in gram-negative bacteria*. Bio-Technology, 1983. **1**(9): p. 784-791.
242. Biomedal, *E.coli S17-1 lambda pir*. 2018.
243. Agilent, *E.coli XL-1 Blue*. 2018.
244. NEB, *Making your own chemically competent cells*. 2018.
245. Center, M.U.a.G.Q.I., *Massively Parallel Sequencing - PacBio requirement*. 2016.
246. Bussmann, I., et al., *Preferential cultivation of type II methanotrophic bacteria from littoral sediments (Lake Constance)*. FEMS Microbiology Ecology, 2004. **47**(2): p. 179-189.
247. Puri, A.W., et al., *Genetic tools for the industrially promising methanotroph Methylocystis parvus*. Appl Environ Microbiol, 2015. **81**(5): p. 1775-81.
248. Pieja, A.J., E.R. Sundstrom, and C.S. Criddle, *Poly-3-Hydroxybutyrate Metabolism in the Type II Methanotroph Methylocystis parvus*. Applied and Environmental Microbiology, 2011. **77**(17): p. 6012-6019.
249. Rahnama, F., et al., *PHB production by Methylocystis hirsuta from natural gas in a bubble column and a vertical loop bioreactor*. Biochemical Engineering Journal, 2012. **65**: p. 51-56.
250. Hoefman, S., et al., *Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria*. Microbial biotechnology, 2012. **5**(3): p. 368-378.
251. Bourne, D.G., I.R. McDonald, and J.C. Murrell, *Comparison of pmoA PCR Primer Sets as Tools for Investigating Methanotroph Diversity in Three Danish Soils*. Applied and Environmental Microbiology, 2001. **67**(9): p. 3802-3809.

252. Auman, A.J., et al., *Molecular characterization of methanotrophic isolates from freshwater lake sediment*. Applied and Environmental Microbiology, 2000. **66**(12): p. 5259-5266.
253. Jhala, Y., et al., *Rapid methods for isolation and screening of methane degrading bacteria*. J. Bioremed. Biodeg, 2015. **7**(1): p. 1-5.
254. Stepniewska, Z., et al., *Enrichment culture and identification of endophytic methanotrophs isolated from peatland plants*. Folia Microbiol (Praha), 2017. **62**(5): p. 381-391.
255. Ali, H., et al., *Duplication of the mmoX gene in Methylosinus sporium: cloning, sequencing and mutational analysis*. Microbiology, 2006. **152**(10): p. 2931-2942.
256. Hakemian, A.S. and A.C. Rosenzweig, *The Biochemistry of Methane Oxidation*. Annual Review of Biochemistry, 2007. **76**(1): p. 223-241.
257. Auman, A.J., et al., *Molecular characterization of methanotrophic isolates from freshwater lake sediment*. Appl Environ Microbiol, 2000. **66**(12): p. 5259-66.
258. Pacheco-Oliver, M., et al., *Detection of methanotrophs with highly divergent pmoA genes from Arctic soils*. FEMS Microbiol Lett, 2002. **209**(2): p. 313-9.
259. Kalyuzhnaya, M.G., et al., *Highly efficient methane biocatalysis revealed in a methanotrophic bacterium*. Nature Communications, 2013. **4**: p. 2785.
260. Yang, Y.M., et al., *Production of 3-hydroxypropionic acid in engineered Methylobacterium extorquens AM1 and its reassimilation through a reductive route*. Microb Cell Fact, 2017. **16**(1): p. 179.
261. Holmes, A.J., N.J. Owens, and J.C. Murrell, *Detection of novel marine methanotrophs using phylogenetic and functional gene probes after methane enrichment*. Microbiology, 1995. **141**(8): p. 1947-1955.
262. Ryan, T., T. Callahan, and S.R. King, *Engine Knock Rating of Natural Gases—Methane Number*. Journal of engineering for gas turbines and Power, 1993. **115**(4): p. 769-776.
263. Álvarez-Flórez, J. and E. Egusquiza, *Analysis of damage caused by siloxanes in stationary reciprocating internal combustion engines operating with landfill gas*. Engineering Failure Analysis, 2015. **50**: p. 29-38.
264. Jensen, P.R. and K. Hammer, *Artificial promoters for metabolic optimization*. Biotechnology and Bioengineering, 1998. **58**(2 - 3): p. 191-195.
265. Ali, H. and J.C. Murrell, *Development and validation of promoter-probe vectors for the study of methane monooxygenase gene expression in Methylococcus capsulatus Bath*. Microbiology, 2009. **155**(3): p. 761-771.
266. Phornphisutthimas, S., A. Thamchaipenet, and B. Panijpan, *Conjugation in Escherichia coli*. Biochemistry and molecular biology education, 2007. **35**(6): p. 440-445.

267. Crombie, A. and J.C. Murrell, *Chapter eight - Development of a System for Genetic Manipulation of the Facultative Methanotroph Methylocella silvestris BL2*, in *Methods in Enzymology*, A.C. Rosenzweig and S.W. Ragsdale, Editors. 2011, Academic Press. p. 119-133.
268. Jennert, K.C.B., et al., *Gene transfer to Clostridium cellulolyticum ATCC 35319*. *Microbiology*, 2000. **146**(12): p. 3071-3080.
269. Pieja, A.J., K.H. Rostkowski, and C.S. Criddle, *Distribution and selection of poly-3-hydroxybutyrate production capacity in methanotrophic proteobacteria*. *Microb Ecol*, 2011. **62**(3): p. 564-73.
270. Medvedkova, K., et al., *Synthesis of melanin by a moderately thermophilic methanotroph Methylocaldum szegediense depends on cultivation temperature*. *Microbiology*, 2008. **77**(1): p. 112-114.
271. Jahn, M., et al., *Copy number variability of expression plasmids determined by cell sorting and Droplet Digital PCR*. *Microbial Cell Factories*, 2016. **15**(1): p. 211.
272. Puri, A.W., et al., *Genetic Tools for the Industrially Promising Methanotroph *Methylomicrobium buryatense**. *Applied and Environmental Microbiology*, 2015. **81**(5): p. 1775-1781.
273. Shishkina, V.N. and Y.A. Trotsenko, *Multiple enzymic lesions in obligate methanotrophic bacteria*. *FEMS Microbiology Letters*, 1982. **13**(3): p. 237-242.
274. Wood, A.P., J.P. Aurikko, and D.P. Kelly, *A challenge for 21st century molecular biology and biochemistry: what are the causes of obligate autotrophy and methanotrophy?* *FEMS Microbiol Rev*, 2004. **28**(3): p. 335-52.
275. Hou, C.T., A.I. Laskin, and R.N. Patel, *Growth and Polysaccharide Production by Methylocystis parvus OBBP on Methanol*. *Appl Environ Microbiol*, 1979. **37**(5): p. 800-4.
276. Nies, D.H., *Microbial heavy-metal resistance*. *Appl Microbiol Biotechnol*, 1999. **51**(6): p. 730-50.
277. Park, I., E.H. Jho, and K. Nam, *Optimization of Carbon Dioxide and Valeric Acid Utilization for Polyhydroxyalkanoates Synthesis by Cupriavidus necator*. *Journal of Polymers and the Environment*, 2014. **22**(2): p. 244-251.
278. Stackebrandt, E. and B. Goebel, *Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology*. *International Journal of Systematic and Evolutionary Microbiology*, 1994. **44**(4): p. 846-849.
279. Edgar, R.C., *Updating the 97% identity threshold for 16S ribosomal RNA OTUs*. *Bioinformatics*, 2018. **34**(14): p. 2371-2375.
280. Campbell, A., et al., *Nomenclature of transposable elements in prokaryotes*. *Plasmid*, 1979. **2**(3): p. 466-473.
281. Martínez-García, E., et al., *pBAM1: an all-synthetic genetic tool for analysis and construction of complex bacterial phenotypes*. *BMC Microbiology*, 2011. **11**(1): p. 38.

282. Naumann, T.A. and W.S. Reznikoff, *Trans catalysis in Tn5 transposition*. Proceedings of the National Academy of Sciences, 2000. **97**(16): p. 8944-8949.
283. Stalker, D.M., M. Filutowicz, and D.R. Helinski, *Release of initiation control by a mutational alteration in the R6K pi protein required for plasmid DNA replication*. Proc Natl Acad Sci U S A, 1983. **80**(18): p. 5500-4.
284. Bhasin, A., I.Y. Goryshin, and W.S. Reznikoff, *Hairpin Formation in Tn5 Transposition*. Journal of Biological Chemistry, 1999. **274**(52): p. 37021-37029.
285. Kulasekara, H.D., *Transposon Mutagenesis*, in *Pseudomonas Methods and Protocols*, A. Filloux and J.-L. Ramos, Editors. 2014, Springer New York: New York, NY. p. 501-519.
286. Puri, A.W., et al., *Genetic tools for the industrially promising methanotroph Methylobacterium buryatense*. Applied and environmental microbiology, 2015. **81**(5): p. 1775-1781.
287. Schulz, V.P. and W.S. Reznikoff, *Translation initiation of IS50R read-through transcripts*. J Mol Biol, 1991. **221**(1): p. 65-80.
288. Pastwa, E. and J. Błasiak, *Non-homologous DNA end joining*. Acta Biochimica Polonica, 2003. **50**(4): p. 891-908.
289. Haber, J.E., *Partners and pathways: repairing a double-strand break*. Trends in Genetics, 2000. **16**(6): p. 259-264.
290. Chayot, R., et al., *An end-joining repair mechanism in Escherichia coli*. Proceedings of the National Academy of Sciences, 2010. **107**(5): p. 2141-2146.
291. Chang, H.H.Y., et al., *Non-homologous DNA end joining and alternative pathways to double-strand break repair*. Nature Reviews Molecular Cell Biology, 2017. **18**: p. 495.
292. Cary, R.B., et al., *DNA looping by Ku and the DNA-dependent protein kinase*. Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(9): p. 4267-4272.
293. Pang, D., et al., *Ku proteins join DNA fragments as shown by atomic force microscopy*. Cancer Res, 1997. **57**(8): p. 1412-5.
294. Yaneva, M., T. Kowalewski, and M.R. Lieber, *Interaction of DNA-dependent protein kinase with DNA and with Ku: biochemical and atomic-force microscopy studies*. EMBO J, 1997. **16**(16): p. 5098-112.
295. Feldmann, E., et al., *DNA double-strand break repair in cell-free extracts from Ku80-deficient cells: implications for Ku serving as an alignment factor in non-homologous DNA end joining*. Nucleic Acids Res, 2000. **28**(13): p. 2585-96.
296. Weller, G.R., et al., *Identification of a DNA Nonhomologous End-Joining Complex in Bacteria*. Science, 2002. **297**(5587): p. 1686-1689.
297. Aravind, L. and E.V. Koonin, *Prokaryotic homologs of the eukaryotic DNA-end-binding protein Ku, novel domains in the Ku protein and prediction of a prokaryotic double-strand break repair system*. Genome Res, 2001. **11**(8): p. 1365-74.

298. Doherty, A.J., S.P. Jackson, and G.R. Weller, *Identification of bacterial homologues of the Ku DNA repair proteins*. FEBS Lett, 2001. **500**(3): p. 186-8.
299. Weller, G.R. and A.J. Doherty, *A family of DNA repair ligases in bacteria?* FEBS Lett, 2001. **505**(2): p. 340-2.
300. Krejci, L., et al., *Homologous recombination and its regulation*. Nucleic Acids Research, 2012. **40**(13): p. 5795-5818.
301. Camerini-Otero, R.D. and P. Hsieh, *Homologous recombination proteins in prokaryotes and eukaryotes*. Annual review of genetics, 1995. **29**(1): p. 509-552.
302. Moeller, R., et al., *Role of DNA Repair by Nonhomologous-End Joining in Bacillus subtilis Spore Resistance to Extreme Dryness, Mono- and Polychromatic UV, and Ionizing Radiation*. Journal of Bacteriology, 2007. **189**(8): p. 3306-3311.
303. Sun, B., et al., *A CRISPR-Cpf1-Assisted Non-Homologous End Joining Genome Editing System of Mycobacterium smegmatis*. Biotechnol J, 2018. **13**(9): p. e1700588.
304. Zheng, X., et al., *An efficient system for deletion of large DNA fragments in Escherichia coli via introduction of both Cas9 and the non-homologous end joining system from Mycobacterium smegmatis*. Biochem Biophys Res Commun, 2017. **485**(4): p. 768-774.
305. Stephanou, N.C., et al., *Mycobacterial Nonhomologous End Joining Mediates Mutagenic Repair of Chromosomal Double-Strand DNA Breaks*. Journal of Bacteriology, 2007. **189**(14): p. 5237-5246.
306. Park, J.M., et al., *Development of a gene knockout system for Ralstonia eutropha H16 based on the broad-host-range vector expressing a mobile group II intron*. FEMS Microbiology Letters, 2010. **309**(2): p. 193-200.
307. Wasels, F., et al., *A two-plasmid inducible CRISPR/Cas9 genome editing tool for Clostridium acetobutylicum*. Journal of Microbiological Methods, 2017. **140**: p. 5-11.
308. molbiol-tools.ca, *Online analysis tools - promoters*. 2017.
309. Li, Y., et al., *Metabolic engineering of Escherichia coli using CRISPR-Cas9 mediated genome editing*. Metabolic Engineering, 2015. **31**: p. 13-21.
310. Stock, M., et al., *Exploration and prediction of interactions between methanotrophs and heterotrophs*. Research in Microbiology, 2013. **164**(10): p. 1045-1054.
311. Hrsak, D. and A. Begonja, *Growth characteristics and metabolic activities of the methanotrophic-heterotrophic groundwater community*. J Appl Microbiol, 1998. **85**(3): p. 448-56.
312. Hršak, D. and A. Begonja, *Possible Interactions within a Methanotrophic-Heterotrophic Groundwater Community Able To Transform Linear Alkylbenzenesulfonates*. Applied and Environmental Microbiology, 2000. **66**(10): p. 4433-4439.
313. van der Ha, D., et al., *Selection of associated heterotrophs by methane-oxidizing bacteria at different copper concentrations*. Antonie Van Leeuwenhoek, 2013. **103**(3): p. 527-37.

314. Malashenko, I., V. Romanovskaia, and V. Bogachenko, *Method of isolating pure cultures of mesophilic, thermotolerant and thermophilic methane-utilizing bacteria*. Mikrobiologiya, 1975. **44**(4): p. 707-713.
315. Yan, X., et al., *Electroporation-Based Genetic Manipulation in Type I Methanotrophs*. Applied and Environmental Microbiology, 2016. **82**(7): p. 2062-2069.
316. Baani, M. and W. Liesack, *Two isozymes of particulate methane monooxygenase with different methane oxidation kinetics are found in *Methylocystis* sp. strain SC2*. Proceedings of the National Academy of Sciences, 2008. **105**(29): p. 10203-10208.
317. Su, Z., et al., *Methanol Production from Biogas with a Thermotolerant Methanotrophic Consortium Isolated from an Anaerobic Digestion System*. Energy & Fuels, 2017. **31**(3): p. 2970-2975.
318. Lamsen, E.N. and S. Atsumi, *Recent progress in synthetic biology for microbial production of C3-C10 alcohols*. Front Microbiol, 2012. **3**: p. 196.
319. Mukhopadhyay, A., *Tolerance engineering in bacteria for the production of advanced biofuels and chemicals*. Trends in Microbiology, 2015. **23**(8): p. 498-508.
320. López, J., et al., *Biogas-based polyhydroxyalkanoates production by *Methylocystis hirsuta* : A step further in anaerobic digestion biorefineries*. Vol. 333. 2017.
321. Byrne, R.T., et al., **Escherichia coli* Genes and Pathways Involved in Surviving Extreme Exposure to Ionizing Radiation*. Journal of Bacteriology, 2014. **196**(20): p. 3534-3545.
322. Claeys Bouuaert, C. and R. Chalmers, *Hsmar1 Transposition Is Sensitive to the Topology of the Transposon Donor and the Target*. PLOS ONE, 2013. **8**(1): p. e53690.
323. Garg, S., J.M. Clomburg, and R. Gonzalez, *A modular approach for high-flux lactic acid production from methane in an industrial medium using engineered *Methylobacterium buryatense* 5GB1*. Journal of Industrial Microbiology & Biotechnology, 2018. **45**(6): p. 379-391.
324. Kenney, G.E., M. Sadek, and A.C. Rosenzweig, *Copper-responsive gene expression in the methanotroph *Methylosinus trichosporium* OB3b*. Metallomics : integrated biometal science, 2016. **8**(9): p. 931-940.
325. Wang, Y., et al., *A 'suicide' CRISPR-Cas9 system to promote gene deletion and restoration by electroporation in *Cryptococcus neoformans**. Scientific Reports, 2016. **6**: p. 31145.
326. Carvalho, N.D.S.P., et al., *Expanding the ku70 toolbox for filamentous fungi: establishment of complementation vectors and recipient strains for advanced gene analyses*. Applied microbiology and biotechnology, 2010. **87**(4): p. 1463-1473.

327. Song, L., et al., *Efficient genome editing using tRNA promoter-driven CRISPR/Cas9 gRNA in Aspergillus niger*. PLOS ONE, 2018. **13**(8): p. e0202868.

10 Appendix

10.1 pMTL90882 plasmid sequence

GGCAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCCTTCGTTTTATCTGTTGTTTGTGCGGTGAACGCTCTCCTGAGTAGGACAAATCAGGAAACAGCTAT
GACCGCGGCCGAGACGTGCGGGTCGAAACTTGTGTAACGGAGGGGCGTTTGTCTAAGTTAGGCGCCAGCCAAGCGCTACGATACTCAAAGGGGAGGC
GGCATATGACCATGATTACGAATTCGGTACCCGGGGATCCTAGAGTCAGAGCTCAGCGTCCATGGAGATCTCGAGGCCTCGAGACATGCAAGCTTGGCACT
GGCCGTCGTTTTACACGTCGTGACTGGGAAAAACCTGCGCTTACCCAATTAATCGCCTTGACGACATCCCCCTTCGCCAGCTGGCGTAATAGCGAAGAG
GCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTAGCGACTCCTGTTGATAGATCCAGTAATGACCTCAGAATCCATCTGG
ATTTGTTTCAGAACGCTCGGTTGCGCGCGGGCGTTTTATTGTTGAGAATGGCGCGCCGACTGGGCGGCCATGTCAGCTACTGGGCTATCTGGACAAGGGA
AAACGCAAGCGCAAGAGAGAAAGCAGGTAGCTTGACGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAAGCGAACCGGAATTGCCAG
CTGGGGCGCCCTCTGGTAAGTTGGGAAGCCCTGCAAGTAACTGGATGGCTTTCTTCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAG
AGACAGGATGAGGATCGTTTCGATGATTGAACAAGATGGATTGACGCGAGGTTCTCCGCGCGCTTGGGTGGAGAGGCTATTCCGCTATGACTGGGCACAAC
AGACAATCGGCTGCTGATGCCGCGGTGTTCCGGCTGTACGCGCAGGGGCGCCCGTTCTTTTGTCAAGACCGACCTGCTCCGGTGCCTGAATGAATGCA
GGACGAGGCGCGCGGCTATCGTGGCTGGCCACGACGCGGCTTCCTTGCGCAGCTGTGCTGACGTTGCTCACTGAAGCGGGAAGGGACTGGCTGCTATTGG
GCGAAGTGCCGGGGCAGGATCCTGTCTCATCTCACTTGTCTCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGC
TACCTGCCATTTCGACCAACGCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCA
GGGGCTCGCGCCAGCCGAATGTTGCCAGGCTCAAGGCGCGCATGCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCTGCTTCCGCAATATCAT
GGTGGAATAGGCGCTTTCTGGAATCATCGACTGTGGCGGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGA
GCTTGGCGGCGAATGGGCTGACCGCTTCTCGTCTTACGGTATCGCCGCTCCGATTTCGACGCGCATCGCCTTCTATCGCCTTCTGACGAGTTCTTCTGAG
CGGGACTCTGGGTTGCAATAGTAAACGCCGGAAGTACCCACAAAGGCCCTAGCGTTTGAATGCACGAGTATCATTTGACCCAGGCGTGTCCACC
AGGCGCTGCTCGCAACTCTTCGACGGCTTCGCCGACCTGTGCGGCCACTTCTTACGCGGGTGGAATCCGATCCGACATAGGCGGGAAGGTTTCCAGCT
TGAGCGGTACGGCTCCCGGTGCGAGCTGAAATAGTCGAACATCCGTCGGGCGCTCGGCGACAGCTTGGGTACTTCTCCATACGAATTCGTGTAGTGGT
TCGGCAGCAACAGCAGCAGGATTTCTCTGTCGATCAGGACCTGGCAACGGGACGTTTTCTTCCACGCTCCAGGACGCGGAAGCGGTGACGACGCAACCG
ATTCAGGTGCCAACGCGGTGCGACGTGAAGCCATCGCGCTCGCTGATAGCGCGACAGGCATTCTCGGCCTTCGTGTAATACCGGCCATTGATGACCA
GCCCAGGTCTCGCAAGCTCTGTAAGCTGAAGGTGATCGGCTCGCGGATAGGGGTGCGCTTCCGCTACTCCAACACCTGCTGCCACACCAAGTTCGTGATCG
TCGGCCGCGAGCTCGACGCCGGGTGAGGTGATCTTACGTCTTGTGACGTGGAAAAATGACCTTGTGTCAGCGCTCGCGCGGATTTCTTGTGCGCGT
GGTGAACAGGGCAGAGCGGGCGGTGCTGTTGGCATCGCTCGCATCGTTCGGGCCACGGCGCAATATCGAACAAGGAAAGTGCATTTCTTGTATCTGCTG
CTTCTGTGTTTCAGAACGCGGCTGCTTGGCTGCTGACCTGTTTTGCCAGGTCTCGCGCGGTTTTTCGCTTCTTGGTCGTATAGTCTCTCGCTGTC
GATGGTCATGACTTCGCCAAACCTGCCGCTCTGTTTCGAGACGACGCGAACGCTCCACGGCGGCGGATGGCGGGCAGGGCAGGGGGAGCCAGTTGCA
CGCTGTGCGCTCGATCTTGGCCGTAGCTTGTGGACCATCGAGCCGACGGAAGTTTCGCGGGGCGCAGCATGACGGTGGCGCTTGGATGTTTT
CGGCATCTCGGCGGAAAAACCGCGTGCATCAGTTCTTGCCTGTATGCTTCCGGTCAAACGTCGGAATCATTACCCCTCTTGGCGGATGCCCCGACTCAC
GCCGGGGCAATGTGCCCTATTCTGATTTGACCCGCTGGTGCCTTGGTGTCCAGATAATCCACCTTATCGGCAATGAAGTCGGTCCCGTAGACCGCTGGCC
GTCCTTCTCGTACTTGGTATTCGAATCTTGCCTGCAAGTAACAGCTCCGCGAAGTGCCTCTTCTTATGAGCGCATGGGGACGTCTTGGCAATCAGCG
GCACCCCGGCGGTTTTAGCGGCTAAAAAGTCATGGCTCTGCCCTCGGCGGACCAACGCCCATCATGACCTTGCAAGCTCGTCTGCTTCTTCGATCTT
CGCCAGCAGGGCGAGGATCGTGGCATCACCGAACCGCGCGTGGCGGGTCTGCTGAGCCAGAGTTTTCAGCAGGCCGCCAGGCGGCCAGGTGCGCAT
TGATGCGGGCAGCTCGCGACGTGCTCATAGTCCAGCAGCCGCTGATTTGTAGCCCTGGCGGACGCGCCAGGATAGGCTACAGGCTATGCGCGGCT
CCGCCGCAATTTCTCAATCGCTTCTGTTCTGCTGGAAGGCAGTACACTTGATAGGTGGGTGCGCCTTCTGTTGGCTTGGTTTATCAGCCATCGCCTTGC
CCTCATCTGTTACGCGCGGTAGCCGCGCAGCTCGCAGAGCAGGATTCGCTTGAACCGCCAGGTGCGAATAAGGGACAGTGAAGAAAGAACCCCG
CTCGCGGTGGGCTACTTCACTATCTGCCCCTGACGCGGTGGATACACCAAGGAAAGTCTACACGAACCTTTGGCAAAATCTGTATATCGTGCGA
AAAAAGGATGGATATACGAAAAAATCGTATAATGACCCGGAAGCAGGGTTATGACGCGGAAAAAGATCCGTGACCTTCCGACGCTCACCGGGCTGGTTG
CCCTCGCGCTGGGCTGGCGGCGCTATGCGCCTGCAAAACGCGCAGAAACGCCGTCGAAGCGGTGTCGAGACACCGCAGCGCGCGGCTTGTGGATACC
TCGCGGAAAACTTGGCCCTCACTGACAGATGAGGGGCGGACGTTGACACTTGAAGGGCGGACTCACCCGCGCGGCGTTGACAGATGAGGGGACAGGCTCGA
TTTCGCGCGGCGACGTGGAGCTGGCCAGCTCGCAATCGCGGAAAAACGCTGATTTACGCGAGTTTCCACAGATGATGGACAAGGCTGGGGAATAGT
GCCCTGCGGTATTGACACTTGAGGGGCGGCACTACTGACAGATGAGGGGCGCGATCCTTGACACTTGAGGGGCGAGTGTGACAGATGAGGGGCGCACCT
ATTGACATTTGAGGGGCTGTCCACAGGCGAGAAAAATCCAGCAATTTGCAAGGGTTTCCGCCGTTTTTCGGCCACCGCTAACCTGTCTTTAACTGCTTTAAACC
AATATTTATAACCTTGTTTTAAACAGGGCTGCGCCTGTGCGGTGACCGCGCACGCCGAAGGGGGGTGCCCCCTTCTCGAAACCTCCCGGCCGCTAAC
GCGGGCTCCCTATCCCCCAGGGGCTGCGCCCTCGGCGCGAACGCGCTCACCCAAAAATGGCAGCCAAGCTGACCACTTCTGCGCTCGGCCCTTCCGGCT
GGCTGTTTTATTGCTGATAAATCTGGAGCCGTGAGCGTGGGTCTCGCGGTATCATTGACGACTGGGGCAGATGGTAAGCCCTCCGATATCGTAGTTATCT
ACACGAGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCTCACTGATTAAGCATTGGTAACGTGACAGCAAGTTTACT
CATATATACTTTAGATTGATTTAAACTTCATTTTTAATTTAAAGGATCTAGGTGAAGATCCTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTC
GTTCACTGAGCGTCAGACCCCGTAGAAAAAGATCAAAGGATCTTCTGAGATCCTTTTTTCTGCGCGTAATCTGCTGCTTCAAAACAAAAAACCCGCTAC
CAGCGGTGGTTTTGTTGCGGATCAAGAGCTACCAACTCTTTTCCGAAGGTAACCTGGCTTACGAGAGCGCAGATACCAAACTGTCCTTCTAGTGTAGCCG
TAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCCTACATACCTGCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGCGGATAAGTCGTGCTTACC
GGGTTGGACTCAAGCAGATAGTTACCGGATAAGGCGCAGCGGTGCGGCTGAACGCGGGGTTCTGTGACACAGCCAGCTTGGAGCGCAACGACCTACACCGA
ACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCACGCTTCCGAAGGAGAAAGCGGACAGGTATCCGGTAAGCGCGAGGGTCGGAACAGGAGAG
CGCAGAGGGAGCTTCCAGGGGAAACGCTGATCTTTATAGTCTGCGGTTTCGCCACCTGACTTGAGCGTCGATTTTGTGATGCTGTCAGGGG
GGCGGAGCTATGAAAAACGCGCAGCAACGCGGCTTTTACGGTCTCGGCCCTTTGCTGCGCTTTGCTCAGATGGTACCCCTGCA

10.2 pMTL71401 Plasmid sequence

CCTGCAGGATAAAAAAATTGTAGATAAATTTTATAAAATAGTTTATCTACAATTTTTTATCAGGAAACAGCTATGACCGCGGCCGCTGTATCCATATGACCAT
GATTACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACGTCACGCTCCATGGAGATCTCGAGGCTGCAGACATGCAAGCTTGGCACTGGCCGT
CGTTTATCAACGTCGCTGACTGGGAAAAACCTTGGCGTTACCCAACTAATCGCTTGCAGCACATCCCCCTTTCCGCACTGGCGTAATAGCGAAGAGGCCCGC
ACCGATCGCCCTTCCCAACAGTTGCGCAGCTGAATGGCGAATGGCGCTAGCATAAAAATAAGAAGCTGCATTTCAGGCTTCTATTTTTATGGCGCCGCG
GCCGCGCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGAAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCT
AGACTGGGCGGTTTTATGGACAGCAAGCGAACCGAATTGCCAGCTGGGGCGCCCTCTGTTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTCTT
GCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGATGATTGAACAAGATGGATTGCACGCAAGTTCTCC
GGCCGCTTGGGTGGAGAGGCTATTTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCGTGTTCGGCTGTCAGCGCAGGGGCGCCCG
TTCTTTTTGTCAAGACCGACCTTCCGGTGCCTGAATGAATGCAAGACGAGGACGCGCGCTATCTGGTGGTGGCCACGACGGGCGTTCTTTCGCGAGCTGT
GCTCGACGTTGTCACTGAAGCGGGAAGGAGTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCTGTCACTCTCACCTTGTCTCTCGCGAGAAAGTATC
CATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGTATCCGGCTACTGCCATTTCGACCACCAAGCGAAACATCGCATCGAGCGAGCAGTACTCGGATG
GAAGCCGCTTGTGCTGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAAGTTTCGCCAGGCTCAAGGCGCGCATGCCGACGGCGA
GGATCTCGTGTGACCCATGGCGATGCCTGCTTCCGAATATCATGTTGGAATAATGGCCGCTTTCTGGATTATCGACTGTGGCCGCTGGGTGTGGCGGAC
CGCTATCAGGACATAGCTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGCGAATGGGCTGACCGCTTCTCGTGTTCACGGTATCGCCGCTCCGATT
CGCAGCGCATCGCCTTCTATCGCTTCTTGACGAGTTCTTCTGAGCGGAGCTCTGGGTTTGAATGACCGACCAAGCGCAACCTGCAATCAGGAT
TTCGATTCCACCGCGCCTTCTATGAAGGTTGGGCTTCGAATCGTTTTCGGGACGCGGCTGGATGATCTCCAGCGCGGGATCTCATGCTGGAAGTTCTT
CGCCACCCCATGGGCAAAATATATACGCAAGGCGACAAGGTGCTGATGCCGCTGGCGATTGAGTTTATCATGCGGTTTGTGATGGCTCCATGTCGCGCAG
AATGCTTAAATGAATACAAAGTTTTATGCATGCGCCAATACGCAAAACCGCTCTCCCGCGCTTGGCCGATTCAATAGTCAGCTGGACGACAGGTTTC
CCGACTGGAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGTCTACTCATTAGGCACCCAGGCTTACACTTTATGCTTCCGGCTCGTATGTTGTG
GGAATTGTGAGCGGATAACAATTTACACAGGAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCTCTACTAAAGGGTTTAAACGCTGCTACGCT
GAATAAGTGATAATAAGCGGATGAATGGCAGAAATTCGAAAGCAATTCGACCCGCTCGTGGTTACGGGCGAGGTCGTTAAATAGCCGCTTATGTCTATTG
CTGGTTTAACTGATTTTATTGACTACCGGAAGCAGTGTGACCGGTGCTTCTCAAATGCTTGAAGGCTGAGGCGAATTTGCTCAGGCTCTCCCGTGGAGGTAATAATTGACG
ATATGATCATTTATCTGCTCCAGAGCTGATAAAACGGTGAATTCGTTAGCGAGGTGCCGCGCTTCCATTAGGTCGAGGTGGCCCGCTCCATGCA
CCGCGACGCAACGCGGGGAGGCGACAAAGGTATAGGGCGCGCAGGCGGCTCAGCCGATAGTCTGGAACAGCGCACTTACGGGTTGCTGCGCAACCCAAAG
TGCTACCGGCGCGGCGAGCGTGACCCGTGTCGGCGCTCCAAACGGCTCGCCATCTGTCAGAAAAACAGGCTCATCGGGCATCGGCAGGCGCTGCTGCCCGCGC
CGTTCCTTCTCCGTTTCGGTCAAGGCTGGCAGGTCTGGTTCATGCCGGAATGCCGGCTGGCTGGGCGGCTCTCGCCGGGGCGGTCGGTAGTTGCT
GCTCGCCCGGATACAGGGTGGGATGCGCGCAGGTGCGCATGCCCAACAGCGATTCTGCTCTGGTCTGCTGATCAACACCAACGCGCGCACTGAACACCG
ACAGCGCAACTGGTCGCGGGGCTGGCCCCACGCCACGCGTATTGACACGTAGGCCGACACGGTGGCCGGGCGGTTGAGCTTACGACGGAGATCCAG
CGCTCGGCCACCAAGTCTTACTGCGTATTGGACCGTCCGCAAGAACGCTCCGATGAGCTTGGAAAGTGTCTTCTGGCTGACCCACACGCGGCTTCTGGTGGC
CCATCTGCGCCACGAGGTGATGCAGCAGCATTGCCGCGTGGGTTCTCTGCAATAAGCCCGCCACGCTCATGCGCTTTCGCTTCGCTTTCGACCCAGTG
ACCGGGCTTGTCTTGGCTGAATGCCGATTCTCTGACTGCGTGCCATGCTTATCTCATGCGGTAGGGGTGGCCGACGGTGGCGCACCATGCGCAATC
AGCTGCAATTTTTCGGCAGCGCGACAACAATTATGCGTTGCGTAAAGTGCAGTCATTAACAGATTTCCTTAACTACGCAATGAGCTATTGCGGGGGTG
CCGCAATGAGCTGTTGCGTACCCCCCTTTTTAAGTTGTTGATTTTTAAGTCTTTCGATTTCGCCCTATATCTAGTTCTTGGTGCCAAAGAGGGCACCCCTG
CGGGGTTCCCCACGCTTTCGGCGCGCTCCCTCCGGCAAAAGTGCCCTTCGGGGCTTGTGATCGACTGCGCGGCTTTCGGCTTCCCAAGGTGGC
GCTGCCCTTGAACCCCGCACTCGCCCGTGAAGTCTGGGGGCGAGGCGGGCGGCTTCGCTTCACTGCCCCACTCGCATAGGCTTGGGTCTTTC
CAGCGCGCTCAAGGCCAAGCGCTGCGCGTCTGCGGAGCTTGAACCGCTTCACTTGGTGTCCAACCGGCAAGCGAAGCGCAGGCGCGAGGCC
GGAGGCTTTTCCCAAGAGAAAATTAAAAAATTGATGGGCAAGGCCGCAAGCCGCGCAGTTGGAGCGGTGGGTATGTGTCGAAGGCTGGGTAGCCGG
TGGGCAATCCCTGTGGTCAAGCTCGTGGGCGAGGCGCAGCTGTCCATCAGTTTCCAGCAGGGTGTCCACGGGCGAGCGAAGCGAGCCAGCCGCTG
CGCTCGCGGCTTCTGTCACATATCCACGGCTGGCAAGGAGCGCAGGACCGCGCAGGCGCAAGCCCGGAGCAAGCCGCTAGGGCGCGCGAGCCG
CGTAGGCGGTACAGCTTTGCGAAGCAAAGTCTAGTGAGTATACTAAGCATTGAGTGGCCCGCGGAGGCACCGCTTGCCTGCCCGCTCGAGCGGTT
GGACACCAAAAGGAGGGGAGGCGATGCGCGCATACGCGATCATGCGATGCAAGAAGTGGCGAAATGGGCAACGTTGGCGGCGAGTCTCAAGACCGCT
ACCGCGAGCGAGACGCCAACGCTGACGCCAGCAGGACGCCAGAGAACAGCACTGGGCGGCCAGCAGCACCAGTGAAGCGATGGGCGGCTGCGCGA
GTTGCTGCCAGAGAAGCGGCGCAAGGACGCTGTGTTGGCGTTCGAGTACGTCATGACGGCCAGCCGGAATGGTGGAAGTGGCCAGCCAAAGAACGACG
GCGGCGTTCTTCGAGAAGGCGCAAGTGGCTGGCGGACAAGTACGGGCGGATCGCATCGTGACGGCCAGCATCCACCGTGACGAAACAGCCCGCACAT
GACCGGCTTCTGTTGCCGCTGACGCGAGGACGGCAGGCTGTGCGCCAAAGGATTCTCGGCAACAAAGCGCAGATGACCCGCGACCAACGACGTTTCCG
CCGCTGTGGCCGATCTAGGGCTGCAACGGGGCATCGAGGGCAGCAAGGCGACGTCACACGCGATTTCAGGCGCTTTCAGGCGCTTGGAGCGGCCACCAAGT
GGCCACGTACCATCAGCCCGCAAGCGGTGAGCCACGCGCTATGACCCGAGGGATTGGCCGAAAAGCTGGGAATCTCAAAGCGGCTTGAGACGCCGGA
AGCCGTGGCCAGCGGCTGACAAAAGCGGTTGGCAGGGGATGAGGCTGCCCTCAGGCGCGCGCAGGAGCGGTGAGATGCGCAAGAAGGCCGATCAA
GCCAAGAGACGGCCGAGACCTTCGGGAGCGCTGAAGCCGTTCTGACGCCCTGGGGCGGTTGAATCGGGATATGAGGCCAAGGCGCGCGCATCAT
CAAGGCCGTGGGCAAAAGCTGCTGACGGAACAGCGGGAAGTCCAGCGCCAGAAACAGGCCAGGCCAGCAGGAACGCGGCGCGCACATTTCCCGAA
AAGTGCCACCTGACGCTAAGAAACATTATTATCATGACATTAACTATAAAAAAGGCGTATCACGAGGCCCTTTCGCTTTCGAATAAATACCTGTGACGGA
AGATCACTTCGAGAATAAATAAATCTGGTGTCCCTGTTGATACCGGGAAGCCCTGGGCCAACTTTTGGCGAAAATGAGACGTTGATCGGCACGTAAGAG
TTCCAACCTTACCATAATGAAATAAGATCACTACCGGGCGTATTTTTGAGTTATCGAGATTTCAGGAGCTAAGGAAGCTAAAAATGGAGAAAAAATCACTG
GATATACCAACCGTTGATATATCCCAATGGCATCGTAAAGAACATTTTGAAGCATTCAGTCAGTTGCTCAATGTACCTATAACCAAGACCGTTACGCTGGATATT
ACGGCCTTTTATGCTTTTGCCTTCTACCGGATTGAGTCTGCTCATGTTGATTTCTCACTTGATAACCTTATTTTACGAGGGGAAATTAATAGGTTGTATT
GATGTTGGACGAGTCGGAATCGCAGACCGATACCAAGATCTTGCCATCTATGGAATGCTCGGTTGAGTTTCTCCTTATTACAGAAACGGCTTTTCAAAA
ATATGTTGATGATAATCTGATGAATAAATGCAAGTTTCATTTGATGCTCGATGAGTTTTCTAATCAGAATTGGTTAATGGTTGTAACACTGGCAGAGCAT
TACGCTGACTTGACGGGACGGCGGCTTGTGTAATAATCGAACTTTTGTGAGTTGAAGGATCAGATCACGCATCTTCCGACACGCGAGACCGTTCCGTGG
CAAAGCAAAAGTTCAAATACCAACTGCTCCACCTACAACAAAGCTCTCATCAACCGTGGCTCCCTCACTTTCTGCTGGATGATGGGGCGATTACGGCCTG
GTATGAGTCAGCAACACCTTCTCACGAGGACAGCTCAG

10.3 pMTL90882_EYFP-P3 plasmid sequence

TTGGTCGTACATAGTTCCTCGCGTGTGCATGGTTCATCGACTTCGCCAAACCTGCCGCTCTGTTTCGAGACGACGCGAACGCTCCACGGCGGCGGATGGCGCGG
GCAGGGCAGGGGAGCCAGTTGCACGCTGTCGCGCTCGATCTTGGCCGTAGCTTGTGGACCATCGAGCCGACGGACTGGAAGGTTTCGCGGGGCGCACGC
ATGACGGTGGCGCTTCGCGATGCTTCCGCATCTCGGCGGAAACCCCGCGTGATCAGTTCTTGCCTGTATGCCTTCCGGTCAAAACGTCCGATTCATTACCCC
TCCTTGCGGGATTGCCCGACTCACGCCGGGCAATGTGCCCTTATTCTGATTGACCCGCTGGTGCCTTGGTGTCCAGATAATCCACCTTATCGGCAATGA
AGTCGGTCCGCTAGACCGCTCGGCCGCTCTCTGCTACTTGGTATTCGGAATCTTGGCTGACGCAATACCAAGCTCCGCGAAGTCGCTCTTCTGATGGAGCGC
ATGGGGACGTGGGCAATCACGCGCACCCCGCGCGCTTTTAGCGGCAAAAGTCATGGCTGTGCCCTCGGCGGACGACGCCCATGACCTTGCCTAC
AGCTCGTCTGCTTCTCTCGATCTTCCAGCAGAGGGCGAGGATCGTGGCATCACGAACCGCGCCGTGCGCGGGTGTGCGGTGAGCCAGAGTTTCAGCAGG
CCGCCAGGCGGCCAGGTGCGCATTGATGCGGGCCAGCTCGCGGACGTGCTCATAGTCCACGACGCCCCGTGATTTTGTAGCCCTGCCCAGCGCCAGCAGG
TAGGCCTACAGGCTCATGCCGGCCGCCGCCCTTTTCTCAATCGCTCTTCTGTTCTGTGAAGGCGAGTACACCTTGATAGGTGGGCTGCCCTTCTGTTGTG
CTTGGTTTCATCAGCCATCCGCTTGCCCTCATCTGTTACGCCGGCGGTAGCCGGCCAGCCTCGCAGAGCAGGATTCCCGTTGAGCACCGCCAGGTGCGAATAA
GGGACAGTGAAGAAGGAACACCCGCTCGCGGTGGGCTACTTCACTATCTCTGCCCGGTGACGCCGTTGGATACACCAAGGAAAGTCTACACGAACCTT
TGGCAAAATCTGTATATCTGTGCGAAAAAGGATGGATATACCGAAAAATCGCTATAATGACCCCGAAGCAGGGTTATGACGCGGAAAAAGATCCGTCGACCC
TTTCCGACGCTCACGGGCTGGTTGCCCTCGCGCTGGGCTGGCGCGCTATGCGCCCTGCAAAACGCGCCAGAAACGCCGTCGAAAGCCGTGTGCGAGACAC
CGCAGCCGCCGCGTGTGTGATACCTCGCGGAAACTTGGCCCTACTGACAGATGAGGGGCGGACGTTGACACTTGAGGGGCGGACTCACCCGCGCGGC
GTTGACAGATGAGGGGAGGCTCGATTTGCGCCGCGCAGCTGGAAGCTGGCCAGCCTCGCAAAATCGCGCAAAACGCTGATTTTACGCGAGTTTCCACAGAT
GATGTGGACAAGCCTGGGGATAAGTGCCCTGCGGTATTGACACTTGAGGGGCGGACTACTGACAGATGAGGGGCGGATCCTTGACACTTGAGGGGCGAGA
GTGCTGACAGATGAGGGGCGCACCTATTGACATTTAGGGGCTGTCCACAGGCAGAAAAATCCAGCATTTTCAAGGGTTTCCGCCGTTTTTCCGCCACCGCTA
ACCTGTCTTTTAACTGCTTTTAAACCAATATTTATAAACCTTGTTTTAAACAGGGCTGCGCCCTGTGCGCGTGACCCGCGACGCCGAAGGGGGGTGCCCCCC
CTTCTCGAACCTTCCCGGCCGCTAACGCGGGCTCCCATCCCCAGGGGCTGCGCCCTCGGCCGCGAACGCGCTCACCCAAAAATGGCAGCCAGCTGA
CCACTTTCGCGCTCGGCCCTTCCGGCTGGGCTGTTTATGCTGATAAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTTGACGACTCGGGGCGAGAT
GTAAGCCCTCCGATCTGATGTTATCTACACGACGGGAGTCAGGCACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGC
ATTGGTAACGTGCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAACTTCATTTTTAAATTTAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCAT
GACCAAAATCCCTTAACTGAGTTTTCTTCACTGAGCGTCAGACCCGTAGAAAAGATCAAAGGATCTTTTCTGAGATCCTTTTTTCTGCGCGTAATCTGCTG
CTTGCAAAACAAAAAACCCGCTACCAGCGGTGGTTTTGTTGCCGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATA
CCAAATACTGCTCTTAGTGATAGCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCTACATACCTCGCTCTGCTAATCTGTTACAGTGGCTGCT
GCCAGTGGCGATAAGTCGTGTTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTGCGGCTGAACGGGGGTTCTGTGCACACGCG
CAGCTTGGAGCGAACGACCTACACGAACTGAGATACCTACAGCTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAAGAGCGGACAGGTATCCGG
TAAGCGCGAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAGCGCTGATCTTTATAGTCTGTGCGGTTTCCGCACTCTGACTTGAGC
GTCGATTTTTGTGATGCTGTCGAGGGGGCGGAGCCTATGGAAGAACGCGCAGCAACGCGGCTTTTACGTTCTGCGCTTTTGTGCGCTTTTGTGCATG
GTACCCCTGACAGCAATAAACCGAAAGGCTCAGTCGAAAGACTGGGCTTTCGTTTTATCTGTTGTTGTGCGGTGAACGCTCTCCTGAGTAGGACAAATCAG
GAAACAGCTATGACCCGGCGCGCGTTTTCTTAAAGCTGAGGGAAGTACCCAAAAATTCATCCTTCTCGCTATGCTCTGGGGCTCGGCAGATGCCAGCGC
TGCATACCGTCCGGTAGGTGCGGAAGCGTGCAGTGCCGAGGCGGATTAATCGATGAGCTGTTGACAATTAATCATCGGCTCGTATAATGAATTCAGTGTTA
ACTTTAAGAAGGAGATATACATATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCTGGTCGAGCTGGACGGCGACGTAACCGGCCACA
AGTTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCTGAAGTTTATCTGCACACACCGGCAAGCTGCCGTGCCGCACTCC
TCGTGACCACCTTCGGCTACGGCTGCACTGCTCGCCGCTACCCCGACCATGAAGCAGCAGCAGCTTCTCAAGTCCGCCATGCCGAAGGCTACGCTCAG
GAGCGCACCATCTTCTTCAAGGACGACGGCACTACAAGACCCGCGCGGAGGTGAAGTTGAGGGCGACACCTGTTGAACCGCATCGAGCTGAAGGGCAT
CGACTTCAAGGGAGGGCAACATCTGGGGCACAACTGGAGTACAACTACAACAGCCACAACGCTCTATATCATGACGCAAGCAAGAAAGCATCA
AGGTGAATCTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCATCGCGACGCGCCCGTGTGCTGC
CCGACAACCACTACCTGAGCTACCACTCCGCCCTGAGCAAGACCCCAACGAGAGCGGATCAGATGCTGCTGCTGAGTTCGTGACCGCCGCCGGATCA
CTCTCGCATGACGAGCTGTACAAGTAAGCTTGGCACTGGCCGTGTTTTACAACGTCGTGACTGGGAAAAACCTGGCGTTACCAACTTAATCGCCTTGCA
GCACATCCCTTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCGACCGATCGCCCTTCCCAACAGTTGCGCAGCTGAATGGCGAATGGCGTACGCACTCC
TGTTGATAGATCCAGTAATGACCTCAGAATCCATCTGGATTGTTGAGAACGCTCGGTTGCCGCGGGCGTTTTTATTGGTGAGAAATGGCGCGCCGACTGG
GCCGGCATGTGAGCTACTGGGCTATCTGGACAAGGGAAGCGCAAGCGCAAGAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTG
GGCGGTTTTATGGACAGCAAGCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGTTAAGGTTGGGAAGCCCTGCAAAAGTAACTGGATGGCTTTCTGCCGCC
AAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTTCGATGATTGAACAAGATGGATTGCACGAGGTTCTCCGGCCG
CTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTGATGCGCGCGTGTTCGGGTGTGACGCGAGGGGCGCCCGTTCTTT
TTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAAGTGCAGGACGAGGCGCGGCTATCGTGGCTGGCCACGACGCGGCGTTCTTTCGCGAGCTGTGCTCG
ACGTTGTCACTGAAGCGGGAAGGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCTGTATCTCACCTTGCTCTGCGGAGAAAGTATCCATCAT
GGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCATTGACCAACAGCGAAACATCGCATCGAGCGAGCAGTACTCGGATGGAAGC
CGGTCTTGTGATGATGATGATGATGAGCAAGAGCATCAGGGGCTCGCGCCAGCCGAAGTGTTCGCGAGGCTCAAGGCGCGCATGCCGACGCGGAGGATC
TCGTCGTGACCCATGGCGATGCTGCTGCTGCCGAATATCATGGTGAAAAATGGCCGCTTTTCTGGAATTCAGACTGTGGCCGGGTGGGTGTGGCGGACCGCTA
TCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCTCTGCTGTTTACGGTATCGCCGCTCCCGATTGCGAG
CGCATCGCCTTCTATCGCCTTCTGACGAGTTCTTCTGAGCGGACTCTGGGTTTCAAAATGAGTTTAAACGCGGAACTGACCCCAAGGCCCTAGCGTTTG
CAATGCACCAAGGTCATATTGACCCAGGCGTGTTCACACAGGCCGCTGCCTGCAACTCTTCGACGCTTCCGCCGACCTGTCTGCGCCACTTCTTACGCGGGT
GGAATCCGATGCGCAGCATGAGGCGGAAGGTTTTCCAGCTTGAGCGGGTACGGGCTCCGGTGGAGCTGAAATAGTGAACATCCGTCGGGCGCTGCGCGACA
GCTTGGCGTACTTCTCCATACGAATTTCTGTAGTGGTGCACGAAACAGCAGCAGATTCTCGTGCATCAGGACTGGCAACGCGGAGCTTTCTTGCCA
CGGTCCAGGACGCGGAAGCGGTGCGAGCAGCGACACCGATTCCAGGTGCCCAACCGGTCGAGCGTGAAGCCCATCGCCGTGCGCTGTAGGCGCGACGAGCA
TTCCGCGCCTTCTGTGTAATACCGGCCATTGATCGACCAAGCCAGTCTGCTGCGAAAGCTCGTAGAAGCTGAAGGTGACGCTCGCGGATAGGGGTGCTGTC
GCGTACTCCAACACCTGCTGCCACACCAAGTTCGTATCGTGGCGCCGAGCTCGACGCGGCTGTAGGTGATCTTACGCTCTTGTGACGTGGAATAATGACCTT
GTTTTGAGCGCCTCGCGCGGATTTCTTGTTCGCGTGGTGAACAGGGCAGAGCGGGCGGTGCTGTTTGGCATCGCTCGCATCGTGTCCGCCACGCGCGC
AATATCGAACAGGAAGCTGCATTTCTTGATCTGCTGCTTCTGTGTTTTCAGCAACGCGGCTGCTTGGCTCGCTGACCTGTTTTGCCAGGTCCTCGCCGG
CGGTTTTTCGCTT

10.4 pBBR1-2USER_NPR3-EYFP

GTCACGACTTTGCGAAGCAAAAGTCTAGTGAGTATACTCAAGCATTGAGTGGCCCGCGGAGGCAACCGCTTGCCTGCCCCGTCGAGCCGGTTGGACACCA
AAAGGGAGGGGAGGATGGCGGCATACGCGATCATGCGATGCAAGAAAGTGGCGAAAAATGGGCAACGTGGCGGCCAGTCTCAAGCAGCCTACCGGAG
CGCGAGACGCCCAACGCTGACGCCAGCAGGACGCCAGAGAAACGAGCACTGGCGGCGCAGCAGCACCAGTGAAGCGATGGGCGGACTGCGCGAGTTGCTGC
CAGAGAAGCGGCGCAAGGACGCTGTTGGCGGTGAGTACGTATGACGCCACGCCGGAATGGTGGAAGTCGGCCAGCCAAGAACAGCAGGCGCGGTT

CTTCGAGAAGGCGCACAAAGTGGCTGGCGGACAAGTACGGGGCGGATCGCATCGTGACGGCCAGCATCCACCGTGACGAAACAGCCCGCACATGACCGCGT
TCGTGGTGCCGCTGACGCAGGACGGCAGGCTGTCCGCCAAGGAGTTTATCTCGGCAACAAAGCGCAGATGACCCGCGACCAGACCAGTTTGGCGGCGCTGTG
GCCGATCTAGGGCTGCAACGGGCTCGAGGGCAGCAAGGCACGTCACACGCGCATTCAGGCGTTCTACGAGGGCCTGGAGCGGGCCACAGTGGGGCCAGCT
CACCATCAGCCCGCAAGCGGTCGAGCCACGCGCTATGACCCGAGGGATTGGCCGAAAGCTGGGAATCTCAAAGCGCGTTGAGACGCCGGAAGCCGTGG
CCGACCGGCTGACAAAGCGGTTCCGCGAGGGGTATGAGCCTGACGCGCCGCGCAGGAGCGCGTGAGATGCGCAAGAGGCGGATCAAGCCCAAGA
GACGCGCCGAGACCTTCGGGAGCGGCTGAAGCCGTTCTGGACGCGCTGGGGCCGTTGAATCGGGATATGACAGGCCAAGGCCGCGCATCATCAAGGCCG
TGGGCGAAAGCTGCTGACGGAAACGCGGGAAGTCCAGCGCCAGAAACAGGCCAGCGCCAGCAGGAACGCGGGCGCGCACATTTCCCCGAAAGTGCCA
CCTGGGATGAATGTCACTACTGGGCTATCTGGACAAGGAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAAGTGGGCTTACATGGCGATAGCTAGA
CTGGGCGGTTTTATGGACAGCAAGCGAAACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAACTGGATGGCTTTCTTGCC
GCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGG
CCGCTTGGGTGGAGAGGCTATTCCGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCGGTTCCGGCTGTACGCGCAGGGGCGCCCGGTTT
TTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACCTGACGAGCAGGACGCGCGGCTATCGTGGCTGGCCACGACGCGGCGTTCTTGCGCAGCTGTGC
TCGACGTTGTCACTGAAGCGGGAAGGGAAGTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCTGTCACTCACCTTGCTGCTGCGGAGAAAGTATCCA
TCATGGCTGATGCAATGCGGCGGCTGCATACGCTGATCCGGCTACCTGCCATTGACACCACCAAGCGAAACATCGCATCGAGCGAGCAGTACTCGGATGG
AAGCCGCTCTTGTGATCAGGATGATCTGGACGAAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCCGCGAGGCTCAAGGCGCGCATGCCGACGCGAG
GATCTCGTGTGACCATGGCGATGCGCTGTTGCCGAATATCATGGTGGAAGTGGCCGCTTTTCTGGATTATCGACTGTGGCCGGCTGGGTGTGGCGGACC
GCTATCAGGACATAGCGTTGGCTACCCGTGATATTCTGAAGAGCTTGGCGGCGAATGGGCTGACCCGTTCTCGTGCTTACGCTTACCGGCTCGCGATT
GCAGCGCATGCGCTTCTATCGCTTCTTGACGAGTTCTTGACGCGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCGCCAACTGCCATCAGGAGATT
CGATTCCACCGCCGCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTC
GCCACCCCATGGGCAAAATATTATACGCAAGGCGACAAGTGCTGATGCCGCTGGCGATTCAAGTTTCATCATGCCGTTTGTGATGGCTTCCATGCGGCAGA
ATGCTTAATGAATTACAACAGTTTTATGCATGCGCCCAATACGCAAAACCGCTCTCCCGCGCGTTGGCCGATTCAATATGACGCTGGCAGCAGAGGTTTCC
CGACTGGAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCAACCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTGTGT
GGAATTGTGAGCGGATAACAATTTACACAGGAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCTCTACTAAGGGAACAAAAGCTGGGTACC
AAATTAAGCAGAAAGGCTCTGACGGATGGCTTTTTGCGTTTTCTTAAGGCTGAGGGGAAAGTACCCAAAAATTCATCCTTCTGCGCTATGCTCTGGGGCTCG
GCAGATGCGAGCGTGATACCCGTCGGTAGGTCGGGAAGCGTGCAAGTCCGAGGCGGATTAATCGATGAGCTGTGACAATTAATCATCGGCTCGTATAAT
GAATTCAGTATTTAATTTAAGAAGGAGATATACATATGGTGAGCAAGGGCGAGGAGCTGTTACCCGGGGTGGTGCCATCCTGGTGCAGCTGGACGGCGA
GCTAAACGGCCACAAGTTACGCGTGTCCGGCAGGGCGAGGGCGATGCCACTACGGCAAGCTGACCCGTAAGTTTCATCTGCACACCGGCAAGCTCCCGT
GCCCTGGCCACCTCGTGACCACCTTCGGCTACGGCCTGCAAGTCTTCCGCGCTACCCCGACCATGAAGCAGCAGCACTTCTCAAGTCCGCCATGCCCG
AAGGCTACGTCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCGGAGGTGAAGTTTCGAGGGGCGACACCTGGTGAACCGCATC
GAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCTGGGGCACAAGCTGGAGTACAACAGCCAGCACAACGCTATATCATGCGCGACAAGCA
GAAGAACGGCATCAAGGTGAACCTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGAGCTCGCCGACCACTACCAGCAGAAACCCCATCGCGCAGC
GCCCCGTGCTGCTGCCGACAACCACTACCTGAGCTACCAAGTCCGCCCTGAGCAAAAGACCCCAACGAGAAGCGCGATCAGATGCTCTGCTGGAGTTCTGAGC
CGCCGCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAGCTTGGATCCATGCTCTCTCAGCTAGCATCAATAAAACGAAAGGCCCTTCGGGCTTTTCG
TTTTATCTGTTGTTACCGGTTTATTGACTACCGGAAGCAGTGTGACCGTGTGCTTCTCAAATGCCTGAGGCCAGTTTGCTCAGGCTCTCCCGGTGGAGTAAT
AATTGACGATATGATCATTTTCTGCTCCAGAGCTGATAAAAACGGTGAATCCGTTAGCGAGGTGCCCGCGCTTCCATTAGGTCGAGGTGGCCCGGC
TCCATGACCCGCGACCAACGCGGGGAGGCAGACAAGGTATAGGGCGGCGAGGCGGCTACAGCCGATAGTCTGGAACAGCGCACTACGGGTTGCTGCGC
AACCAAGTGCTACCGGCGCGCAGCGTGACCCGTGTGCGCGGCTCCAACGGCTGCCATGCTCCAGAAAAACACGGCTCATCGGCATCGGCAGGCGCTGCT
GCCCCGCGCGTTCCCATTTCTCGGTTTCGGTCAAGGCTGGCAGGTCTGTTTCCATGCCCGGAATGCCGGGCTGGCTGGGCGGCTCTCGCCGGGGCGGTCG
GTAGTTGCTGCTCGCCCGATACAGGGTCGGGATGCGGCGCAGGTGCGCATGCCCAACAGCGATTGCTCTGCTGCTGCTGATCAACACACGCGCGCAC
TGAACACCGACAGCGCAACTGGTCGCGGGGCTGGCCCCACGCGCAGCGGCTATTGACCAGTAGGCGGACACGGTGCCGGGGCGGTTGAGCTTACGACG
GAGATCCAGCGCTCGGCCACCAAGTCTTGACTGCGTATTGGACGCTCCGCAAAAGAACGTCCGATGAGCTTGGAAAGTGCTTCTGCTGACCAACACGCGCT
TCTGGTGGCCCATCTGCGCCACGAGGTGATGCAGCAGCATTGCCGCGTGGGTTTCTCGCAATAAGCCCGGCCACGCGCTCATGCGCTTTGCGTTCCGTTTGC
ACCCAGTGACCGGCTTGTCTTGGCTTGAATGCCGATTCTCTGACTGCGTGCGCATGCTTATCTCATGCGGTAGGGTGCCGACGCTTGGCGCACCATG
CGCAATCAGCTGCAACTTTTCGGCAGCGCGACAACAATTATGCGTTGCGTAAAGTGCGCAGTCAATTACAGATTTTCTTAACTACGCAATGAGCTATTGCGG
GGGGTCCGCAATGAGCTGTTGCGTACCCCCCTTTTTAAGTTGTTGATTTTTAAGTCTTTCGATTTCCGCTATATCTAGTTCTTTGGTGCCAAAGAGGGC
ACCCCTGCGGGGTTCCCCACGCTTCGCGCGGCTCCCCCTCCGGCAAAAGTGCGCCCTCCGGGGCTTGTGATCGACTGCGCGGCTTCGCGCTTGCCCA
AGGTGGCGCTGCCCTTGGAAACCCCGCACTCGCGCGGTGAGGCTCGGGGGCAGGCGGGCGGGCTTCGCTTTCGACTGCCCCACTCGCATAGGCTTGG
GTCGTTCCAGGCGGCTCAAGGCCAAGCCGCTGCGCGGTGCTGCGGAGCCTTGACCCGCTTCACTTGGTGCCAACCGGCAAGCGAAGCGCGCAGGGCG
CAGGCCGAGGCTTTTCCAGAGAAAAATAAAAAATTGATGGGGCAAGCGCGCAGGCCGCGCAGTTGAGCCGGTGGGTATGTGTGAAGGCTGGGT
AGCCGGTGGGCAATCCCTGTGTTCAAGCTCGTGGGCGAGGCGCAGCTGTCCATCAGCTTGTCCAGCAGGGTTGTCCAGGGCGGAGCGAAGCGAGCCAGCC
GGTGCCGCTCGCGGCATCGTCCACATATCCAGGGCTGGCAAGGAGCGCAGCGACCGCGCAGGGCGAAGCCCGGAGAGCAAGCCGTAGGGCGCGCG
AGCCCGGTAGGCG

10.5 Minitrn5Kan_R6K plasmid sequence

TACTCGGATGGAAGCCGGTCTTGTGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCCGCAAGGCTCAAGGCGCGCATGCC
CGACGGCGAGGATCTCGTGTGACCATGGCGATGCTGCTTCCGCAATATCATGGTGGAATAATGGCCGCTTTTCTGATTATCATGACTGTGGCCGGCTGGGT
GTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCTCGTGCTTTACGGTATCGCC
GCTCCGATTGCGAGCGCATCGCTTCTATCGCTTCTTGACGAGTTCTTCTGAGCGGGAAGTGGGGTTGCAAAATGAGTTTAAACGCCGGAACCTGACTAATTA
AATGGGGACCCAGAGGTCCCTTTTTATTTTGTGAGATGTGTATAAGAGACAGACTAGTTACACAAGTACGCTCTGAACGGAACCTTCCCGTTTCCAG
AATCTGATGTTCCATGTGACCTCTAACATGGTGACCTTCATGATTACCAAGTGCATCGTGCGCGGATTGGGCGAAAGCGGTGTTTTCTAGTGTGCGC

TGGGTGATCCGCGCTGTACCGCGCTCTGGTGAATGTTGCGGCGCAACTGGCCAAATATAGCGGCAAAAGCATTACCATTAGCAGCGAAGGCAGCAAAGCCA
TGCAGGAAGGCGCGTATCGTTTATTTCGTAATCCGAACGTGAGCGCGGAAGCGATTTCGTAAGCGGGTGCCATGCAGACCGTGAAACTGGCCAGGAATTTTC
CGGAACTGCTGGCAATTGAAGATACCACCTCTCTGAGCTATCGTCACTCAGGTGGCGGAAGAACTGGGCAAACTGGGTAGCATTGAGGATAAAAGCCGCTGGTT
GGTGGGTGCATAGCGTGCTGCTGCTGGAAGCGACCACCTTTCTGACCGTGGGCTGCTGCATCAAGAATGGTGGATGCGTCCGATGATCCGGCGGATGCG
GATGAAAAAGAAAGCGGCAAACTGGCTGGCGCTGCTGCAACTTCGCGTCTGAGAATGGGCAGCATGATGAGCAACGTGATTGCGGTGTGCGATCGTGAAGC
GGATATTCTAGCGTATCTGCAAGATAAACTGGCCATAACGAACGTTTTGTGGTGCGTAGCAAAACATCCGCTAAAGATGTGGAAGCGCGCTGTATCTGTAT
GATCACCTGAAAAACAGCCGGAACCTGGGCGCTATCAGATTAGCATTCCGAGAAAGGCGTGGTGGATAAACGTGGCAACGTAAAAACCGTCCGGCGCG
TAAAGCGAGCCTGAGCCTGCGTAGCGGCCGTATTACCTGAAACAGGGCAACATTACCTGAACGCGGTGCTGCGCCGAAGAAATCAATCCGCCGAAAGGCCA
AACCCGCTGAAATGGCTGCTGCTGACGAGCGAGCCGGTGAAGTCTGGCCAAAGCGCTGCGTGTGATTGATATTTATACCCATCGTTGGCGCATTGAAGA
ATTTCAAAAGCGTGGAACCGGGTGGGGTGGGAACGTGAGCGTATGGAAGAACCGGATAACCTGGAACGTATGGTGAGCATTCTGAGCTTTGTGGCGG
TGCGTGTGCTGCAACTGCGTGAATCTTTTACTCCGCCGCAAGCACTGCGTGGCGAGGGCTGCTGAAAGAAGCGGAACAGTTGAAAGCCAGAGCGCGGAAA
CCGTGCTGACCCCGATGAATGCCAACTGCTGGGCTATCTGGATAAAGGCAACGCAACGCAAGAAAAAGCGGCGAGCTGCAATGGGCGTATATGGCG
ATTGCGGTCTGGGCGGCTTTATGGATAGCAACGTACCGGCATTGCGAGCTGGGGTGGCGTGTGGGAAGGTTGGGAAGCGCTGCAAGCAAACTGGATGG
CTTTCTGGCCGGAAGACCTGATGGCGAGGGCATTAAATCTAATCGTAGTAGCCCGCTAATGAGCGGGCTTTTTTTGGCCGCCAGTGGGCAAGTTGA
AAAAATCACAAAATGTGTATAATATCTTTGTTCAATTAGAGCGATAAACTTGAATTTGAGAGGGAACTTAGATGTTATTTGAAAAAATGATAAAAAAGTTG
GAACAGAAAAGATTTTTGACCACTACTTTGCAAGTGTACCTTGACCTACAGCATGACCGTTAAAGTGGATATCACACAAATAAGGAAAAAGGGAATGAAA
CTATATCTGCAATGCTTTTATTATATTGCAATGATTGTAACCGCCATTACAGATTTAGGACGGCAATCAATCAAGATGGTGAATTGGGGATATATGATGAGAT
GATACCAAGCTATACAATATTCAATGATACTGAAACATTTTCAGCCTTTGGAGTGAAGTGAAGTCTGACTTTAAATCATTTTTAGCAGATTATGAAAGTGA
TACGCAACCGTATGGAAAAATCATAGAATGGAAGGAAAGCAAAATGCTCCGGAAAAACATTTTTAATGTATCTATGATACCGTGGTCAACCTTCGATGGCTTT
AATCTGAATTTGCAGAAAGGATATGATTATTTGATTCTATTTTACTATGGGAAATATTATAAAGAAGATAACAAAATTACTTCCCTTTGGCAATTCAGATT
CATCACGCAGTATGTGACGGATTTACATTTGCCGTTTTGTAACGAATTGCAGGAATTGATAAATAGTTAACTTCAGGTTTGTCTGTAACAAAAACAAGTAT
TTAAGCAAAAACATCGTAGAAATACGGTGTTTTTTGTTACCTAAGTTTGTGTTAAACGCAAGTTCACCTGTTGATAGTACGTACTAAGCTCTCATGTTTCACGTA
CTAAGCTCTCAAGTTTAACGTAAGCTCTCATGTTTAACGAACTAAACCTCATGGCTAACGTAAGCTCTCATGGCTAACGTAAGCTCTCATGTTTC
ACGTAATAAGCTCTCATGTTTGAACAATAAAATTAATATAAATCAGCAACTTAAATAGCCTCTAAGGTTTTAAGTTTTATAAGAAAAAAAAGAAATATAAGGC
TTTTAAGCTTTTAAGGTTTAACGGTTGTGGACAACAGCCAGGGATGTAACGCAGTGAAGAAGCCCTTAGAGCCTCTCAAAGCAATTTTGAAGTACACAGGAA
CACTTAACGGCTGACAACCGCGGCGGCCCTCTCACTTCCTGTAAAGTATCTTCTGGCATCTTCCAGGAAATCTCCGCCCCGTTCGTAAGCCATTTCCGCTCGC
CGCAGTCGAACGACCGAGCGTAGCGAGTGGGCCCCCTGCTTCGGGGTCAATTATAGCGATTTTTTCGGTATATCCATCTTTTTTCGACGATATACAGGATTTTG
CCAAAGGGTTCGTGTAGACTTTCTTGGTGTATCCAACGGCGTCAGCCGGGCAGGATAGGTGAAGTAGGCCACCCGCGAGCGGGTGTCTCTTCTCACTGTC
CCTTATTCGCACTGGCGGTGCTCAACGGGAATCCTGCTCTGCGAGGCTGGCCGGCTACCGCCGCGTAACAGATGAGGGCAAGCGGATGGCTGATGAAACC
AAGCAACAGGAAGGGCAGCCACCTATCAAGGTGACTGCCTTCAGACGAACGAAGAGCGATTGAGGAAAAGCGCGGGCGGCCGCGATGAGCCTGTC
GGCCTACCTGCTGGCGTGGCCAGGGCTACAAAATCACGGGCGTCTGGGACTATGAGCAGTCCGCGAGCTGGCCCGCATCAATGGCGACCTGGGCGCCCT
GGGCGGCTGCTGAACTCTGGCTCACGACGACCCGCGCACGGCGCGTTCGGTGTATGCCACGATCTCGCCCTGCTGGCGAAGATCGAAGAGAAGCAGG
ACGAGCTTGGCAAGGTATGATGGGCGTGGTCCGCCGAGGGCAGAGCATGACTTTTTAGCCGCTAAACGGCCGGGGGGTGGCGGTGATTGCCAAGCA
CGTCCCCATGCGCTCCATCAAGAAGAGCGACTTCGCGGAGCTGGTGAAGTACATCACCAGCAGCAAGGCAAGACCGATCGGGCCCCCTGCAGGGGGGGCTG
TCTCTATACACATCTCAACCATCATCGATGAATTCGAGCTCAAGCTTCCCGGGGATCATCTTATTAATCATATGTGACGTCCATCAAGCTACGATGAACGAGCT
TGGCTAGCTTTAATTAATCTAGACAAAGCCCGCCGAAAGGCGGGCTTTCTGTGCGCCGACTGGGCGGCCATGTGAGTACTGGGCTATCTGACAAAGGG
AAAACGCAAGCGCAAGAGAGAAAGCAGGTAGCTTGCAAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGACAGCAAGCGAACCGGAATTGCCA
GCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAAGTAAACTGGATGGCTTTCTGCGCCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAA
GAGACAGGATGAGGATGTTTTGCGATGATTGAACAAGATGGATTGCACGAGGTTCTCCGCGCCGCTGGGTGGAGAGGCTATTCCGGCTATGACTGGGCACAA
CAGACAACTCGGCTGCTGATGCCCGCTGTTCCGGCTGTACGCGCAGGGGCGCCCGGTTCTTTTGTCAAGACCGACCTGTCGGGTGCCCTGAATGAAGTGC
AGGACGAGGCGAGCGCGCTATCGTGGCTGGCCACGACGGGCGTTCTTGTGCGAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTG
GGCGAAGTGGCGGGCAGGATCTCTGTATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGGCGCTGCATACGCTTATCCGG
CTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACG

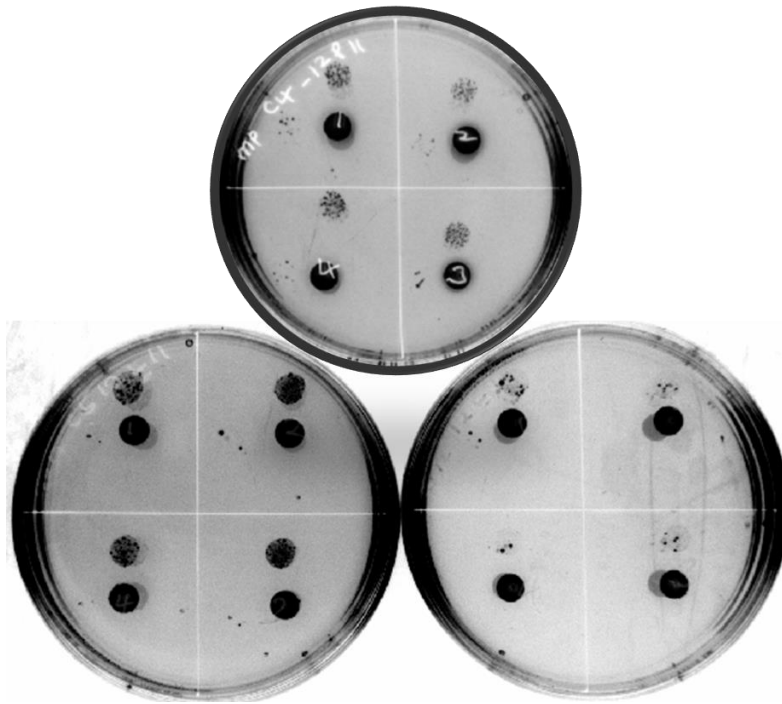
10.6 pMTL90882_12L11 plasmid sequence

CGCGCCGACTGGGCGGCCATGTACGCTACTGGGCTATCTGGACAAGGGAACGCAAGCGCAAGAGAGAAAGCAGGTAGCTTGCACTGGGCTTACATGGCG
ATAGCTAGACTGGGCGTTTTATGACAGCAAGCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAAGTAAACTGGATGG
CTTTCTTGGCCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGATGATTGAACAAGATGGATTGCACGCAG
GTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTGATGCCGCCGTGTTCCGGCTGTACGCGCAGGGGC
GCCCAGTTCTTTTTGTCAAGACCGACCTGTCCGGTGGCTGAATGAACTCGAGGACGAGGCAAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCTTTCG
CAGCTGTGCTCGACGTTGTCACTGAAGCGGAAGGGACTGGCTGCTATTGGGCGAAGTCCCGGGCAGGATCTCTGTCACTCTCACTTGTCTCTGCCGAGA
AAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGTATCCGGCTACCTGCCATTTCGACCAACAGCGAAACATCGATCGAGCGAGCAGTAC
TCGGATGGAAGCCGGTCTGTGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGGCCAGCCGAACTGTTCCGAGGCTCAAGGCGCGCATGCCCG
ACGGCAGGATCTCGTGTGACCCATGGCGATGCTGCTTCCGAATATCATGGTGGAAAAATGGCCGCTTTTCTGGATTATCGACTGTGGCCGCTGGGTGT

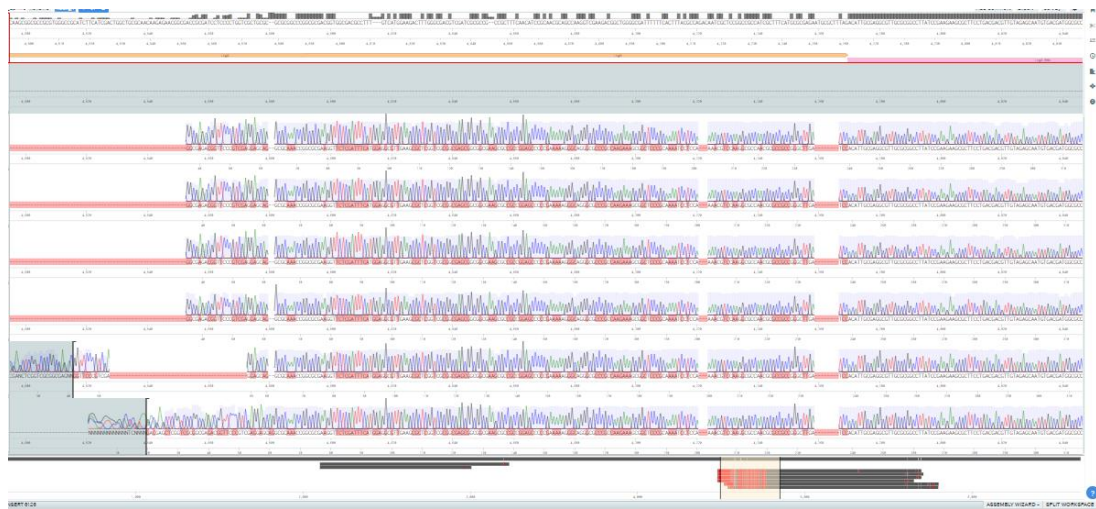
GGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCTCGTGTCTTACGGTATCGCCGCT
CCCCGATTCGACGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCCTTGAGCGGGAGCTTGGGGTTCGAAATGAGTTTAAACGCCGGAAGTACCCCAACAAG
GCCCTAGTTCGCAATGACACGAGGTGATCATTGACCCAGGCGTGTTCACACGCGCGCTGCTCGCAACTCTTCGCAAGCTTCGCGACGCTGCGGCCACT
TCTTCACGCGGGTGAATCCGATCCGACATGAGGCGGAAGGTTCCAGCTTGAGCGGGTACGGCTCCCGGTGCGAGCTGAAATAGTCGAACATCCGTCGGG
CCGTGCGGCGACGCTTGGCGGTACTTCTCCCATACGAATTCGCTGAGTGGTGCGCCAGCAAAACAGCACGACGATTCTCTGTCGATCAGGAGCTGGCAACGGGA
CGTTTCTTCCGACGGTCCAGGACGCGGAAGCGGTGCAGCAGCGACGAGTTCACAGGTGCCAACGCGGTGCGGAGCCCATCGCGCTCGCTGTAG
GCGCGACAGGCATTCTCGGCCTCGTGTAAATACCGGCCATTGATCGACACGCGGCTCTGGCAAAGCTCGTAGAACGTGAAGGTGATCGGCTCGCGGAT
AGGGGTGCGCTTCGCGTACTCCAACACCTGCTGCCACACGAGTTCGTCATCGTCGCGCCGCGAGCTCGACGCGCGGTGATAGGTGATCTTACGCTCTTGTGACGT
GGAAAAAGACCTTGTTCGAGCGCTCGCGGGGATTTTCTGTTGCGCGTGGTGAACAGGGCAGAGCGGGCCGTGTCGTTGGCATCGCTCGCATCGTGTG
CGGCCACGCGCAATATCGAAACAGGAAAGCTGCAATTCCTTGATCTGCTGCTTCGTGTGTTTCAGCAACGCGCGCTGCTTGGCCTCGCTGACCTGTTTGCCA
GGTCTCGCGCGGCTTTCGCTTCTTGGTGTGATAGTTCCTCGCGTGTGATGGTCATCGACTTCGCCAAACCTGCCGCTCTGTCGAGACGACGCGAA
CGCTCCACGCGCGCGATGCGCGGGGAGGGCAGGGGGAGCCAGTTGACGCTGTCGCGCTCGATCTTGGCCGTAGTGTGCGACCATCGAGCCGACGGA
CTGGAAGGTTTCGCGGGGCGACGATGACGCTGCGGCTTGCATGTTTCGCGATCCTCGGCGGAAACCCCGCTCGATCAGTTCCTGCTGTATGCTTTC
CGGTCAAACGTCCGATTCACTACCTCTCTGCGGGATTGCCCCGACTCACGCCGGGGCAATGTGCCCTTATTCCTGATTGACCCGCTGGTGCTTGTGTG
CAGATAATCCACCTTATCGGCAATGAAGTCGGTCCCGTAGACCGTGTGGCGCTCTTCTCGTACTTGGTATTCCGAATCTTGGCTGACGAAATACAGCTCCGC
GAAGTCGCTCTTCTGATGGAGCGCATGGGGACGTGCTTGGCAATCACGCGACCCCCCGCGTTCCTAGCGGTAAAAAGTCATGGCTCTGCCCTCGGGC
GGACCAAGCCCTGACTTTCGCAAGCTTCTTCTGCTATCTCGCATCTCGCGAGCGGGGAGGATCGTGGCATCACGGAACCGCGCGCTGCGCGGCTC
GTCGGTAGCCAGAGTTTCAGAGCGCGCCAGCGCGCCAGGTGCGCATTGATGCGGGCCAGCTCGCGGACGTGCTCATAGTTCACGACGCGCGGTGATTTT
GTAGCCCTGGCCGACGCGCAGAGGTAGGCTACAGGCTCATGCGCGCGCGCGCGCTTCTCTCAATCGCTCTCGTTCGTGGAAGGCGAGTACACCTTG
ATAGGTGGCTGCGCTTCTGTTGGCTTGGTTTTCATCAGCCATCCGCTTCCCATCTGTTACGCGCGCGGTAGCGCGGCTGCGACGAGGATTCCTC
GTTGAGCACCAGGTGCGAATAAGGGACAGTGAAGAAGGAACACCGCTCGCGGGTGGGCTACTTCACTATCTCGCCGCTGACGCGCTTGGATACA
CCAAGGAAAGTCTACAGAACCTTTGGCAAAATCTGTATATCGTGCAAAAAGGATGGATATACCGAAAAATCGCTATAATGACCCGGAAGCAGGGTTAT
GCAGCGAAAAAGATCCGTGACCTTTCCGACGCTCACCGGCTGGTGGCTCGCGCTGGGCTGGGCGCGCTATGCGCTGCAACGCGCCAGAAAG
CCGTGCAAGCCGTGCGAGACCGCAGCGCGCGGCTTGGATACCTCGCGGAAAACTTGGCCCTCACTGACAGAGGGCGGACGTTGACCTTG
AGGGGCGGACTACCCGGCGCGGCTTGACAGATGAGGGGCGAGGCTGCAATTCGCGCGGCGACGTGGAGCTGGCCAGCTCGCAAACTCGCGAAAAACGCT
GATTTACGCGAGTTTCCACAGATGATGTGGAACGCTGGGGATAAGTGCCCTGCGGTATTGACACTTGAAGGGCGGCACTACTGACAGATGAGGGGCGC
GATCTTTCGACACTTGAAGGCGAGAGTGTGACAGATGAGGGGCGCAGCTTATGACATTGAGGGGCTGTCACAGGAGCAAACTTTCGCAAGGTTT
TCCGCGCTTTTCGCGCCACCGCTAACCTGTCTTTAACCTGCTTTTAAACCAATATTTATAAACCTTGTTCCTAACAGGGCTGCGCCTGTGCGGTGACCGG
CAGCGCAAGGGGGGTGCCCCCTTCTCGAACCTTCCGCGCGCTAACGCGGGCTCCATCCCCGAGGGGCTGCGCCCTCGGCGCGCAACGGGCTCAC
CCAAAAATGGCAGCAAGCTGACACTTCTGCGCTCGGCCCTTCGGCTGGCTGTTTATTGCTGATAAATCTGAGCCGGTGAGCGTGGGTCTCGCGGTAT
CATTCGACACTGGGGCAGATGGTAAGCCCTCCGCTATCTGATGTTTACACGACGGGAGTCAAGGCAACTATGGATGACAGAAATAGACAGATCGCTGA
GATAGTGCCTCACTGATTAAGCATTGGTAAGTGTGACAGCAAGTTTACTCATATATCTTAGATTGATTTAAACTTCACTTTTAAATTTAAAGGATCTAGGT
GAAGATCCTTTTGATAATCTCATGACCAAAATCCCTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCGTAGAAAAAGATCAAGGATCTTCTGAGATC
CTTTTTTTCGCGTAATCTGCTGCTTGCACAAACAAAAACACCGCTTACCGCGGTGTTTGTTCGCGGATCAAGAGCTACCAACTCTTTTCCGAAGGTAA
CTGGCTTCAGCAGAGCGCAGATACCAAACTACTGCTTCTAGTGTAGCGGTAGTTAGGCCACCCTTCAAGAACTCTGTAGCAGCCCTACATACCTCGCTCG
CTAATCTGTTACAGTGGCTGCTGCCAGTGGCGATAAGTGTGCTTACCGGTTGGAATCAAGACGATAGTTACCGGATAAGGGCGACGGCTGCGGGTGA
ACGGGGGTTTGCACACAGCCAGCTTGAGCGAACGACCTACCCAGATGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCAGCTTCCGAAAG
GAGAAAGGCGGACAGGTATCCGTAAGCGCGAGGGTCGGAACAGGAGAGCGCAGGAGGAGCTTCAGGGGGAAACGCTGGTATCTTTATAGTCTGTG
GGGTTTCGCGACCTCTGACTTGAAGCGTGTATTTGTGATGCTGCTCAGGGGGCGGAGGCTATGAAAAACGCGCAGCAACGCGGCTTTTACGGTTCCTGG
CCTTTGCTGGCTTTTGTCTACATGGTACCCCTGACGGCAAAATAAACGAAAGGCTCAGTCGAAAGACTGGGCTTTCGTTTATCTGTTGTTGTCGGTGA
CGCTCTCCTGAGTACGAAATCAGGAACAGCTAGACCGCGGCGCAGTATGACCTATGACCATGATTACGAATCTCAGTCACTCTGACTGACTCAAAATC
AATGCGTGTTCATAAAGACAGTATGATGATTGATGGATAAGAGTGGCATCTAAAACTCTTTTGTAGACGTATATCGTTTACGATCAATTTGTATCAAAAT
ATTTAAAGAGCGGGAGCTCAAGATTCTGCAACGTAAATAAATGAATAATATTTCTGCTGTTTACGATTTGGTTGTCTATGTTGTTATATGACCTAA
GAACCTTATCTAAATGGCATCTGTAAATAAACCGCTTAGAAAACTCAGTATTGCTCAATAATCTCATCTAAATAGCTTATGCTCTCAACAAACAACT
GTTTTTGTCTGTTATCTTCTGGACTACCTTCAACTTTTATAATGACTAGTAAATAAAAAATTCACATATTTGCTTGGCAGAGCCAGCTCATTTCTTTTGT
AATTCTCCGCGACTAGCGAGCATCCGTTTACGACCGTTTTCTAACTCAAAAAGACTATATTTAGGTAGTTAATGATTAAAGCTTTTTTAACTCTTATATCCTT
AGCTTCAAAAAGTCAATCGGATTTTTTCAAAGGAACCTTTTCCATAATTTGATCCCTAGTAACCTTTTAAAGGATTTAACTCTTCGATTTCCCTTTTCCA
CCTTAGCAACCACTGAGACTGAATAAGCTACCGTTGGACTATCAAAACCCACCATATTTTTTGGATCCGAGCTTTTTTACGAGCAATAAGCTTTCGCAATTC
TTTTTGGTAAATGACTCCTTGGAATCCGCTGCTGTAATCTGTTTTCTTGACAAATGACTTGGGCGATGGACAATACTTTCGCGACTGTGGCAAAAT
CTGCGCTTATCCGAGACAATTTCTCAGTTTCCCATAGTTTCGATTAGAGAGCGGTTTGCAGATCTCCTATTGCAAGGTAAATTTCTGTTTGAAGAAGT
CATGATAATAGTAGTAAAGAAATATTTGCGGTTGCTTGGCTATTTCTGCTCAGACTTAGCAATCATTTTACGAACATCATTAATGCTTATGCTGCTCAGACA
AACTCCGATTCAAGTTTTGGATATTTCTAATCAAAGCAGTTCACACGCGCATTTAGATACGATCATGGGCGATGATGGTAATTTGTAATCTCACGTACTTTA
TAGAATTGAAAACTTTTCGGAAGTCAGAACTAATTTAGATTTAAGGTAATCACTTTAACCTCTCGAATAAGTTTATCATTTTTCATCGATTTAGTATTCATGC
GACTATCAAAATTTTGCCACATGCTTAGTGATTGGCGAGTTTCAACCAATTTGGCGTTTGATAAAAACGAGCTTATCAAGTTCACTCAAACTCCACGTTAC
CATTGTTAAATTTACAACTTACGTTGAGTGATTAACTTGGCGTTTGAAGTTTCCCAATAGTTTTTCACTCTTTTGACTCTTGGAACTGGAAGTTATCC
GATTTACACGAGTTTTATCAGAACGCGTTAAGACCTATTGTCTAATGAATCGTCTTAAAGGAACTTTGGAACAAATGTGATCGACATCATTAATCACTTAA
CGATTAATATCAATTTCTGGTCCACATACATGCTCTTCCATTTTGGAGATAATAGAGATAGAGCTTTTCAATTTGCAATTTAGTATTTTCAACAGGATGCTCT
TAAGAACTCTACTAATTTCTTGATACCTTCTCGATTGCTTTCATCGCTTCCGCAATTTTCTGGCCCTTTTGAATTTGCTGATTTGCTGATTTTCA
ATAACGATATTTTCTGGCTATGCCGCCCTTACTTTGACCAATTCATCAACAACTTTTACAGTCTGTAATAACCTTTTATAGCAGGGCTACCGCTAAAT
TTGCAATATGTTATGTAACATATCGCCTTGTCCAGACACTGTGCTTTTTGAATGCTCTTTTAAATGTCAAACATCATCATGATCAGCTGCATAAAATGGC
ATTGGCAAAACCATCTGATTTCAAAAAATCTAATATTGTTTGGCAGATTGCTTATCCCTAATACCATTAATCAATTTTTCGAGACAAACGTCCTCAACAGTATA
CGGCGAGCTTTAAGCTGTTTATCACCTTATCATCAAGAGGTGAGCATATTTTAAAGTCTTCTCAATCATCTCCATCTTCAAAATAGGTTTGTGATAA
CAATATCCTCTAAGATATCTTCAATTTCTTATATCCAAAAATCTTATCTTAAATAATTTTGTAGCAATCATGGTAGTACCAATGAAGCATTAATCTATCT
TCAACTCCTGAAATTTCAACACTATCAAAACATCTTATTTTTGAATAATCTTCTTTAATTGCTTAAACGGTTACTTTTCGATTGTTTGAAGAGTAAATCAAC
AATGGCTTTCTTCTGTTCACTGAAAGAAATGCTGGTTTTGCAATCTTCAGTACACATATTTGACCTTTGTCAATTCGTTTAAACCGTAAATCATATAAGC
AAACTAGTTTTGGTAGTACTTTTTCAATTTGGAAGATTTTTATCAAAGTTTGTGATGCGTTCAATAAATGATTGAGCTGAAGCACCTTTATGACAACTTCTTCAA
AATTCATGGGGTAATTTGTTTTCAGACTTCCGAGTCATCCATGCAAAACGACTATTGCCACGCGCAATGGACCAACATAAAGGAATTCGAAAGTCAAG
ATTTTTCAATCTCTCAGGATTGCTTTTTAAAAATGGATAAAAGTCTTCTGTTCTTCAAAATAGCATGCGACTACCCAAAGTGAATTTGATGGGGAATAGAG
CCGTTGTCAAAGGTCGTTGCTGCGCAGCAAACTTCAAGATTAGTTTACCAATTAATCTCAGTACCATCCATTTTCTTAAATTTGTTTGAATAATTTAT
AAAAATCTCTTGGTAGCTCCCATCAATATAACCTGCATATCCGTTTTTGTGATGATCAAAAAAGATTCTTATATCTTTTGTGAAGTTGTTGCGAATCA
AGCTTTTAAAGAGTCAAGTCTTGATGATGTTTCATCGTAGCGTTTAACTATTGAAGCTGATAGGGGAGCCTTAGTATTTCAGTATTTACTCTTAGGATATCTGA
AAGTAAATAGCATCTGATAAATCTTAGCTGCCAAAAACAATCAGCATCTTCAATTTGCGCAATAAATATCAATCATCATGTAAGTATCTT
TGAAAGCTGAATTTAGCATCTTCTGCCAAATCAAAATTTGATTTAAATTTAGGGGTCAAACCAATGACAAAGCAATGAGATTCCCAATAAGCCATTTTCTT
CTCACGGGGAGCTGAGCAATGAGATTTCTAATCGTCTGATTACTCAATCGTGAGAAAGAAATCGCTTAGCATCTACTCACTTGGCTTAATAGGGTTTTCT
TTCAAAATATTGCTGAGTTGTACCAACTGGATAAATAGTTGTCACATCACTAATTATCAGGATTTAAATCTCCCTCAATCAAAAAATGACCAAACT
AATCAATGTTGCTAAGGCCAAATAGATTAAGCGCAAAATCCGTTTATCAGTAGAACTTACCAATTTTTCGAGATAGATGATTGTTGATTTCTGATGATA
AGCAACTTCACTACTATATTTCAAAAAATAGGATGACGTTTCATGCTCTTGTCTTCTTCCACCAAAAAAGACTCTTCAAGTCGATGAAGAAACTATCATCTACT
TTGCGCATCTCATTTGAAAAATCTCCTGTAGATAACAAATACGATTCTTCCGACGTGTATACCTTCTACGAGCTGCTGGTTGAGACGAGTCGCTTCCGCTGTC
TCTCCACTGTCAAATAAAGAGGCCCTATAAGATTTTTTTGATACCTGCTGGCGGTGTGATTTCCGAGAACCTTGAATTTTAGACGAACTTATCTATCATAG
TGATACCGGCCATCCGACGCTATTGTGCGGATATCTAAGCCTATTGAGTATTTCTATCCATAGACGAGTCTCCTGCGACGCTCTGCAAGGCCGCTCATGGC
TTCTTGAAGGATGGCGTGCCTCGTATAGACGCTCACTCTTTTGAATTGGAGCTGCCGGTTCGTTGAAGGTGCGCTTCTTGGGGGCGCTGCGCGCTT

TATGGGAGAGACGATCCGACCGCATATAAATTTACGGCGCGCGACCTGTCTTACGATTTATCGGTAATGTGCGGCGGGAAGATCGGCATATCTCCTCCCG
TATCTAGACACCCAGGGATGGTCCGGCGGCACGGTTTTGACCTTGCGGCATGCTGGACACGGCGCTGGCCCGTTTTCCGCGGCGGAAGGAGCTTACGGCGG
GTGCGGGGGCACTGAGGCGGCGACCATGACCGGCGGCGGCATCGCAGGAAAGCGACCCGAGCTTTCCAAACCTGTTCTTGACGTAAACAGAAAGTTCCGGGT
ACGGTTCTCCACTTTTCACTTGGCGCTCCCACTACTGGAGCGGTTTGCAACGGGCGCCCGTAAGCGCCTGATCAGTAAAGAGGGGCCGAAGCTTAATTAACCA
GTAAAGGAGATCGAAAGTTTTAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGCGACCGAGTCGGTGCTTTTTTATAA
AAATAAGAAGCCTGCATTTGACAGGCTTCTATTTTTATGCGATTTTCTTTTTGCGTATCTCCGGCTTCGCCGCCGCCGGGCGCTTCGCGCCGCCGCTTATGT
AAAGTGATGCTATCGCGCTGCAGGAGCAAAATCATGGCCGAGTCGGCGCGCACTTCTGGAAGGGCCATCTGCGTCTGGCCCTGGTTTCGATCCCCGTCC
GCCTCATCGCGGCTGAAAAGGCCGAATCGGAGATCCGTTTCATCAGTTCGACCGCAAGTCGAAGCAGCGCATCCGCTATCTCAAGGTCGCGCTCGGCAAGG
GCGAGGTCAAGAAAGAGGATATTGTCTCGGTTATGAGGTGGAGCCGGGAAATTACGTGTTTCATGGAGGACGAGGAGCTCGATACGCTGAAGCTTCTCTCG
CGTCATACGATCGAGCTGTCTCAATTCGTCGACGCGGACGAAATCGAGCCGCTTTATTTCAACCGGCCCTATTATGTTCTGCCGACGGCGAAGTGCGGAGG
AGGGCTATCGCGTTTTGCGCGACGCCCTCTGGTCCAAGCGAAAGGTTGGAATCGGCCAGCTCACATTGCGCGACGCGAGAATCTCTGCGCGCTGCTGCCGG
CCGGCGACGGGCGAGGGGCTCGTCTCGACACGCTCGGTTATGAGAGCGAACTCAAGGACGCCGAGGAAATTTCTCTCCATCGGCCGCGACAAGCCGCGC
GAGGACATGGTGCAGATGGCGGAGGATCTGATCGAACGGCGCAGCGAGCCCTTCGACGCCGCTAAGTTCCACAACCATACGACAGAGGCGCTGCGCGACCT
CGTAAAGACGAAGCTCGGTGCGGCGAGACGGTCCCGTCGAGGAGCAGGCGCAAACCGGCGCAAGGTTCTCGATTTTCATGGAGGCGTTGAAGCGCTCGG
TCGCGGCGAGCGGCGGCGAAGCGCCGCGAGCCCCGAAAAAGGGCAGGGCGCCCGCCCAAGAAAGCGGCTCCCGCAAAATCTCCAAAACGTCCAAAGGC
GCCAACGCGCCGCGGGCTTGATCCACATTGCGAGGCGTTGCGCGGCTTATCCGAAGAAGCGCTTCTGACGACGTTGTAGAGCAATGTGACGATGGCGCC
GGTGACGCCGCCGCCACGGCTTGCCCAAGCAGAACGCCGAGGTGCAAGCCCCCGCCGCTCGCGGCGCTGGCGAGCGAGGGGATGACGGCGCTGAGG
ATCTGGCCGCAACTCCGCCGCGAGGCGCGCTAATGAGGTGCGCAAGCGCAATTGTCGCTTTTGTGCGCTTGCCCGCGCTTTGCCGCAAGGGCGCCCG
CGACGACTTGAATGAGCAGGTGCGTGATGGACATCAAAAATCGCTCCAGATAAAGGCTTCCGCCCCCTAGATAAGCGCAATGGAGCCGATTGGTTCCCTGGG
CGGCTCAGGCCGCGAGGTGCGCGACGACGGCTCGAGGACAGGGAAGCCTTCGAGCTCACGCGCAAACGTTTTTCGCGGGTGATTTTCGACGAGCCCGTCG
CCGATCAATTGCTGATTGCGGATTGCGAAAGCTTTTGCCGGTGAGCAGGAAATAACGTTGCGGGTCAATGCCTTCCCGAGGCGCAGCCCCATCAGCAGAA
ATTCGTCGCCCTCTGCTCCGAATTCAGCAATTCGTCTCAACGATCCCGTGCCCTCCGTTTCGACGACGAGTGAGCCACATCTCGGGGTGTTTCTCGACAGGCC
TGGGCGCGGCGCGCGAGGTGACGATGCGGCCATGGGCGCCGGGGCCGACGCCGCGCATATCTCCGTAGCGCCAATAGAGGAGATTGTGCCGGCACT
CTGCGCCGGGGCGCGCATGGTTTCGACACTTCGTAGGAGGGCAGGCCGTGGCGGGCGGCGACCTCCTGCGTCACGTCCCATAAAGCGCGCTGCGTGTCTGATG
TCGGGCGAGCCCATCTTGCCCGTATTCACCATGCGCTCGAACAGCGTGTCCGGTTCGATCGTAAGCTGATAGAGCGACACATGCTCGGGCGCGGGCGAGC
GCGAGGT

10.7 *ligD* gene knockout plates for *M.parvus* (OBBP)



10.8 *ligD* knockout sequencing



10.9 Materials and equipment list

10.1 Materials used in this study

Methanotroph growth:

Liquid culture – 60, 125, 145, 250 and 500 mL serum bottles. Gas-tight box used for liquid plates

Solid agar – Anaerocult, Oxoid Anaerojar, Thermo Scientific Oxoid Anaerobic Jar, Tesco KlipFresh Airtight Tupperwares

Microscopy:

Nikon Eclipse C (Phase Contrast Microscope)

Incubators:

New Brunswick Innova 42 (shaking), New Brunswick Innova 44 (shaking), Thermo Scientific Heratherm Incubator (static)

Centrifuge:

Fisher Scientific AccuSpin Micro 17R

Beckman Coulter Allegra X-30R Centrifuge

DNA quantification:

SimpliNano Spectrophotometer

Thermo Scientific Qubit 4 Fluorometer

Culture growth quantification:

Thermo Scientific Biomate 3S UV-Visible Spectrophotometer

Storage:

Revco Scientific Inc (-80°C)

Freezer (-20°C)

Cold room (4°C)

Fume cupboard:

ThermoElectron Corporation Hersafe KS 12 laminar flow cabinet

ThermoElectron Corporation Biomat 2 Class II Microbiological Safety Cabinet

PCR Thermocycler:

TProfessional TrioThermocycler – Biometra Product Line

Weighing Balance:

Denver Instrument SI-234

Stuart Heat-stir

Autoclave:

Classic Prestige Medical Autoclave

pH Meter:

Mettler Toledo FE20

Electroporation:

Biorad Genepulser Xcell

Biorad Micropulse

Imaging:

Biorad Molecular Imager GelDoc XR+ Imaging System

Electrophoresis:

Biometra Compact XS/S gel Tank

Biorad Powerpac Basic Electrophoresis Machine

Syngene Blue Light Illuminator

Freeze Drying

Thermo Micromodulyo Freeze Dryer

Trace GC

Trace 1310 Gas Chromatography

Cryo storage

Microbank™ Bacterial and Fungal Preservation System, Pro-Lab Diagnostics, UK

NMS + 15% glycerol

Antibiotic MIC

Lifolichem MIC strips

Teddlar bags

Sigma-Aldrich 2 L Teddlar bags