Isolation and Characterization of Lytic and Lysogenic Phages for Methanotrophic Bacterial Systems

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Chemical Engineering

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Abstract

Methanotrophic bacteria have the potential to act as a platform for several novel bio industrial processes, due to their capacity to convert methane, often from impure sources, into valuable products. These products can include, but are not limited to, isoprenoids for the development of bio-jet fuels and polyhydroxybutyrate (PHB) for the production of bio-plastics. However, before these processes can be implemented at commercial scale, several concerns need to be resolved. For instance, the majority of valuable products produced by these microorganisms are intracellular, and the current means of recovery are costly and/or show poor efficiency. Moreover, they often require the use of noxious chemicals such as chloroform, which are costly to dispose of and can be problematic in the environment. Therefore, alternative means of product recovery need to be established to effectively scale these processes up to commercial level.

Bacteriophages (phages) are viruses capable of infecting bacteria. Following infection, they can undergo either lytic (virulent) or lysogenic life cycles. During virulent life cycles the phage will overtake the host replication machinery to make copies of itself, eventually lysing the cell, releasing progeny which are then free to infect subsequent hosts present in the surrounding environment. On the other hand, phages that undergo lysogeny integrate their genetic material into host genomes. The resulting prophages can be induced into the lytic life cycle in response to environmental stimuli.

Little is currently known about phages of methanotrophic bacteria or the impact they may pose to the scale-up and commercialization of methanotroph-based bioprocesses. Therefore, by studying phage life cycles in the context of methanotrophic bacteria we hope to achieve the following. First and foremost, the discovery of a virulent phage capable of consistently infecting

and lysing methanotrophic bacteria could provide a novel solution to product recovery and aid in the downstream processing of novel biomaterials. Secondly, although lysogeny can beneficially confer many valuable traits to the host bacteria, it can also carry a significant metabolic burden to the cell and the unintentional induction of a prophage could result in disruption of entire production batches with negative consequences. Thus, by better understanding the role prophages play in methanotroph systems we can prevent their premature induction, or potentially even learn how to control this process to again aide in product recovery.

This thesis is dedicated to my mother and father,
Their career dedications to the educational sector
And the reminder that the pursuit of happiness
far exceeds anything else in this world

Acknowledgements

This manuscript could not have come to fruition without the support of a team of people, both inside and outside the academic world. Throughout this degree I have had the privilege of crossing paths with some amazing people, I wish I could name you all.

First and foremost, I would like to thank my primary supervisors Dr. Dominic Sauvageau and Dr. Lisa Stein, who in a time when I was about to turn my back on research gave me the space where I could rekindle my love for science. Your unwavering support has been invaluable over the years as I have discovered what I can bring to the scientific community. The guidance and mentorship you have given me since my undergrad degree has helped me accomplish everything I have today and has made me feel like I have something to contribute to the scientific community.

Secondly, I need to thank the members of the Sauvageau and Stein Lab groups for their support and encouragement throughout the years. Kieran, Mark, Melissa, Bea and Catherine as well as the rest of the group, our daily lunches, random beer outings, and insane lab social events were often the highlight of my days in the lab. The friendship, laugher, and M&M's you all provided throughout this degree were invaluable. Liam Reider I see a lot of my undergrad self in you: dedication, creativity and innovative spirt – your commitment and aptitude for this field has far exceeded any undergrad I have mentored throughout this program. When people say jump you simply respond with, "how high?" This manuscript would not have been completed on time without your hard work, and willingness to go along with every time I said, "okay, so I have a plan…" You're going to do great things, I can't wait to see what they are. Diana Martinez, I am forever indebted to you for a conversation we had back in spring 2016, where you urged me to reach out, to get help for myself, and fight for the path I was wanted in life. I would not have returned to academia without your friendship, encouragement, and confidence in my skills as a scientist.

I would also like to thank: Arlene Oatway, and the Biological Science Microscopy Unit for the support and guidance in imaging samples, Dr. Tariq Siddique, for generously providing environmental samples, Dr. Andrew Millard and the group in Leicester for sharing their expertise and equipment, and Sara Almas who assisted on this project. Additionally, thanks to

Dr. Marina G. Kalyuzhnaya, and David Collins for the continued collaboration and for providing *Methylobacter marinus* A45 with its prophage.

Next, I need to thank the collective of friends who have helped me navigate this life, from undergrad to Hide and Seek, the "TWOSE Crew" has been a tremendous source of support over the years. It's been amazing to watch you all evolve through your own degrees and what we've all accomplished since our demonstrator days is amazing. A specific mention to Heather Capel the lifeboat I held onto through the darker times of my degree. Your constant reminder that this isn't everything I am has propelled me to do amazing things in life. It will be an honor to graduate with you this year – we've earned this. Drew Delbaere, you're albeit sometimes crass life advice, has always helped me stay grounded and your persistence need to keep out the lab although decreased productivity, has kept me sane. To the new friends met at Beakerhead, who helped me push through the final chapter of this manuscript I thoroughly promise "*to go forth and not bore the world with the greatest adventure story which ever existed, science.*"

Finally, Mom and Dad. I don't even know where to begin here. Thank you so much for willingly encouraging me to go on this adventure, for letting me make my own mistakes, and celebrating my successes with me. I know you both watched me struggle to find my home in academia, in science, and most importantly this crazy world, but knowing that I could always come home for Sunday dinner somehow made all of that so much easier. Know that everything I do is to make you proud, and that I will remember you both said you would willing do this all again for a PhD someday.

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1 Introduction

Methanotrophs are a unique class of bacteria capable of metabolizing methane as their sole source of carbon and energy and convert it into valuable bioproducts. This conversion is initiated through the methane monooxygenase (MMO) pathway(Hanson & Hanson, 1996). As only bacterial and archeal methanotrophs are known to harbor a fully active MMO pathway for the conversion of free methane into methanol, methanotrophs have been proposed as a platform for several biotechnological applications (Kwon, Ho, & Yoon, 2019). For instance alphaproteobacterial methanotrophs, a subclass of methanotrophs which channel methanol into the serine cycle, have the ability to produce polyhydroxy-alkenoates (PHAs) – specifically polyhydroxybutyrate (PHB) – which can in turn be made into biodegradable alternatives to plastics of petrochemical origin (Kwon et al., 2019; Rostkowski, Pfluger, & Criddle, 2013). A recurring problem with the majority of high value products synthesized by methanotrophic bacteria is that they are predominantly intracellular, and, due to the unique membrane characteristics of these organisms, their extraction and recovery are not straightforward (Anderson & Dawes, 1990; Strong, Xie, & Clarke, 2015).

The current means of product recovery, particularly for high value molecules such as PHAs, is quite arduous and costly, and involves the use of harsh chemicals such as chloroform (Koller, Niebelschütz, & Braunegg, 2013). Not only is the disposal of chloroform quite costly, but the compound can accumulate in bodies of water downstream from processing plants such as rivers and reservoirs (Bedding, McIntyre, Perry, & Lester, 1982). Therefore, establishing alternative, ecofriendly and cost-effective means of product recovery is highly desirable and can help in the implementation of commercial methanotroph-based technologies. This is not to say this is the only obstacle to large scale exploitation of methanotrophs. For instance, many aspects of their physiology remain elusive at best, and common threats to industrial processes such as the presence of bacteriophages (phages) have been predominantly overlooked.

Phages are viral entities capable of infecting bacterial hosts. Following infection, they undergo one of two life cycles. The first is the lytic (virulent) life cycle – whereby they hijack the host replication machinery and make several copies of themselves, which are released by lysing and effectively killing their host (Herskowitz & Hagen, 1980). The second option, which

is encountered in temperate phages, is to undergo a lysogenic life cycle. In this mode, the phage incorporates its genome into the host cell's genome, where it can remain dormant as a prophage and be replicated along with host DNA under standard cell division (Casjens & Hendrix, 2015; Herskowitz & Hagen, 1980). Under the right environmental stimulus, prophages excise themselves from the hosts' genome and resume a lytic course of action until signaled otherwise (Howard-Varona, Hargreaves, Abedon, & Sullivan, 2017).

Both life cycles can impact the industrialization of methanotrophic strains. For instance, in theory a strictly virulent phage could be used in downstream processing as a chemical-free alternative means for cell lysis and product recovery. This would attenuate some of the foreseeable environmental problems associated with current means of cell lysis for product recovery (Bedding et al., 1982; Koller et al., 2013). Temperate phage life cycles provide their own unique dichotomy to the industrialization of methanotrophs. On one hand, induction of a prophage could completely jeopardize production schemes, and on the other reliable, controlled induction of a prophage by an applied stimulus could, again in theory, be adapted as a means of cell lysis for product recovery. This is incongruent with the current ideologies surrounding phage biology, whereby phages are often seen as pests in industrial processes, such as in the dairy industry whereby the unintentional induction of a prophage can result in the loss of an entire fermentation batch (Kilic, Pavlova, Ma, & Tao, 1996). The field, however, does acknowledge some of the potential benefits of carrying a prophage. For instance phage often confer valuable genetic information to their host, such as coding for advantageous genes or preventing subsequent phage infections (Susskind, Botstein, & Wright, 1974; Wang & Wood, 2016).

The truth is that, presently, little is known about the prevalence or consequences of phages in methanotrophic systems. Therefore, this study aims to take a two-pronged approach to examine the impact phages have on their methanotrophic hosts, and what this could mean in terms of developing large-scale industrial processes. The first objective aligns with previous works (Tyutikov, Bespalova, Rebentish, Aleksandrushkina, & Krivisky, 1980; Tyutikov et al., 1983), whereby we look to isolate from natural environments of methanotrophs novel virulent phages capable of infecting in-house bacterial strains. The second involves the identification of potential prophages in in-house methanotrophic bacterial strains using a series of bioinformatic software. This can provide information on the frequency of lysogenized phages within methanotroph genomes, and the nature of potential prophages in these bacteria. Chemical

induction of one or more of these potential prophages is then attempted using Mitomycin C and characterization of the resulting phage(s) is performed. By doing so we hope to gain insight to the nature of phage infection within methanotrophs and use this knowledge to move forward in the context of commercializing methanotroph-based technologies.

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2 Literature Review

This section details the relevant background information for the context of this manuscript. Specifically, the unique physiology and industrial relevance of methanotrophic bacteria, and how phages can be incorporated into their industrial processes.

2.1 Methanotroph Background

First described by Kaserer and Söhngen, methanotrophs are a unique class of bacteria known for their ability to use single carbon compounds such as methane as their primary carbon and energy source (Kaserer, 1905; Söhngen, 1906; Trotsenko & Murrell, 2008). In recent years, they have become of keen interest to bioindustry, particularly since they can convert methane from waste streams into high value compounds such as isoprenoids precursor molecules for biojet fuels, and biopolymers, such as poly-β-hydroxybutyrate (PHB) (Fei et al., 2014; Reshetnikov et al., 2011; Zhang, Zhou, Wang, & Zhang, 2017).

Phylogenetically, aerobic methanotrophs are predominantly split between two phyla: *Proteobacteria*, which are then classified into *Alpha-* and *Gammaproterobacteria*, and *Verrucomicorbia* (Ho et al., 2013). *Verrucomicorbia* are a unique subset of methanotrophs capable of growing under extremely acidic conditions, and at elevated temperatures (higher than 65°C) (Op den Camp et al., 2009). Recently a third phylum, NC10, has been characterized for methanotrophs which demonstrate the capacity to anaerobically oxidize methane coupled with nitrogen reduction (He et al., 2016; Raghoebarsing et al., 2006). It should also be noted that methanotrophy is not exclusively limited to bacteria, and archaic anaerobic methane oxidizers have been characterized amongst Euryarchaeota, including: *Methanomicrobiales, Methanobacteriales, Methanococcales, Methanopyrales, Methanocellales* (Welte, 2018).

For the purpose of this manuscript we will be focusing predominantly on the proteobacterial methanotrophs, characterized in more detail below.

2.1.1 Physiology of Proteobacterial Methanotrophs

Methanotrophs can be roughly divided into three overarching classifications – type I, type II and type X – based on morphology, membrane structure and rough physiological characteristics. Type I are Gammaproteobacterial methanotrophs distinguished by their intracytoplasmic membranes and their ability to use the ribulose monophosphate (RuMP) pathway to absorb carbon. Type II are *Alphaproteobacterial* methanotrophs with membranes arranged around the cellular periphery, and are characterized by their use of the serine pathway for carbon assimilation (Hanson & Hanson, 1996). Type X methanotrophs are a combination of the above, aligning much closer with the type I methanotrophs, but also possessing some serine cycle enzymes (Park & Lee, 2013). They also notably carry RuBisCO form I type IA and demonstrate the capacity to fix carbon dioxide (Baxter et al., 2002; Elsaied, Kaneko, & Naganuma, 2006).

2.1.2 Industrial Impact/Potential Applications of Methanotrophs

In terms of industrial potential, some Type II methanotrophs, such as *Methylosinus trichosporium* OB3b, have been thoroughly explored due to their capacity to synthesize PHB through the serine cycle (Rostkowski et al., 2013). PHB is a high value polymer with many properties similar to polypropylene that can serve as a biodegradable substitute for many carbon-based plastics currently derived from fossil fuels. Presently the production of polyhydroxylalkanoates (PHAs) – the class of polymers to which PHB belongs – is costly and can be difficult to scale up (Anderson & Dawes, 1990; Braunegg, Lefebvre, & Genser, 1998). However, as type II methanotrophs can, under the right conditions, produce these molecules naturally, the large-scale fermentation of these organisms using waste methane as a feedstock has been suggested as an effective and environmentally sustainable way to produce PHAs, and in particular PHB.

In brief, when a nutrient such as nitrogen is limited, and methane and oxygen are abundant in the environment, type II methanotrophs will synthesize and accumulate intracellular PHB granules through an extension of the serine cycle (Figure 2.1) (Pieja, Rostkowski, &

Criddle, 2011). In turn, these granules can be extracted and converted into non-woven materials, polymer films, and pharmaceutical products such as sutures or scaffolds for tissue engineering (Chee et al., 2010). Screening assays, have shown that type II methanotrophs have the potential to produce anywhere from 9-44% PHB by dry weight, with production even exceeding 50% under optimal growth conditions (Pieja et al., 2011; Wendlandt, Jechorek, Helm, & Stottmeister, 2001).

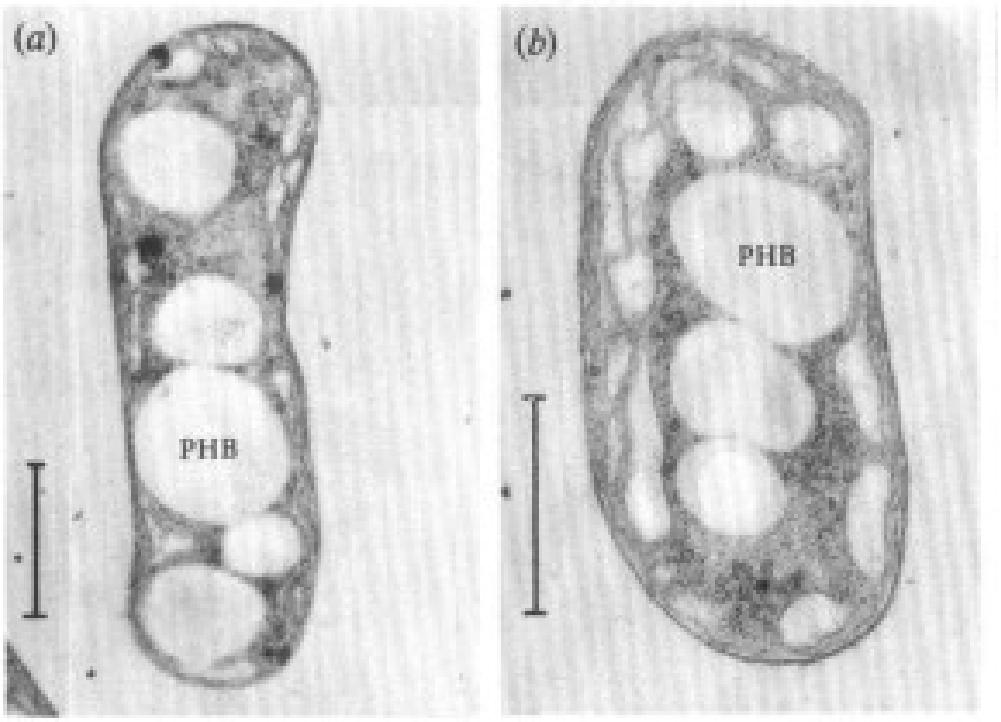

Figure 2.1: Electron micrograph cross-sections of *M. trichosporium* OB3b grown on methanol in batch cultures showing the accumulation of PHB at early-exponential phase, as acquired by Best and Higgins.(Best & Higgins, 1981). Used with permission from publisher.

This is not to write off the industrial potential of type I methanotrophs such as *Methylomicrobium album* BG8, which is a promising strain for bioprocess development due to its ability to grow to high densities under varied methane/methanol conditions and its rapid growth rate in comparison to other strains (Tays, Guarnieri, Sauvageau, & Stein, 2018). Type I strains can natively produce valuable biomolecules or could be genetically modified to produce other valuable biomolecules at high cell densities. For instance, Ye et al. were able to engineer the type I methanotroph *Methylomonas* sp. Strain 16a to produce astaxathin, a commercially

relevant carotenoid used as colorant in fish feed, by introducing a biosynthetic pathway in the organism (Higuera-Ciapara, Felix-Valenzuela, & Goycoolea, 2006; Ye et al., 2007).

2.1.3 Concerns with Current Technology

A few key issues need to be overcome to fully exploit the scope in which methanotrophs can be used in bioindustry. These include low solubility of methane into culture media resulting in slow growth and low-density cultures, competitive inhibition between metabolites and growth substrates, potential toxicity from products or co-metabolites, as well as an overall ambiguity about the organisms genetic makeup and evolution (H. Jiang et al., 2010).

However, one of the predominant issues in the field is the recovery and isolation of intracellular products, such as PHB, from methanotrophs, with high downstream processing costs being the predominant limiting factor to the industrialization of these organisms (Strong et al., 2015). Not only must the cells be recovered and concentrated, and the product extracted and purified, but due to their unique membrane compositions, product recovery has been shown to be difficult in these strains (Hanson & Hanson, 1996; Strong et al., 2015). This problem is exemplified in type I strains which feature several intracytoplasmic membrane invaginations, limiting the access to many products (Hanson & Hanson, 1996).

2.1.4 Current methods for product recovery from methanotrophs

The current means of product recovery for methanotrophs, particularly for PHAs, are quite laborious and costly (Koller et al., 2013). It begins with a pretreatment phase whereby dried biomass is flushed with polar solvents such as methanol, ethanol or acetone – weakening the cell envelopes. This process can be repeated several times if necessary. From there a solvent extraction step is carried out, typically using a halogenated solvent such as chloroform. Next, a low molecular weight alcohol, usually methanol or ethanol (but hexane, acetone or water have also been used), is applied to precipitate the PHA product. In some instances, this precipitation step has also been carried out by lowering the temperature (Koller et al., 2013).

The main concern with this procedure is the use of halogenated solvents such as chloroform, which, at large scale, would be needed in vast quantitates in order to effectively

recover a viable amount of product from batch fermentations (X. Jiang, Ramsay, & Ramsay, 2006). Moreover, the ethanol-chloroform mixture derived from the process is often discarded as the energy demand for solvent recovery and reutilization isn't economical, which undercuts the environmental upsides of the bioproduction of PHAs (Koller et al., 2013). It also should be noted that although at its current environmental levels chloroform poses no risk to human health, it does become highly volatile when mixed with water or exposed to standard environmental conditions (McCulloch, 2003). Moreover, chloroform can be difficult to dispose of and studies have demonstrated that it can accumulate in rivers and reservoirs downstream of disposal sites (Bedding et al., 1982). Additionally, there have been documented cases of workplace-associated chloroform toxicity making the chemical less than ideal for use in large scale industrial processes (Kang, Ahn, & Hwang, 2014).

Other means of product recovery, including non-halogenated solvents, chemical and enzymatic cocktails, and mechanical disruption, have been applied with minimal success.(Koller et al., 2013) Therefore, we need to look for a novel environmentally friendly means of effectively lysing cells to retrieve intracellular products.

2.2 Bacteriophages

Bacteriophages, more simply phages, are viruses capable of infecting bacterial hosts. They are considered one of the most ubiquitous and prevalent biological entities on Earth, estimated to exist in quantities of 10^{31} globally (Clokie, Millard, Letarov, & Heaphy, 2011; Comeau et al., 2008). Despite being originally isolated in 1915 by Frederick William Twort and then again in 1917 by Félix Hubert d'Hérelle, the interest in phages eventually waned in the Western World in favor of pursuing the development of antibiotics as a means to kill bacteria. However, the potential of using phages as therapeutic agents against bacteria has been extensively studied in Eastern Europe for decades. Now, with the upsurge of antibiotic resistant pathogenic bacterial strains, phages have reemerged as valuable agents for treatment of bacterial infections (Clokie et al., 2011; Comeau et al., 2008; Twort, 1915). And, as more is being discovered about phage physiology and genomics, it is becoming clear that phage technologies can be applied far beyond the field of medicine, including in agriculture, food applications and

even large-scale bioprocesses (Clokie et al., 2011; O'Sullivan, Bolton, McAuliffe, & Coffey, 2019).

2.2.1 Phage Classification

Despite their ubiquity, the taxonomic diversity of phages is just beginning to unfurl. This is in part due to advancements in genomic sequencing which are now allowing us to better analyze genetic drift and speciation in phage populations (Glazko, Makarenkov, Liu, & Mushegian, 2007). According to the International Committee on the Taxonomy of Viruses (ICTV) phages can be classified on the foundation of shared physiological/morphological traits and molecular properties such as similarities within key genes and the structure of genomic nucleic acid (Adriaenssens & Brister, 2017).

Currently, the ICTV formally acknowledges one order, 5 families (26 subfamilies), and 363 genus of phages (International Committee on Taxonomy of Viruses (ICTV), n.d.). Generally, phage genomes consist of double stranded (ds) DNA, but a few groups do contain single stranded (ss) DNA or dsRNA. (Ackermann, 2009) In virology as an overarching field, viruses are generally classified by a combination of nucleic acid composition and particle morphology. However, as phage strains are still being elucidated, the ICTV has recommended that every available property be used in phage species delineation.(International Committee on Taxonomy of Viruses (ICTV), n.d.) Therefore phages are usually classified by the following: **viruses with binary symmetry** (*Caudovirales* or tailed phages), **phages with cubic symmetry and DNA** (ssDNA – *Mircroviridae*; dsDNA – *Corticoviridae,* or *Tectiviridae*), **phages with cubic symmetry and RNA** (ssRNA – *Leviviridae*; dsRNA – *Cystoviridae*), **phages with helical symmetry** (ssDNA – *Inoviridae*; dsDNA – *Lipothrixviridae* or *Rudividae*), and **pleomorphic phages** (which do not meet any of the above criteria) (Ackermann, 2006).

Genera within the families are classified via genome structure, however this information is still being explicated as more sequencing information pertaining to phages is becoming available (Ackermann, 2006).

Caudovirales, are arguably the best-known phages with their hallmark tails and symmetrical capsids (Figure 2.2). They are composed of dsDNA and can further be divided into 3 families: *Myoviridae, Siphoviridae* and *Podoviridae.* Both *Myoviridae* and *Siphoviridae*, which

are the focus of this manuscript, have longer tails than *Podoviridae*, which have punctate tails (Figure 2.2). The main difference between *Myoviridae* and *Siphoviridae* phages is that the former has contractile tails, with a distinctive sheath disconnected from the neck, in addition to a central tube structure.

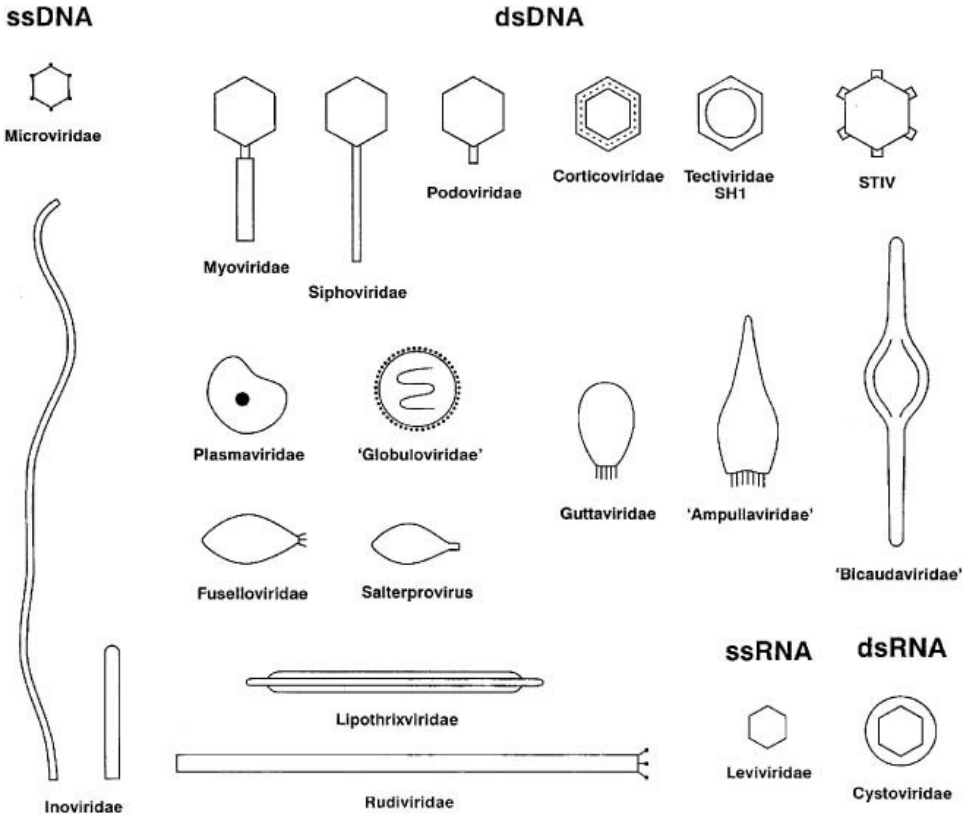

Figure 2.2: Classification of bacteriophages based on physiological features, as described by Ackermann in Methods in Molecular Biology (Ackermann, 2009). Used with permission from publisher.

2.2.2 Phage Life Cycles

Following infection, most phages typically undergo one of two life cycles: lytic or lysogenic. During the lytic cycle the virus hijacks the host replication machinery to produce copies of itself, killing its host by cell lysis imminently after replication. The newly formed phage particles are then free to infect another nearby bacterium, amplifying the phage population through repetitive replication-lysis cycles (Herskowitz & Hagen, 1980; McShan, McCullor, & Nguyen, 2019). Under the lysogenic cycle the phage incorporates its DNA into the host genome as a prophage, usually via specific recombination or random transposition (Casjens & Hendrix,

2015; Harshey, 2014; Herskowitz & Hagen, 1980). No virions – complete viral particles – are produced, and the prophage is replicated along with the host DNA during cell division (Howard-Varona et al., 2017).

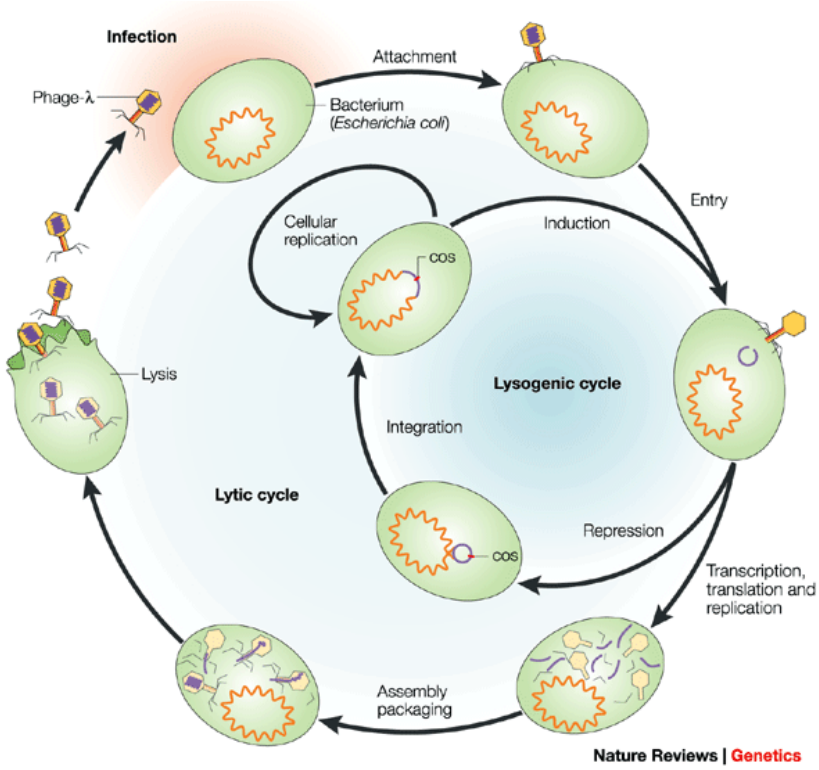

Figure 2.3: Illustration of the life cycles of a typical temperate phage, coliphage λ, demonstrating both lytic and lysogenic life cycles. As featured in a 2003 Nature Review, by Campbell (Campbell, 2003). Used with permission from publisher.

The 'decision' to undergo the lytic or lysogenic life cycle is influenced by both intrinsic bacterial factors and environmental stimuli, such as genetic compatibility (presence of *attB* sites), host physiological state, and environmental phage density (Casjens & Hendrix, 2015; Howard-Varona et al., 2017). Generally, when environmental conditions are favorable for bacterial growth and hosts are abundant, phages undergo a lytic life cycle; lysogeny is generally reserved for situations where propagation may be difficult under the current environmental conditions.

Each life cycle confers the phage and its host with different advantages and disadvantages. Lysogeny for instance can impose a significant metabolic burden on the host cell, with the average phage genome ranging from 30-50 kbp (Hatfull, 2008; Ramisetty & Sudhakari, 2019). Also, carrying a prophage bears with it significant risks as the host will die if the lytic cycle is spontaneously induced (De Paepe et al., 2016). However, the prevalence of putative

prophages in bacterial strains implies a symbiotic benefit to lysogeny for both phage and host (Ramisetty & Sudhakari, 2019). One of the predominant theories is that the resident prophage confers some genetic advantage to the host, such as coding for a multi-drug resistance pump, an outer membrane protease, a small toxic membrane polypeptide, etc. (Wang & Wood, 2016a). It has also been proposed that harboring a prophage can bestow immunity against subsequent infections through superinfection exclusion (Susskind et al., 1974). This ecological immunity is just as advantageous to a resident phage as it is to its host; it allows for the phage to evade harsh environmental conditions that would eliminate the free phage, or to forego conditions in which hosts are exhausted by the phages. The primary disadvantage of lysogeny to the phage is that it allows for only one or two copies of the phage to be replicated through cell division as opposed to the hundreds of copies resulting from lytic propagation (Ramisetty & Sudhakari, 2019).

As alluded to above, lytic cycles offer the advantage of rapid virus propagation through the use of the host replication machinery. This is especially pertinent in environments where phage virions are not numerous, and preferentially 'choosing' a lytic life cycle can help the phage accumulate in number. However, in the later stages of infection, as host numbers begin to decline, a temperate phage may revert back to lysogeny in order to preserve itself in the long term (Erez et al., 2017).

It should be noted, however, that these life cycles are not as black and white as described, and not accessible for all phages. Phages which can undergo both lytic and lysogenic cycles are generally regarded as being temperate. However, phages that cannot enter lysogeny regardless of environmental stimuli are considered to be strictly virulent (Lucchini, Desiere, & Brüssow, 1999). A recent review by Hobbs and Abendon (Hobbs & Abedon, 2016) emphasizes the ambiguous nature of this terminology and the complexity of phage life cycles, and instead proposed that four distinct infection strategies may exist: **lytic and non-temperate** – purely lytic phages which do not exhibit lysogenic cycles; **chronic and non-temperate** – continuous release of phages which do not display lysogeny; **lytic and temperate** – lytic phages which can enter lysogeny; and **chronic and temperate** – phages which are chronically released and can carry out lysogeny. The authors suggest that by taking a multi-factorial approach to the classification of phage lifestyles, phages can be better understood and more appropriately applied to industrial applications (Hobbs & Abedon, 2016). However, for the purpose of this manuscript phages will be referred to using the traditionally "strictly virulent versus temperate" terminology.

2.2.3 Relevance & Applicability of Strictly Virulent Phages

Due to their ability to efficiently lyse and kill bacterial hosts, the medical field has regained interest in using strictly virulent phages for the development of phage therapies. A PubMed search conducted for the year 2018 alone returns hundreds of articles detailing the use of a phage against one particular antibiotic resistant strain or another. Just to name a few, extensive research involves the use of phages against *Staphylococcus aureus, Klesbiella pneumoniae,* and *Pseudomonas aeruginosa* (Ciacci et al., 2018; Nasser et al., 2019; Scarascia, Yap, Kaksonen, & Hong, 2018). Although this work has been carried out successfully *in vitro* for years, Dedrick et al. were recently able to successfully treat a cystic fibrosis patient with an cocktail containing engineered phages that cleared her of a severe antibiotic resistant *Mycobacterium abscesses* infection following a lung transplant.(Dedrick et al., 2019). This is notable as it is not only the first time phage therapy has been used therapeutically against *M. abscesses,* but it is also the first successful record of the therapeutic use of an engineered phage cocktail (Dedrick et al., 2019).

Strictly virulent phages also find applications outside medicine. The same concepts used for phage therapy have been applied in the context of food preservation and biocontrol to prevent pathogen contamination or to improve product longevity by impeding on bacterial overgrowth. For example, Spricigo et al. have suggested that a cocktail of phages UAB_Phi20, UAB_Phi78, and UAB_Phi87 could be used to attenuate *Salmonella* colonization on chicken breast, eggs, lettuce and even pig skin (Spricigo, Bardina, Cortés, & Llagostera, 2013). Another well discussed example of this is the use of a bacteriophage/bacteriocin cocktail used as a preservative treatment to prevent the growth of *Listeria monocytogenes* on fresh-cut produce (Leverentz et al., 2003).

The use of phages in large scale bioindustry is even more limited but is addressed later on in this section.

2.2.4 Relevance & Applicability of Temperate Phages

The use and applications of temperate phages are rare compared to their virulent counterparts. The recent literature highlights how temperate phages with the capacity to undergo lysogeny can be excellent candidates as lateral gene transfer agents (Canchaya, Fournous, Chibani-Chennoufi, Dillmann, & Brüssow, 2003). This stems from the fact that phages are regarded as inherently efficient gene transfer vectors, with specific host recognition and guided DNA entry into the cell (Canchaya et al., 2003; Kanamaru et al., 2002; Molineux, 2001). In this manner, phages have been shown to be agents leading to pathogenicity of their hosts; a notable example being *Shigella*, whereby a prophage encodes the shiga toxin responsible for the virulence of the bacteria as a human pathogen (O'Brien et al., 1984). Theoretically, we can hijack this system and use phages to supply bacteria with accessory genes via plasmids. This was done by Keen et al. whereby two novel lytic coliphages were used to promote plasmid DNA transformation in *Escherichia coli* MG1655 cells (Keen et al., 2017). Insight into lysogeny in the context of horizontal gene transfer not only allows us to insert new genes into bacteria, but it can also allow us to prevent the spread of genes which would be detrimental for our purposes, such as the unintentional transfer of an antibiotic resistance between bacteria as part of a phage therapy protocol (Rodriguez-Rubio, Jofre, & Muniesa, 2017).

Understanding lysogenic cycles has tremendous industrial implications as well. Unintentional induction of a lysogenized phage during batch fermentation can result in significant product loss (Marks & Sharp, 2000). This is particularly problematic in the dairy industry where lactobacilli serve as starter cultures for several fermented dairy products including, yogurt, milk, cheese, etc. (Gilliland, 1990; Kilic et al., 1996). As lactobacilli have probiotic effects inhibiting several foodborne pathogens, having high concentrations of these bacteria present in dairy products is also desirable for health purposes. However, Kilic et al., discovered that a novel phage, Φy8, could be induced from *Lactobacillus acidophilus* Y8 and propagate throughout the culture. This phage is hypothesized to be a cause of the disappearance of *L. accidophilus* in dairy cultures, bringing down the health value of the product (Kilic et al., 1996).

This has also been a prevalent issue in the production of biobutanol by engineered bacteria, where phage infection has been a continued problem; however, in these cases, it's

unclear if the phage existed naturally in the feedstock or were induced prophages (García, Päkkilä, Ojamo, Muurinen, & Keiski, 2011; Jones, Shirley, Wu, & Keis, 2000).

This phenomenon brings with it a unique duality. On one hand, by fully elucidating the prevalence and inducibility of prophages in industrially relevant strains, we can help limit the detrimental effects of unintentional lysis by either knocking out the prophage region in the strain or by limiting the environmental conditions leading to unintentional induction of lysis. On the other hand, induction and lysis could be used to our advantage to, for example, facilitate product recovery.

2.2.5 Current Technology and Industrial Applications of Phages

As mentioned above, the use of phages in bioindustry faces a unique duality, as phages have many desirable characteristics but are also often considered 'pests' in fermentation systems. The current predominant use of phages in bioindustry is in pathogen control. For instance, it has been proposed that phages DT1 and DT6 could be applied to large-scale milk fermentations to attenuate the growth of pathogenic *Escherichia coli*. As phages are highly specific, they only infect their target host, in this instance *E. coli*, and have no detrimental effects on the probiotic strains wished to be maintained in the end product. This is more beneficial then the application of antibiotics, which concomitantly reduce the prevalence of many bacteria, including probiotics (Tomat, Mercanti, Balague, & Quiberoni, 2013).

Similar ideologies have been applied to the beer fermentation industry, whereby phage P1C1 isolated from municipal sewage has been proposed as a biocontrol agent against *Pediococcus damnosus*. As the phage can sustain a lytic life cycle and its host has no identified endotoxins, it could be applied at different stages of the brewing process where *P. damonsus* is present, preventing product spoilage (Kelly et al., 2012). Outside of the food industry this ideology has been applied to the yeast-based bioethanol industry where, phages have been proposed as a means to attenuate contaminant growth in industrial media. Specifically, phage cocktails have been shown to be effective against lactic acid bacteria in yeast fermentation, without causing impacts to the quality of ethanol production (Bertozzi Silva & Sauvageau, 2014).

The use of phages beyond a biocontrol mechanism in industrial settings is limited, but there has been some success using lysogenized phages in the context of recombinant protein production (Oh, Cho, & Park, 2005; Padukone, Peretti, & Ollis, 1990). For instance, lambda prophage has been used to express extremely high levels of β-galactosidase through the process of abortive lysis (Padukone et al., 1990). However, considering the versatility of phages, they could be more broadly applied to various processes, including potentially for product recovery.

2.3 Phages in Methanotrophic Systems

There is minimal knowledge on the prevalence of phages in methanotrophic bacteria. To date there are only two reports of methanotroph phages in the literature (Tyutikov et al., 1980, 1983). The first claims to have isolated several phage strains that are consolidated into two distinct groups, coined gb4-cm4 and cmf1, all capable of infecting and lysing both *Methylosinus sporium* and *Methylosinus trichosporium* (Tyutikov et al., 1980). In that study, samples were retrieved from native methanotroph habitats across various geographical locations of the former USSR, with sources including fermenter culture fluid, cattle rumen, and water from oil and gas installations. Electron microscopy revealed gb-4 to be a short-tailed phage, likely to now be classified as a Podoviridae, and cmfl1 to be a long-tailed phage, likely a Siphoviridae. Due to technological limitations and differences in phage research practices at the time, no sequencing was presented to confirm speciation (Tyutikov et al., 1980).

A second study carried out by some of the same authors successfully isolated from fish two lytic phages, CMF-1-F and 67-F, that were able to infect predominantly *Methylocystis sp.* Phage 63-F was classified as a short-tailed phage, while CMF-1-F a long-tailed phage that was likely a Siphoviridae. Both phages showed unique properties but, again due to technological limitations of the time, were not sequenced (Tyutikov et al., 1983).

2.4 Proposed Hypothesis and Project Overview

As the nature of phage infection in methanotrophic bacteria remains quite elusive, the main objective of this thesis is to provide a knowledge base enabling the use of phages for product

recovery in methanotrophs. This was achieved by, investigating the prevalence and inducibility of lysogenic phage within industrially relevant methanotrophic strains. This has two key implications: firstly, we want to avoid the unintentional induction of prophages during a large scale batch production of valuable bioproducts, and, secondly, we want to establish the conditions to control the induction of the prophage. A second emphasis was placed on isolating and identifying strictly virulent phages of methanotrophic bacteria from environmental samples. The end goal being finding a phage which could be incorporated into future product recovery strategies.

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3 Identification and Induction of a Lysogenic Phage

3.1 Abstract

Methanotrophic bacteria are a unique class of bacteria with imminent industrial potential. Their distinguishing feature is their ability to metabolize single carbon compounds such as methane. They are used to convert methane from impure sources such as industrial waste strains into valuable products, such as biofuels and bioplastics, providing a new platform for the production of biomaterials. However, many aspects of methanotroph biology have yet to be elucidated, including the prevalence and function of lysogenized bacteriophages (phages) within these bacterial strains. Because induction of prophages can be a significant hinderance in industrial processes resulting in the loss of entire batches, understanding phages in the context of methanotroph biology is quintessential to the development of new processes.

Generally, phage infection, specifically lysogenic phage life cycles, are poorly understood within methanotrophic strains. Therefore, the present study uses a series of bioinformatic software (PHASTER, PHIGARO and Island Viewer) to identify potential prophage regions across several methanotrophic strains. Using Mitomycin C, attempts were made to induce these prophage regions and successfully induced phages were characterized using transmission electron microscopy and infection kinetics.

Keywords: prophages, bacteriophages, lysogeny, PHASTER, PHIGARO, bioinformatics, methanotrophs, methanotrophic bacteria,

3.2 Introduction

Methanotrophs are a unique subclass of bacteria with promising industrial potential due to their capacity to convert methane from impure sources, such as industrial waste streams, into valuable products such as isoprenoids for bio-jet fuel production, PHAs for the development of bioplastics, or other high value products such as ectoines (Strong et al., 2015). However, despite being initially characterized in the early 1900s (Kaserer, 1905; Söhngen, 1906), much about methanotroph biology has yet to be elucidated, including the prevalence and impact of lysogenized phages in these strains.

In terms of industrializing methanotrophs, this has two major implications. For one the presence of a prophage in a host can often confer valuable genetic information as the phage incorporates its DNA into the host's genome. Despite being a metabolic burden on the cell (Ramisetty & Sudhakari, 2019), the phage itself often confers an evolutionary advantage to the host, such as the genes for a multi-drug resistant pump, outer membrane protease, or toxic membrane polypeptides etc. (Ramisetty & Sudhakari, 2019; Wang & Wood, 2016b). Therefore, it's not unreasonable to suggest that some of the unique metabolic traits observed in methanotrophs could be of phage origin; this could have implication for the genetic engineering of these strains in the future. Secondly, the unintentional induction of a prophage could result in premature cell lysis and the subsequent loss of an entire production batches, a problem well observed in the dairy industry (De Paepe et al., 2016; Kilic et al., 1996).

Generally, phages are poorly understood in methanotroph systems, with presently only two studies broaching the subject, both focusing on isolating strictly lytic phages (Tyutikov et al., 1980, 1983). Despite the lack of information on lysogenic methanotroph phages, the advancement of bioinformatic software such as PHASTER (Arndt et al., 2016; Zhou, Liang, Lynch, Dennis, & Wishart, 2011) and PHIGARO (Starikova et al., 2019) provides platforms for the investigation of these phages. The results from these tools can then be cross-referenced with more generalized but well characterized software such as Island Viewer (Bertelli et al., 2017) to provide a more in depth picture of prophage genomes within host bacteria.

Using a combination of bioinformatic software and chemical induction by Mitomycin C, prophages were induced and characterized for a range of methanotrophic strains: *Methylobacter marinus* A45, *Methylosinus trichosporium* OB3b, *Methylomicrobium album* BG8,

Methylococcus capsulatus strain Bath, *Methylocystis sp.* Rockwell, and *Methylomonas denitrificans* FJG1. This study provides a basis to better understand the prevalence of prophages in methanotrophic bacteria, prevent the unintentional induction of a phage during batch processes, and possibly control induction to use cell lysis towards bioproduct recovery.

3.3 Materials and Methods

3.3.1 Microorganisms

Several methanotrophic bacterial strains were used for the purpose of this study including: *Methylosinus trichosporium* OB3b, acquired from Alan DiSpirto of Iowa State University; *Methylobacter marinus* A45, isolated by and acquired from Marina Kalyuzhnaya at San Diego State University, California; *Methylomicrobium album* BG8 (ATCC 33003) and *Methylocystis sp.* Rockwell (ATCC 49242), originally obtained from the American Type Culture Collection, and finally *Methylomonas denitrificans* FJG1, acquired from Jay Gulledge, currently at the Oakridge National Laboratory. Cultures were maintained for extended periods at room temperature in liquid stocks, which could be revived after several months of dormancy. Prior to experiments, a "starter" culture was grown from stocks untill early stationary phase to ensure experimental cultures were of prime health.

3.3.2 Prophage Prediction Using Bioinformatic Software

Methanotroph nucleotide sequences were acquired from GenBank and downloaded as .fasta files for the microorganisms *M. marinus* A45, *M. trichosporium* OB3b, *M. album* BG8, *M. capsulatus* strain Bath, *Methylocystis sp.* Rockwell, and *M. denitrificans* FJG1. The sequences were analyzed through the software tools PHAge Search Tool – Enhanced Release (PHASTER), PHIGARO, and Island Viewer. Software runs for were originally performed in January 2018, for PHASTER. They were redone on May 30, 2018 compiling both PHASTER, and IslandViewer results. The final PHIGARO analysis was performed on August 15-16, 2018.

PHASTER, which was adapted from the original programming of PHAge Search Tool (PHAST) (Zhou et al., 2011), uses BLAST+ to run bacterial sequences against a custom phage

protein sequence database developed from the National Center for Biotechnology and Information (NCIB) phage database, in conjunction with a prophage database generated by Srivdhya et. al (Arndt et al., 2016; Srividhya et al., 2006; Zhou et al., 2011). PHASTER is regarded as being much more efficient and robust than its predecessor, using a total of 112 CPUs, versus 32, and has the capacity to handle assembled contig sets from metagenomic data – a caveat of the previous software(Arndt et al., 2016).

PHIGARO is a command line tool developed by Starikova and Pryanichnikov at the Research Institute of Physical and Chemical Medicine, Moscow, Russia in 2017. PHIGARO is a python-based software and uses pVOG hidden Markham models (HMMs) and smoothing algorithm to make predictions about prophage/phage sequences from native bacterial genomes (Starikova et al., 2019).

Meanwhile, IslandViewer works by identifying genomic islands, or distinct segments of DNA showing high similarity, which play a role in horizontal gene transfer and strain evolution (Bertelli et al., 2017). As prophage regions themselves are discrete, the software will often identify them as gene islands; users, can then browse through the annotated genes of these islands to search for essential phage genes such as capsid, tail, baseplate etc. to determine if the region is in fact a potential prophage (Juhas et al., 2009).

3.3.3 Analysis of Identified Potential Prophage Region

Primary analysis of genome regions identified as potential prophages was completed by rendering visualizations using Adobe Illustrator. This tool allowed us to clearly see where regions overlapped between each program and the differences in software specificity, including cut offs.

Identified potential prophage regions were downloaded as .fasta files and processed through BLAST to determine their closest identified phage lineage and/or virus. Alignments were then performed using Geneious 11.1.5 to calculate similarities between each identified region by each program including the closest identified phage relatives.

3.3.4 Media and Solutions

Ammonium mineral salt (AMS) and nitrate mineral salt (NMS) media(Whittenbury, Phillips, & Wilkinson, 1970), both commonly used in the methanotroph field, were used in this study (Tables 3.1 to 3.4). However, to facilitate the growth of the oceanic strain *M. marinus* A45, 100 ml of 1X NMS was supplemented with 6.65 ml 1% NaCl, 2 ml phosphate buffer and 0.5 ml 1 M sodium bicarbonate solution. For all other strains no media modifications were made.

Table 3.1: Whittenbury media recipe for growing methanotrophs

Whittenbury Medium for Growing Methanotrophs
Prior to autoclaving: 100ml 10X AMS or NMS stock 900ml tap distilled water **Post autoclaving:** 10ml sterilized phosphate stock solution

Table 3.2: Preparation of stock 10X Whittenbury stock solutions for methanotroph growth

10X Whittenbury Stock Solutions	
10X Ammonium Stock Solution (AMS)	**10X Nitrate Stock Solution (NMS)**
40.6 mM $MgSO_4$ (4.88 g) or $MgSO_4 \bullet 7\ H_2O$ (10 g) 99mM NH_4Cl (5 g) 15.5 mM $CaCl_2$ (2 g) or $CaCl_2 \bullet 7\ H_2O$ (2.28 g) 10 ml Whittenbury Trace Element Solution 0 ml 0.1% Sodium Molybdate Solution 1 ml FeEDTA solution 0.5 ml 100mM $CuSO_4$ Solution Bring volume up to 1000 ml using tap distilled water	40.6 mM $MgSO_4$ (4.88 g) or 40.6 mM $MgSO_4 \bullet 7\ H_2O$ (10 g) 99mM KNO_3 (10 g) 15.5 mM $CaCl_2$ (2 g) or $CaCl_2 \bullet 7\ H_2O$ (2.28 g) 10 ml Whittenbury Trace Element Solution 0 ml 0.1% Sodium Molybdate Solution 1 ml FeEDTA solution 0.5 ml 100 mM $CuSO_4$ Solution Bring volume up to 1000 ml using tap distilled water.

Table 3.3: Whittenbury trace element solution for the preparation of 10X NMS and 10XAMS stocks

Whittenbury Trace Elements For NMS/AMS (1 L)
$FeSO_4 \bullet 7\ H_2O$ (0.5 g)
$ZnSO_4 \bullet 7\ H_2O$ (0.4 g)
$MnCl_2 \bullet 4\ H_2O$ (0.02 g)
$CoCl_2 \bullet 6\ H_2O$ (0.05 g)
$NiCl_2 \bullet 6\ H_2O$ (0.01 g)
H_3BO_3 (0.015 g)
Na_2EDTA (0.25 g)

Table 3.4: Preparation of phosphate stock solution for preparation of 1X AMS and NMS solutions

Phosphate Stock Solution
26 g KH_2PO_4
33 g $NaHPO_4$
Bring solution up to 1000 ml using tap distilled water

3.3.5 Standard Growth Conditions and Measurements

Working or starter cultures were grown by inoculating 100 ml of 1X AMS or modified 1X NMS with 1 mL of methanotrophic culture from a stock or previous passage in a 250-ml Wheaton bottle sealed with screw-top caps inlaid with butyl rubber plugs. 50 ml of headspace was removed via syringe and replenished with 60 ml of methane (Praxair), pressurizing the bottle to facilitate methane diffusion into the medium. Cultures were allowed to grow to early stationary phase before being used as inoculum for experimental cultures.

Experimental cultures for prophage inductions were grown by inoculating 50 ml of 1X AMS or modified 1X NMS with 0.5-1 mL of methanotrophic culture from a working/starter culture, in a 250-ml sealed Wheaton bottle. Smaller volumes were used relative to the starter cultures to more easily enable centrifugation. As above, 50 ml of headspace was removed via

syringe and replenished with 60 ml of methane. Cultures were allowed to grow to early to mid-log phase prior to conducting experiments.

Cell density was measured using a UV spectrometer (Biochrom Ultrospec 50 UV-Vis) at by measuring optical density at 540 nm (OD_{540})

3.3.6 Prophage Induction Protocol

Cultures that harbored sequences identified as potential prophage were grown until early-mid log phase as determined by OD_{540}. As each strain demonstrated a slightly different growth pattern, this number varied between cultures. On average, an OD_{540} of 0.150-0.300 after ~20 hours post-inoculation was considered appropriate to induce prophage. Prophage inductions appeared less than ideal at $OD_{540} < 0.100$ as there were not enough cells to obtain a substantial pellet when centrifuging, or $OD_{540} > 0.600$, as cells were too old to enable successful induction. Mitomycin C (MitC) was added to cultures to a final concentration of 1or 2 μg/ml. Cultures were left to grow in the presence of MitC for 1 h, with the Wheaton bottles wrapped in tinfoil, as the compound is light sensitive. Following exposure, 30 ml of each culture was centrifuged for 20 min at 20,000 × g at 20°C to remove excess MitC from the media. Cell pellets were resuspended in 30 ml of fresh media, the methane was replenished to the headspace, and cultures were left to grow for 2 days. OD_{540} was measured throughout to confirm prophage induction, which was signified by reduced OD. Other signs of prophage induction included cell clumping, color change in the culture, and the presence of precipitation or debris. These were used concomitantly with OD to determine successful induction.

3.3.7 Phage Isolation and Transmission Election Microscopy (TEM) Preparation

Cultures suspected of having induced prophage were verified using transmission electron microscopy (TEM). Both experimental and control cultures were passage through a 0.2-μm syringe filter to remove whole cells and debris from lysis. In the interim the lysate was stored at 4°C. To prepare samples for TEM, 4 ml of cell lysate was centrifuged at 20,000 × g at 4°C for 1

h. The supernatant was discarded, and the pellets were resuspended in 1 ml of lambda diluent and left to settle overnight at 4°C. Pellets were washed by centrifuging under the above conditions but subsequently resuspended in 100 µl of lambda diluent (Table 3.6) to concentrate the sample. 10 µl of sample was then deposited on copper-coated grids/former films, and left to sit for 4 min, then stained with uracil acetate for 1 min. Using a Morgagni 268 (FEI, Hillsboro, Oregon, USA) TEM, samples were visualized at 110,000× magnification, using a beam intensity of 80 kV. Phage capsid and tail sizes were calculated by measuring a minimum of 3 samples in ImageJ. Additionally, inferences could be made with regards to phage physiology and family from acquired images.

Table 3.5: Lambda diluent recipe for phage TEM preparation

Lambda Diluent
10 ml 1 M TrisCl solution (pH = 7.5) 2.0 g $MgSO_4 \bullet 7H_2O$ 990 ml ddH_2O

3.3.8 Sequence Analysis

Sequence analysis was performed using a combination of NCBI Basic Local Alignment Search Tool (BLAST) (Altschul, Gish, Miller, Myers, & Lipman, 1990) and alignments were performed using Geneious 11.1.5 using the MAUVE alignment plug in (Multiple Alignment of Conserved Genomic Sequence with Rearrangements), and the pairwise/multiple alignment algorithms featured within the Geneious 11.1.5 suite.

3.4 Results

3.4.1 Predictions of potential prophage regions in methanotrophic bacteria

Three different software packages – PHASTER, PHIGARO and IslandViewer – were used to detect and cross-analyze prophage regions from six methanotrophs of interest – *M. marinus* A45, *M. trichosporium* OB3b, *M. album* BG8, *M. capsulatus* strain Bath, *Methylocystis sp.* Rockwell, and *M. denitrificans* FJG1. In the cases where the methanotroph genome remained separated into two or more contigs (*M. marinus* A45 and *Methlyocystis sp.* Rockwell), each genome segment was run through the algorithms individually. Contigs that showed no putative prophage regions are not shown in the results.

The results for each strain investigated are reported visually in Figures 3.1 to 3.6, which are described below. The reference genome sequence is reported at the top of the figures, followed by visualizations of the results obtained by PHASTER with putative prophages highlighted in magenta, PHIGARO with putative prophages highlighted in blue, and the IslandViewer putative prophages highlighted in green, respectively. The location of each putative prophage is indicated by the base pair numbers based on the host genome at the start and end of the putative sequence. Overlapping regions are shown on the reference genome as grey when two predicted regions overlap, and black for sections identified by all three programs.

***M. marinus* A45**

All three algorithms detected prophage regions in *M. marinus* A45 (Figure 3.1). PHASTER detected a single region of 36,793 bp in size located between base pairs 3,831,981 to 3,868,774. This region was also identified by PHIGARO, albeit with the end of the sequence ending earlier, at 3,857,650 bp. This resulted in the sequence being approximately 25,700 bp in length, roughly 10,000 bp smaller than the PHASTER prediction. PHIGARO identified three additional regions, ranging in size from 22,000-37,000 bp earlier in the host genome sequence, which were not detected by PHASTER. IslandViewer identified only one region of potential phage origin, which also differed from the regions identified by the other algorithms. This region, just over 34,000 bp in size, sat near the end of the bacterial genome sequence, between

4,621,050 – 4,655,677 bp. Overall, other than the one overlapping region detected by PHASTER and PHIGARO, only minimal similarity was seen between predictions.

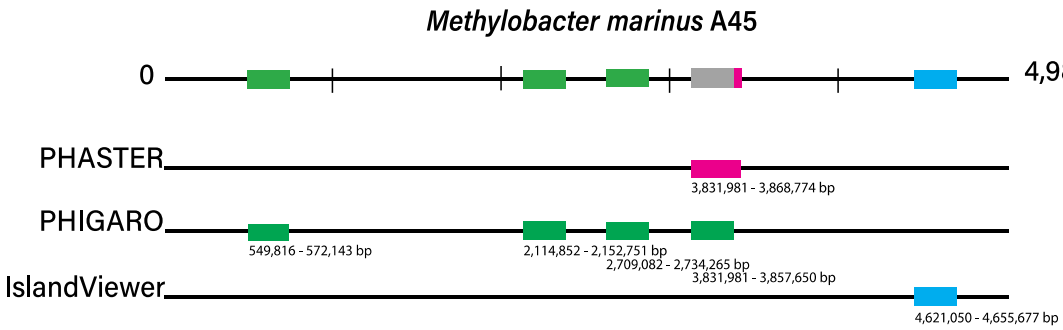

Figure 3.1: Computational prediction of prophage regions in *M. marinus* A45 genome

***M. trichosporium* OB3b**

The predictions from all three algorithms showed more similarities for *M. trichosporium* OB3b (Figure 3.2). PHASTER detected a total of four potential prophage regions, three of which were also detected by PHIGARO. Only the last sequence, found between 1,661,356-1,697,037 bp, was not matched by PHIGARO. However, IslandViewer was able to detect these four regions and identify potential phage genes within them.

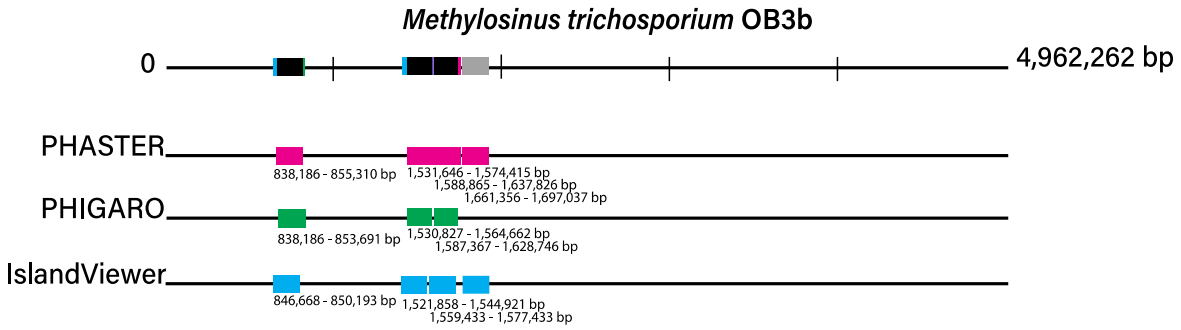

Figure 3.2: Computation prediction of prophage regions in *M. trichosporium* OB3b genome

***M. album* BG8**

PHASTER detected only one putative prophage region in the genome of *M. album* BG8, found between base pairs 1,496,283 and 1,522,820 (Figure 3.3). This sequence was marked as questionable by the program, which usually means it is missing one of the key phage genes used by the algorithm to identify phage regions within gene islands. This region was also detected by IslandViewer but was shifted slightly, sitting between 1,434,717 and 1,469,934 bp, and was a fair bit larger, with over 35,000 bp versus approximately 26,500 bp for PHASTER. PHIGARO did not identify any putative prophage regions in *M. album* BG8.

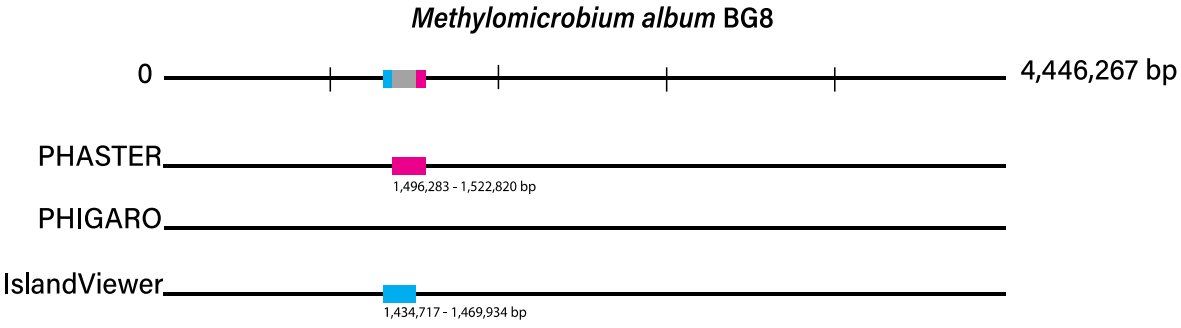

Figure 3.3: Computation prediction of prophage regions in *M. album* BG8 genome

***M. capsulatus* Bath**

All three programs detected two distinct putative prophage regions in *M. capsulatus* Bath, each overlapping over a significant range (Figure 3.4). The first region was similar for all three programs, with a size ranging from 52,000 to 62,000 bp. For the second region, the only notable difference was the shortening of the sequence detected by IslandViewer. While both PHASTER and PHIGARO detected regions of approximately 50,000 bp in length, the region detected by IslandViewer was shorter, at just under 18,000 bp in length.

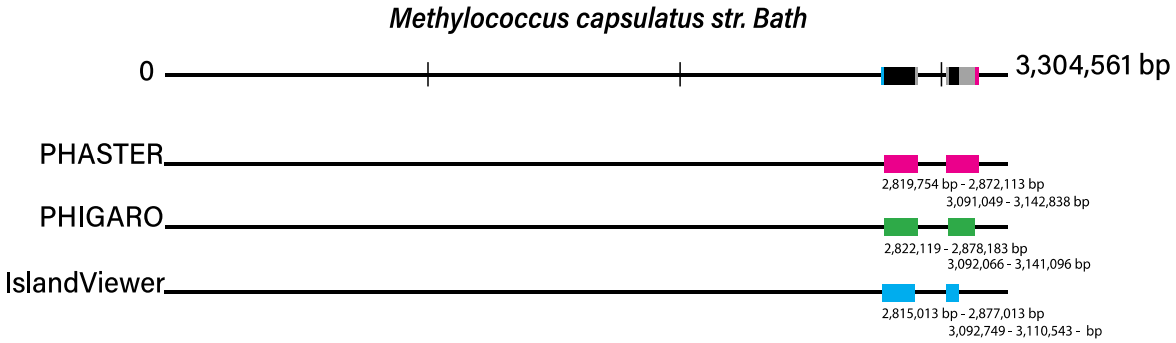

Figure 3.4: Computational prediction of prophage regions in *M. capsulatus* str. Bath

***Methylocystis sp.* Rockwell**

The genome of *Methylocystis sp.* Rockwell was divided into six different contigs (each corresponding to an individual GenBank file). The algorithms detected putative prophage regions in two of these contigs. PHIGARO produced the greatest number of predicted regions, finding a total of five in the first contig, MAH.6 (Figure 3.5A), which also contained the most sizeable amount of genetic material. PHASTER matched three of these regions, finding an extra region at 1,246,731 – 1,263,295 bp. This left two unmatched PHIGARO regions at 2,532,265 – 2,531,861 bp and 3,975,850 -3,977,756 bp. IslandViewer found two regions, one which matched with a prediction from PHASTER and PHIGARO, and a second which was unique at 2,957,691 – 2,690,971 bp (Figure 3.5A). The second contig which had detectable putative prophages, MAH.4

(Figure 3.5B), had one small region which was identified as questionable by PHASTER at 58,594 – 64,882 bp.

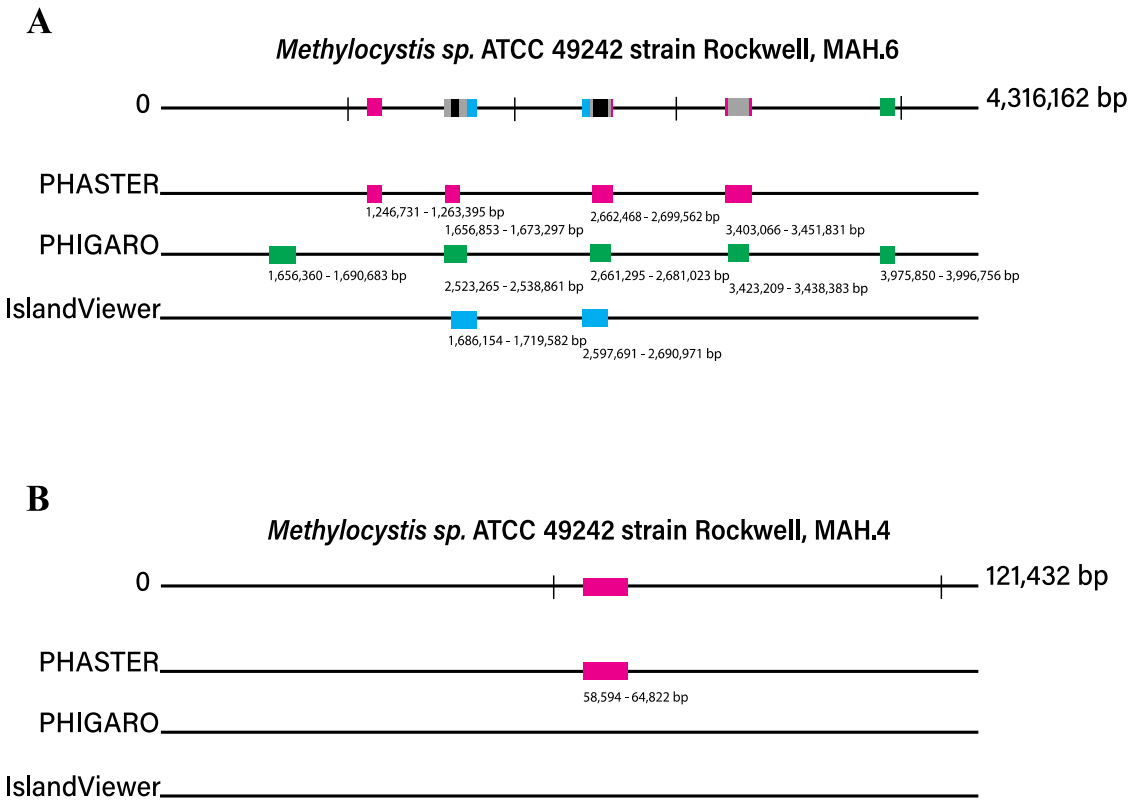

Figure 3.5: Computational prediction of prophage regions in *Methylocytis sp.* Rockwell. The genome was separated in six contigs with the two contigs containing putative prophage regions shown in A and B.

***M. denitrificans* FJG1**

M. denitrificans FJG1 (Figure 3.6) had regions detected only by PHIGARO. In total three regions were identified, each ranging from ~33,000-41,000 bp and located at 796,413 – 831,113 bp, 2,269,762 – 2,303,593 bp and 4,442,751 – 4,484,138 bp.

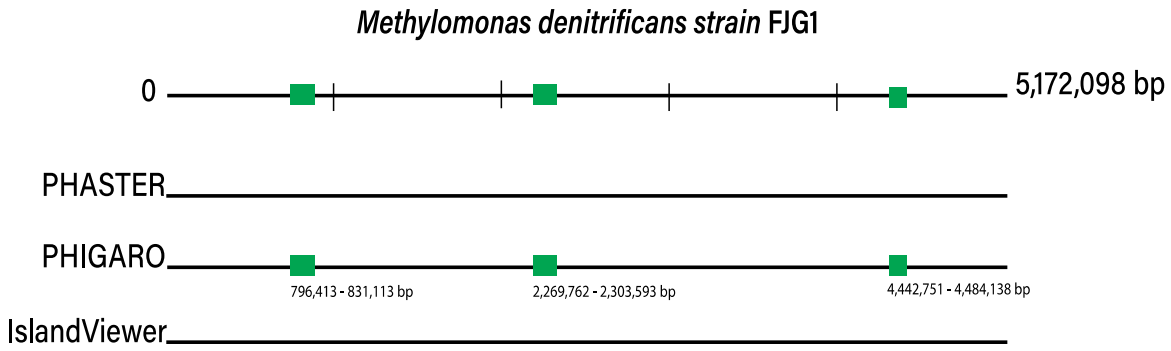

Figure 3.6: Computational analysis of prophage regions in *M. denitrificans* FJG1

3.4.2 Chemical induction of prophages in strains with putative prophage regions

The five strains investigated for prophage induction were *M. marinus* A45, *M. trichosporium* OB3b, *M. album* BG8, *Methylocystis sp*. Rockwell, and *M. denitrificans* FJG1. *M. buryatense* 5GB1 and *M. alcaliphilum* 20ZR were eliminated as the programs used did not detect potential prophage regions. *M. capsulatus* strain Bath failed to consistently grow and was thus also abandoned.

The induction of potential prophages was monitored through dynamic OD_{540} measurements following addition of MitC, with a reduction in OD_{540} indicative of potential prophage activation. Area under the curve of OD_{540} (AUC) was then used to assess the significance of lysis between each experimental condition against the control. A lower AUC value is indicative of reduced growth and, consequently, reduced viability. Results for *M. marinus* A45, *M. trichosporium* OB3b, *M. album* BG8, *Methylocystis sp.* Rockwell, and *M. denitrificans* FJG1 can be found in Figure 3.7. The induction protocol appeared to have no detrimental effect on control cultures, with all of the strains still growing to the maximum OD generally observed for these strains. In most cases, the 1-µg/ml and 2-µg/ml MitC conditions showed stunted growth (Figure 3.7 A, C, E, G and I). *M. album* BG8 was the only exception,

whereby the 1-μg/ml and control cultures showed comparable growth, while only the induction conducted at 2-μg/ml MitC showed impeded growth (Figure 3.7 E).

AUC and two-way ANOVA analysis of raw data validated these results. Specifically, significant differences in cell viability were observed for the 1-μg/ml MitC condition compared to the control for *M. marinus* A45 and *Methylocystis sp.* Rockwell compared to the control, both exhibiting p-values <0.0001. (Figure 3.7 B & H). A less significant but still notable difference was calculated between the control and the 1-μg/ml condition for *M. denitrificans* FJG1 (Figure 3.7 J), with a p-value of 0.003. Both *M. trichosporium* OB3b (Figure 3.7 D) and *M. album* BG8 (Figure 3.7 F), showed no significant difference between the control and the 1-μg/ml MitC condition.

More consistent results could be observed when induction was attempted with 2 μg/ml MitC, as all strains showed a significant difference between the control and experimental conditions (Figure 3.7 B, D, F, H and J). This suggested a reduction in culture viability likely indicative of successful prophage inductions. All strains exhibited p-values <0.0001, except *M. trichosporium* OB3b, which had a p-value of 0.0370 between experimental and the 2-μg/ml MitC condition.

As per significance between the different MitC concentrations, the results varied between strains. *M. marinus* A45 and *M. trichosporium* OB3b showed nominal differences between inductions at 1 μg/ml and 2 μg/ml MitC, suggesting the greater concentration did not further impact viability (Figure 3.7 B and D) and that both concentrations could equally induce a potential prophage region with the same potency. However for the three remaining strains, *M. album* BG8, *Methylocystis sp.* Rockwell, and *M. denitrificans* FJG1 (Figure 3.7 F, H, and J), a clear difference could be observed between the two MitC concentrations, suggesting that the higher 2-μg/ml MitC concentration had a greater impact on cell viability, which could be correlated to an overall more prevalent or faster induction of prophage.

Since none of the induction experiments demonstrated the characteristic "crash" in OD_{540} resulting from population-wide lysis in virulent infection models, additional measures were used to characterize whether an induction was successful or not. Inductions were considered successful if the following was observed: 1) a greater than two-fold difference between the final OD_{540} of the control and an experimental subset – final OD_{540} of the control was based on the time at which the culture reached stationary phase; 2) signs of disrupted or unhealthy cultures,

such as precipitation of cell debris/dead cells at the bottom of the Wheaton bottle, were observed. Overall, it was presumed that the crash in methanotroph growth was not due to a combination of low cell counts of infected cultures (masking reliable reductions in OD_{540}) or interference in OD_{540} from observed cell debris in induced cultures.

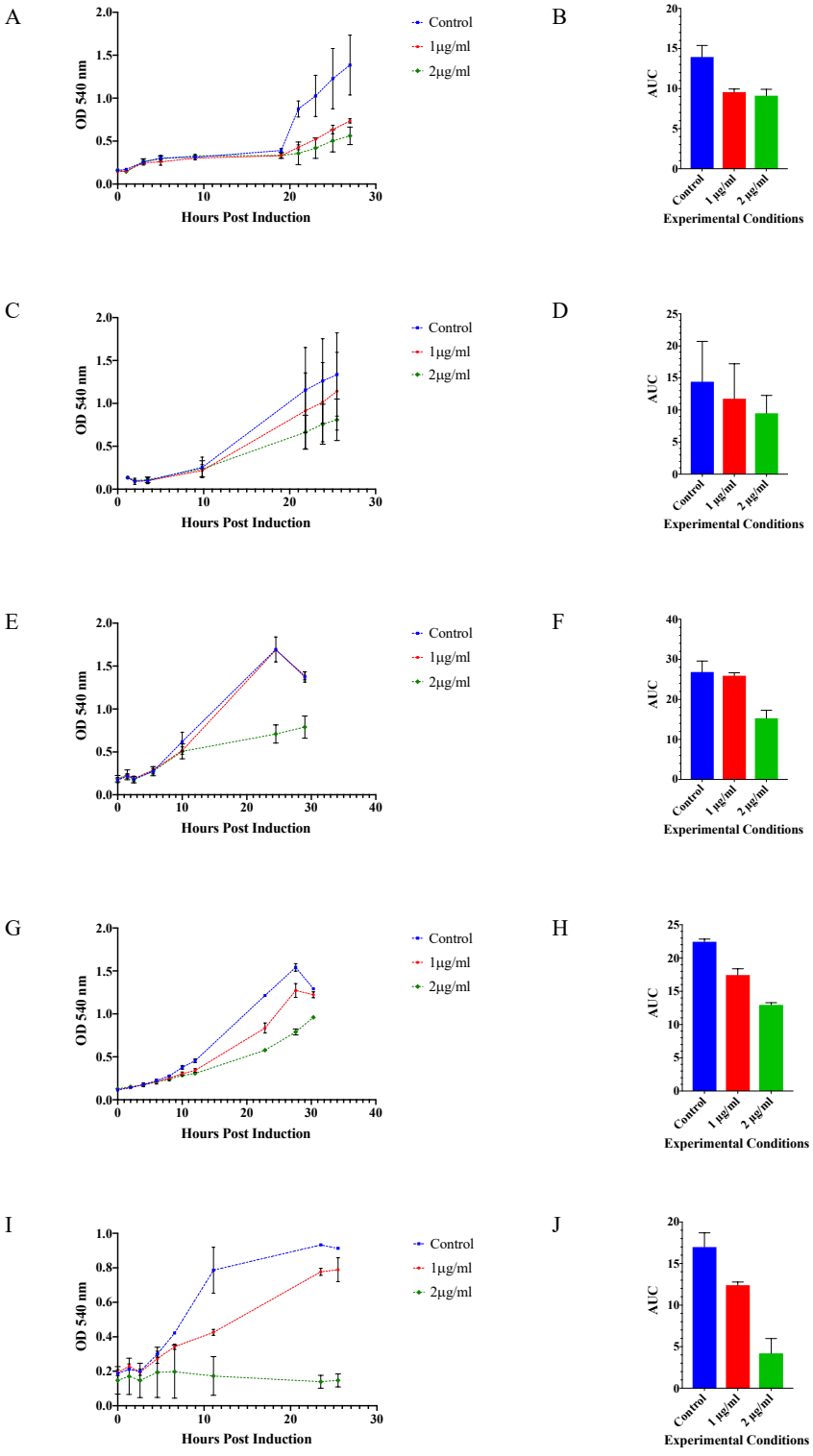

Figure 3.7:Induction of potential prophages by addition of mitomycin C to cultures of different methanotrophs. Optical density (OD_{540}) and the area under the curve of OD_{540} (AUC) are reported for *M. marinus* A45 (A, B), *M. trichosporium* OB3b (C, D), *M. album* BG8 (E F), *Methylocystis sp.* Rockwell (G, H), *M. denitrificans* FJG1 (I, J) after the addition of mitomycin C to concentrations of 1 ug/ml and 2 ug/ml and in control experiments. Error bars denote the standard deviation based on n = 3.

3.4.3 Analysis of Chemically Induced Cultures Under Transmission Electron Microscopy (TEM)

The experimental sample from the 2-µg/ml MitC condition that led to the lowest final OD_{540} for each of the five strains was analyzed under TEM. Phages were only detected in samples from *M. marinus* A45, *M. trichosporium* OB3b, and *M. album* BG8 (Figure 3.8). No phage was observed in samples from *Methylocystis sp.* Rockwell or *M. denitrificans* FJG1.

In terms of the *M. marinus* A45 sample (Figure 3.8 A and B), the phages appeared to be *Caudovirale* in nature, with heads ~51 nm in diameter and long helical tails averaging ~157 nm in length and ~13nm in diameter. (Figure 3.8.A-B, Table 3.1) Visual analysis suggests that these viruses belong to the *Siphoviridae* family as their tails are long and do not appear contractile in nature. However, sequencing is required to confirm their proper classification.

Table 3.6: Dimensions of phage induced from *M. marinus* A45

Measurements *M. marinus* A45, Phage			
Phage	**Head Diameter (nm)**	**Tail Length (nm)**	**Tail Width (nm)**
1	51.8	142.5	13.1
2	51.8	166.3	13.4
3	56.7	144.6	11.3
4	46.1	164.7	12.8
5	49.3	164.9	13.5
Average (nm)	51.1	156.6	12.8
StDev (nm)	3.9	12.0	0.9

Samples from *M. trichosporium* OB3b cultures also appeared to be of the *Caudovirale* order, likely *Siphoviridae.* This is based on their binary symmetry, heads averaging ~58 nm across, and long contractile tails ~156 nm in length and 14 nm in diameter. (Figure 3.8 C-D, Table 3.2). Here again, sequencing is required to confirm their proper classification.

Table 3.7: Dimensions of phage induced from *M. trichosporium* OB3b

Measurements *M. trichosporium* OB3b, Phage			
Phage	**Head Diameter (nm)**	**Tail Length (nm)**	**Tail Width (nm)**
1	55.0	148.0	13.8
2	62.0	162.8	15.9
3	64.3	153.3	13.8
4	57.3	153.2	12.7
5	53.3	164.4	14.9
Average (nm)	58.4	156.3	14.2
StDev (nm)	4.7	7.0	1.2

Finally, samples from *M. album* BG8 also appeared to contain *Siphoviradae*, with both heads and tails showing similar morphology to phages from the other cultures. Average diameter of the heads was 55 nm, while tails measured at ~147 nm in length and 13 nm across (Figure 3.8 E-F, Table 3.3). Again, classification must be confirmed with sequencing.

Table 3.8: Dimensions of phage induced from *M. album* BG8

Measurements *M. album* BG8, Phage			
Phage	**Head Diameter (nm)**	**Tail Length (nm)**	**Tail Width (nm)**
1	54.1	145.2	14.0
2	57.5	149.5	11.8
3	54.0	143.1	12.5
4	58.3	133.7	14.6
5	52.1	161.7	13.5
Average (nm)	55.2	146.6	13.3
StDev (nm)	2.6	10.2	3.9

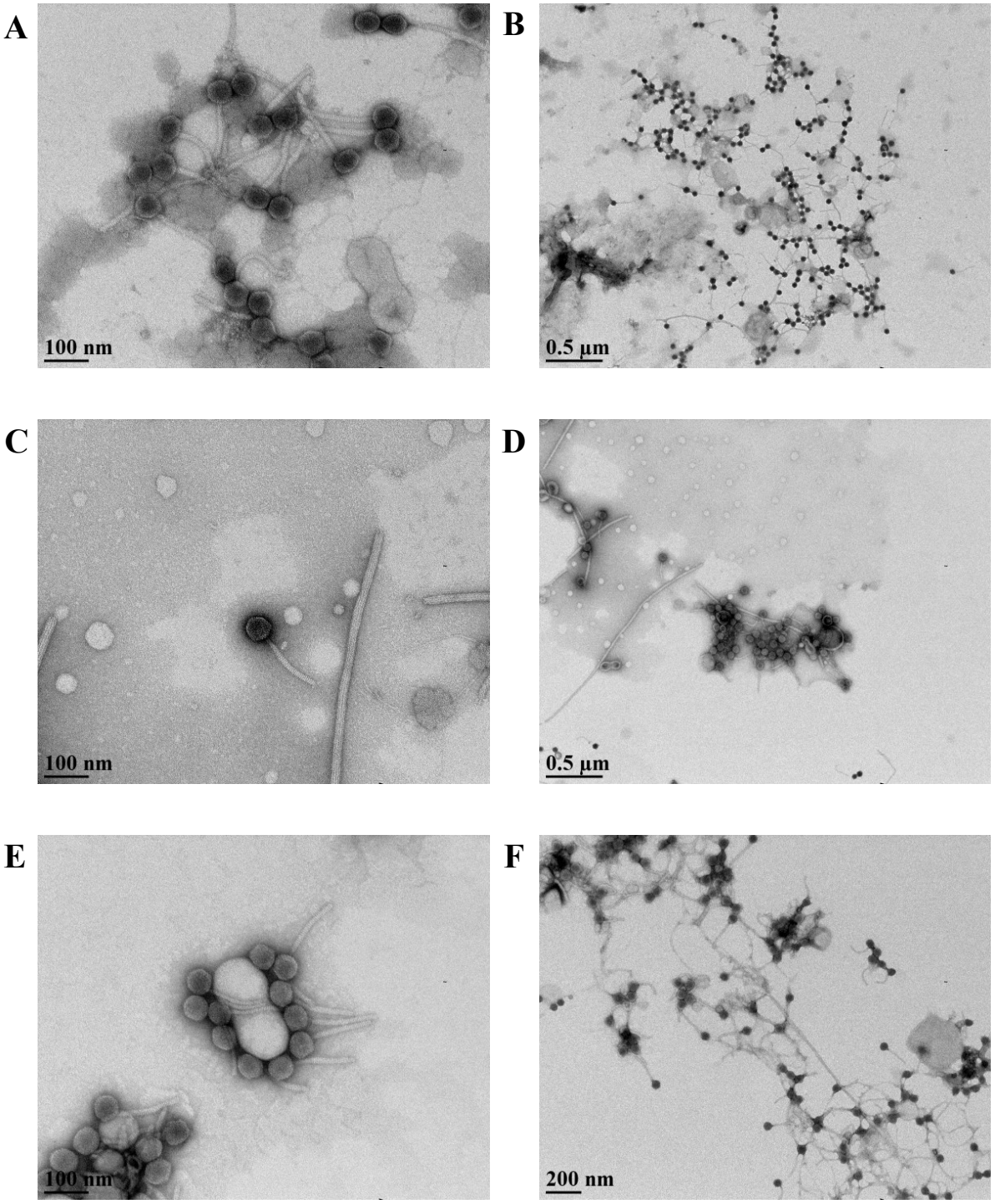

Figure 3.8: TEM visualization of induced cultures of *M. marinus* A45 (A-B), *M. trichosporium* OB3b (C-D), and *M. album* BG8 (E-F) using uracil acetate staining.

3.4.4 BLAST analysis of potential prophage regions of induced strains

Bioinformatic analysis was performed using BLAST and Geneious to compare the putative prophage regions of the six strains investigated using PHASTER and PHIGARO to known phage genomes. The analyses of the three strains leading to phages observed by TEM (*M. marinus* A45, *M. trichosporium* OB3b, and *M. album* BG8) are described below (Figures 3.9 – 3.17). The analyses for the remaining strains (*M. capsulatus* str. Bath, *Methylocystis sp.* Rockwell, and *M. denitrificans* FJG1) can be found in Appendix A. (Figure A.1 – A.5). In the instance where PHASTER and PHIGARO identified sequences with different BLAST relatives, the first known relative to cross over between the two sequences was retained for further analysis. However, it should be noted that this was rare, only occurring for in *M. trichosporium* OB3b sequences 1,531,646-1,574,415bp and 1,588,865-1,637,826bp. With all remaining cases PHASTER and PHIGARO results returned the same BLAST results.

Two different analyses were performed to cross-compare identified prophage sequences and their putative relatives. The first analysis was a global pairwise/multiple alignment in Geneious, whereby two or more sequences are aligned over their entire length and are assumed homologous by the program. The alignment yields a percent identity value, which provides the number of base pairs matches across the aligned regions including gaps. The second analysis was performed in Geneious using the MAUVE alignment plug in (Multiple Alignment of Conserved Genomic Sequence with Rearrangements). Here the software takes a more local alignment approach using the MAUVE algorithm to identify conserved regions of DNA between the two sequences.

***M. marinus* A45**

Using BLAST analysis, the closest identified relative to the PHIGARO region 549,816-572,143 (Figure 3.1) was predicted to be *Streptomyces* phage Toma (Figure 3.9). Geneious aligned the two sequences with 49.75% identity. This is illustrated in Figure 3.9 A, whereby a gold coloration is used to illustrate where the two sequences show homology. The flanking green and solid black regions on either end of this segment show where the sequences were unmatched.

MAUVE found one specific homologous region between the two viruses, which is marked in a red color block outline (Figure 3.9 B). This outline shown on the *Streptomyces* phage Toma is matched to a region on the predicted prophage, which is blocked in the same

color. Specific regions of high homology, if present, are represented by additional peaks within the outlined regions. However, none were identified here. If more than one region of conservation is identified such as in Figure 3.12 (discussed below), then each region will be outlined in a different color.

The region identified by PHIGARO at 2,114,852-2,152,751 (Figure 3.1) was most closely related to *Sphingomonas* phage PAU (Figure 3.10). Geneious aligned the two sequences with 49.9% identity. MAUVE again found one region of similar homology using a global alignment (Figure 3.10 A-B).

Region 2,709,082 – 2,152,751, as identified by PHIGARO (Figure 3.1), was most closely related to an *Escherichia* phage vB_Ecom_G17 (Figure 3.11). The two sequences were aligned in Geneious with a 4.12% identity and using global alignment software MAUVE found one small region of homology between them (Figure 3.11 A-B).

The region identified by PHASTER at 3,831,981-3,868,774, which was also identified by PHIGARO (Figure 3.1), was found to have *Pseudomonas* phage vB_PaesPM105 as its closest relative (Figure 3.12). Geneious aligned the PHASTER/PHIGARO sequences with 69.54% identity, while each aligned with the predicted phage relative with 47.65% identity and 38.96% identity, respectively (Figure 3.12 A). As this is the first time a multiple versus pairwise alignment appears in the document, it should be noted that to accommodate the third sequence the output is modified slightly by the program. As above, the golden region from the BLAST analysis (Figure 3.12 A) shows where all three sequences overlap. The grey regions below this (which were purely black in previous figures) show where two of the three sequences align, which in most instances are the PHASTER/PHIGARO sequences. The black ‘dashes’ within this region are the specific areas where all three sequences match. The red region illustrated on the end shows where only one sequence is present, in this instance at the end of the PHASTER sequences, which was identified as being slightly longer.

MAUVE global alignment was able to map out two regions of synonymous homology between the 3 sequences (Figure 3.12 B), both of which are outlined with two different colors.

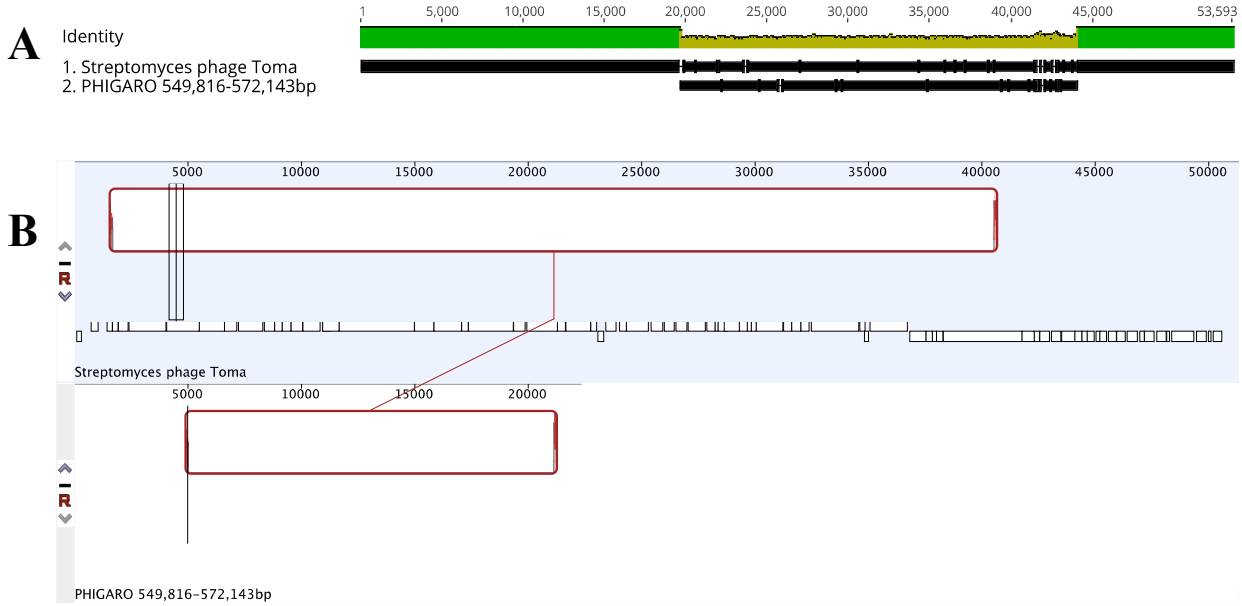

Figure 3.9: Bioinformatic analysis of putative prophage region identified by PHIGARO at 549,816-572,143 in *M. marinus* A45 using the pairwise sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) from Geneious 11.1.5, against closest BLAST relative *Streptomyces* phage TOMA.

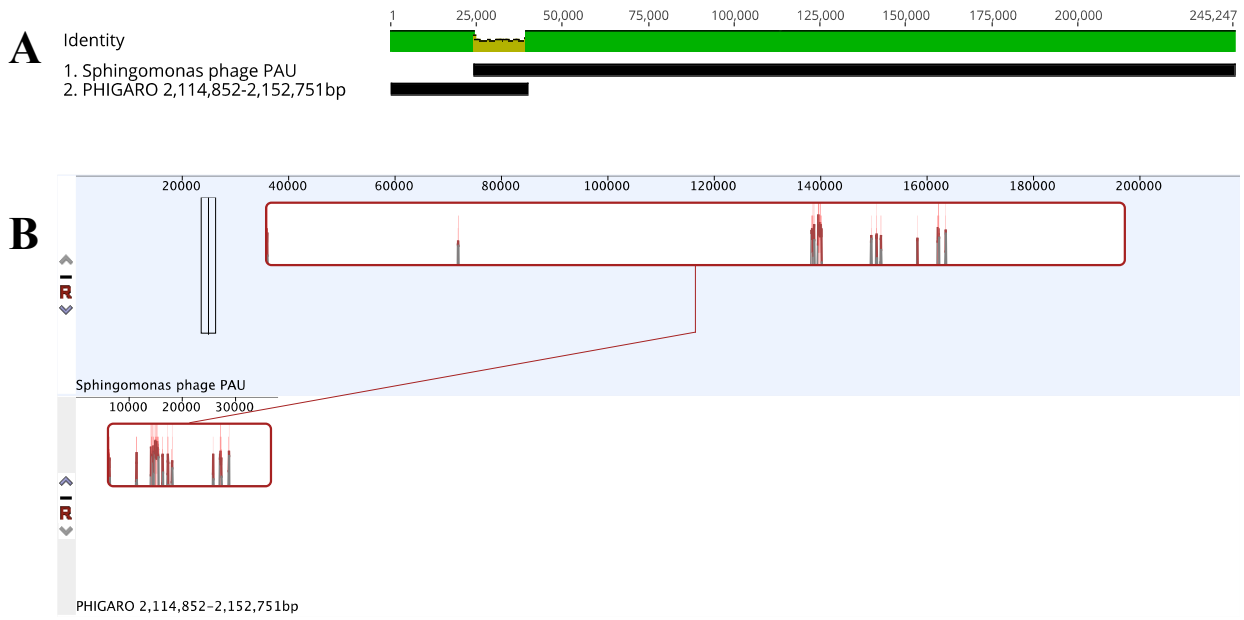

Figure 3.11: Bioinformatic analysis of PHIGARO identified prophage region 2,114,852-2,152,751 bp in *M. marinus* A45 using the pairwise sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative *Sphingomyces* phage PAU.

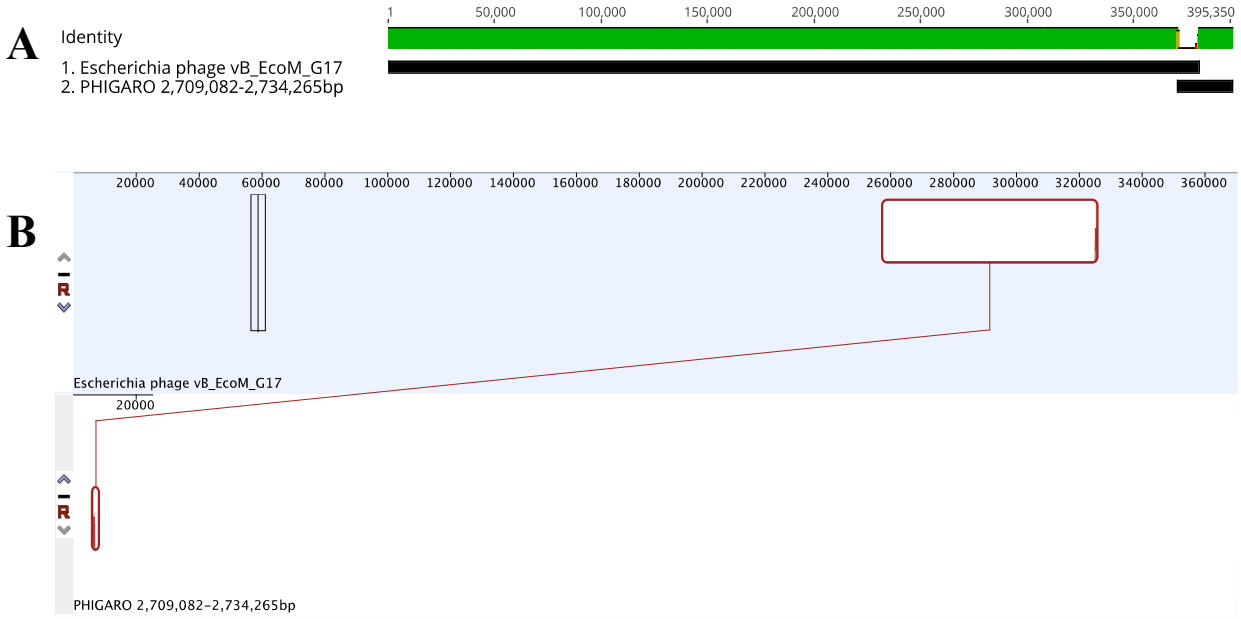

Figure 3.10: Bioinformatic analysis of PHIGARO identified prophage region 2,709,082-2,734,265 in *M. marinus* A45 using the pairwise sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative *Escherichia coli* phage vB_EcoM_G17.

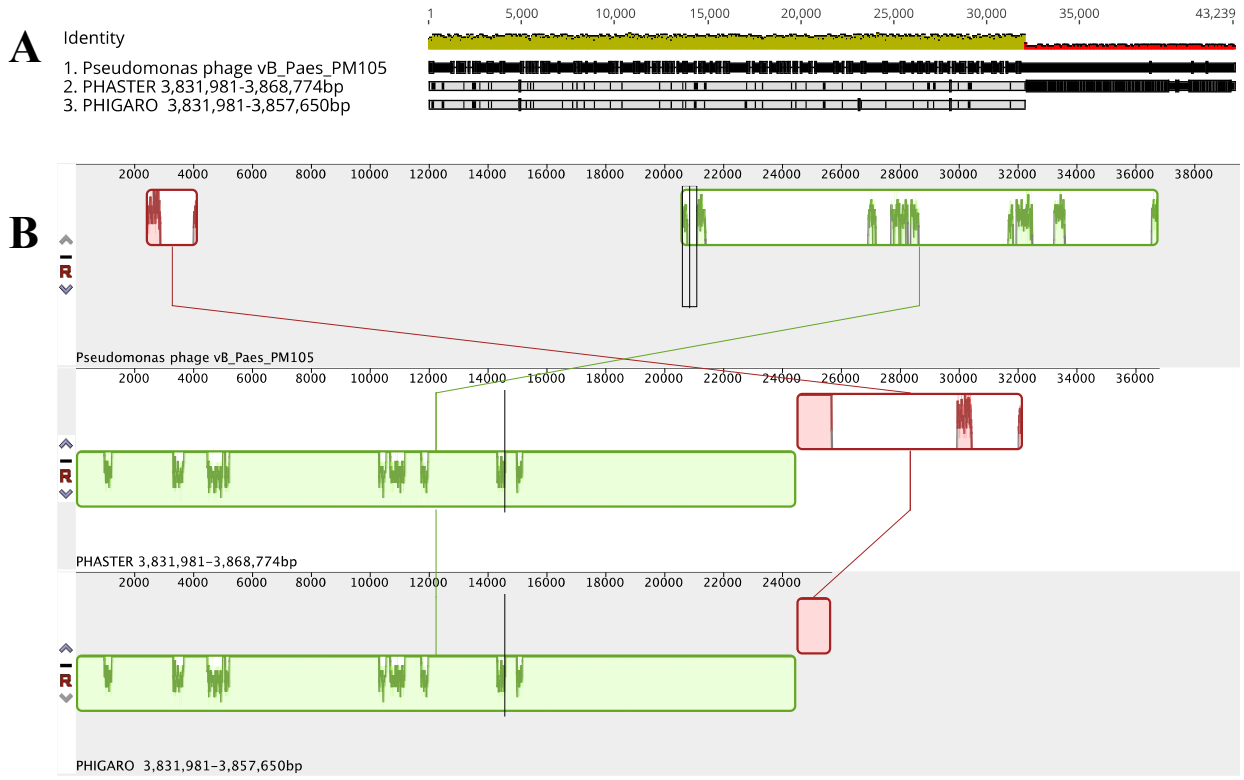

Figure 3.12: Bioinformatic analysis of PHASTER identified prophage region 3,831,981-3,868,776 in *M. marinus* A45 using the multiple sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative *Pseudomonas* phage vB_PaesPM105.

***M. trichosporium* OB3b**

The region at 838,186-885,310, identified by PHASTER as a putative prophage, was also highlighted by PHIGARO (Figure 3.2). BLAST analysis shows the closest relationship to *Cyanobacteria* phage AS-1. Geneious produced an alignment of 90.55% between the PHASTER and PHIGARO sequences, while each aligned with the predicted phage with 9.28% and 10.25% identity, respectively (Figure 3.13 A). MAUVE global alignment saw a small region of homology in the phage that could be matched to each of the respective sequences (Figure 3.13 B).

Region 1,531,646 – 1,574,415, as identified by both PHASTER and PHIGARO (Figure 3.2), was identified as being most closely related to *Pseudomonas* phage JD024. Geneious aligned PHASTER and PHIGARO sequences to each other with 75.68% identity, and to the identified closest related phage with percent identities of 46.17% and 47.74%, respectively

(Figure 3.14 A). MAUVE global alignment found one region of homology between the two predictions that could be aligned to the predicted phage relative.

Region 1,588,865 – 1,637,826, identified by both PHASTER and PHIGARO (Figure 3.2), was found to have an uncultured *Caudovirale* as the closest phage relative between the two strains. Geneious found a 78.97% identity between the PHASTER and PHIGARO produced sequences, and percent identities of 48.54% and 42.77% respectively between the predicted prophage regions and the identified *Caudovirale* relative (Figure 3.15 A). MAUVE global alignment found one homologous region between all three sequences (Figure 3.15 B).

Finally, the region between 1,661,356 -1,697,037 (Figure 3.2), which was only identified by PHASTER, was found most closely related to *Pseudomonas* phage YMC12/01/R24. Geneious aligned the two sequences with 52.63% identity and MAUVE global alignment was able to distinguish two sequences of homology between the two of them (Figure 3.16 A-B).

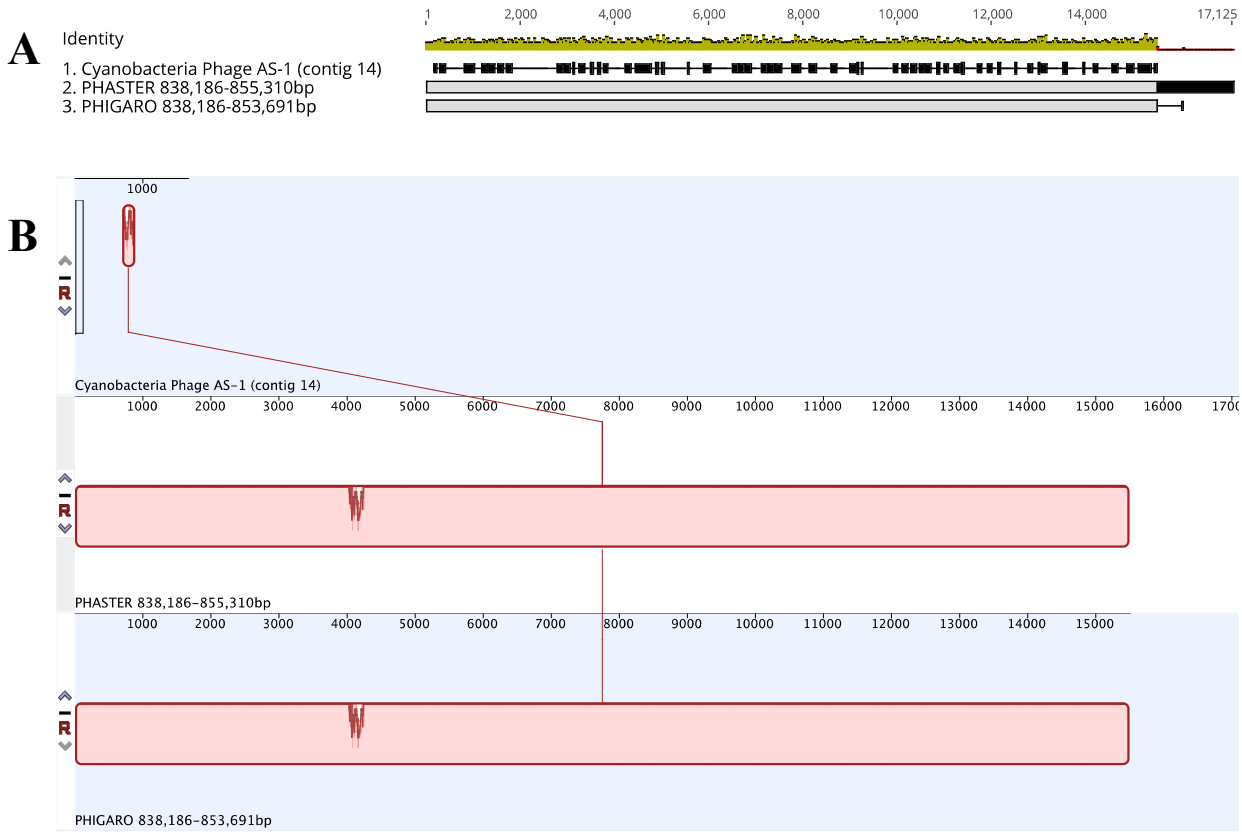

Figure 3.13: Bioinformatic analysis of PHASTER identified prophage region 838,186-855,310 in *M. trichosporium* OB3b using the multiple sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative *Cyanobacteria* phage AS-1(contig 14).

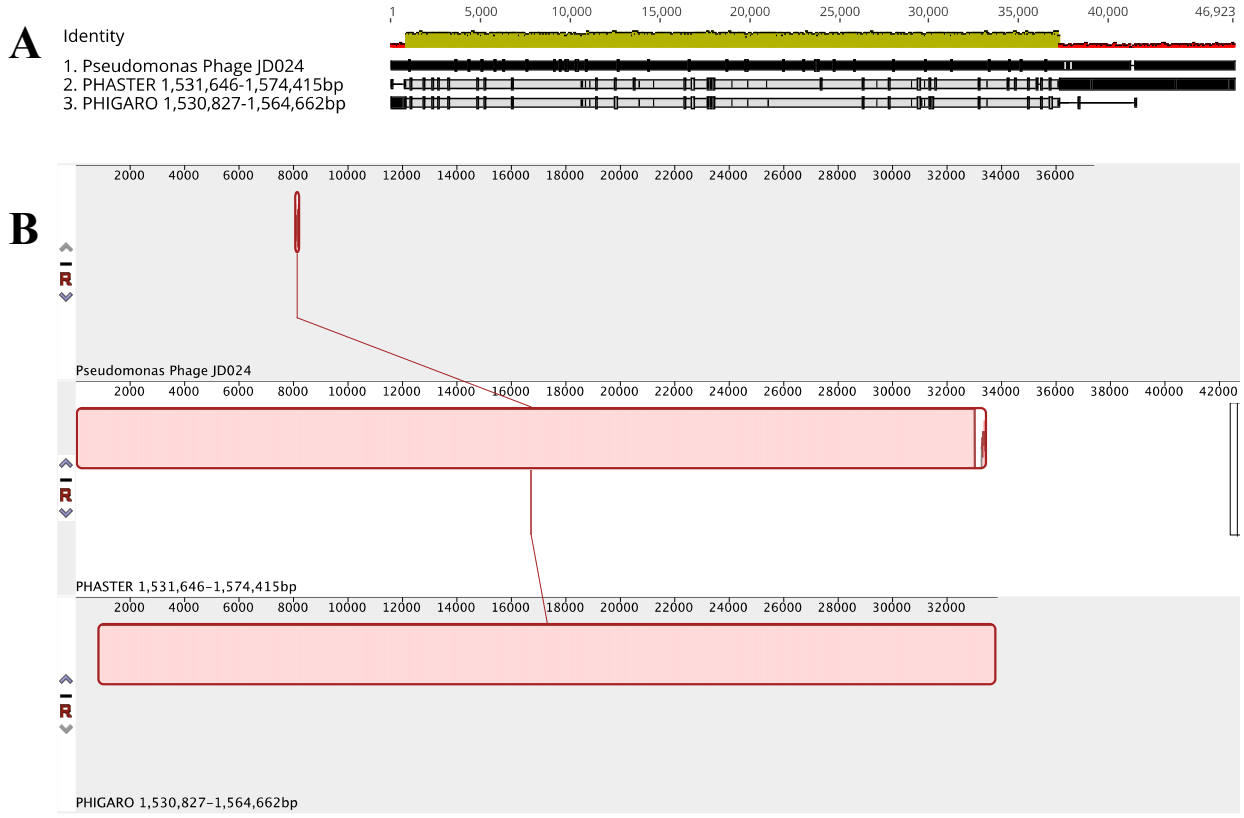

Figure 3.14 Bioinformatic analysis of PHASTER identified prophage region 1,531,646-1,574,415 in *M. trichosporium* OB3b using the multiple sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative *Pseudomonas* phage JD024.

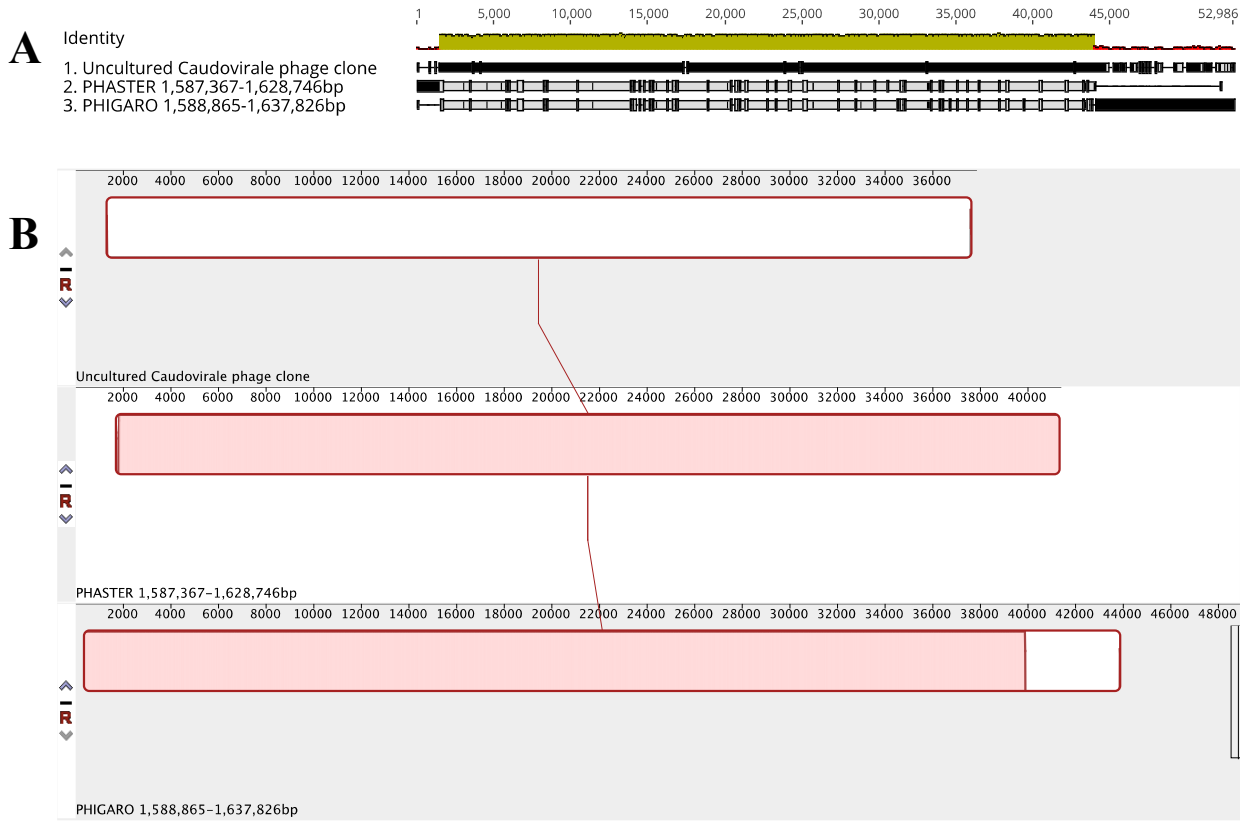

Figure 3.15: B Bioinformatic analysis of PHASTER identified prophage region 1,587,367-1,628,746 in *M. trichosporium* OB3b using the multiple sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative Uncultured Caudovirale Clone.

Figure 3.16: Bioinformatic analysis of PHASTER identified prophage region 1,661,356-1,697,037 in *M. trichosporium* OB3b using the multiple sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative *Pseudomonas* phage YMC12/01/R24.

***M. album* BG8**

Only PHASTER was able to detect a potential prophage region within the *M. album* BG8 genome, at 1,49,283 – 1,522,820 bp (Figure 3.3). BLAST analysis identified *Pseudomonas* phage BR141 as the closest phage relative. Although only a partial genome of this phage is currently available through NCBI, Geneious aligned the two sequences with 50.40% identity and MAUVE was able to classify 3 regions of homology between the two strains using a global alignment (Figure 3.17 A-B).

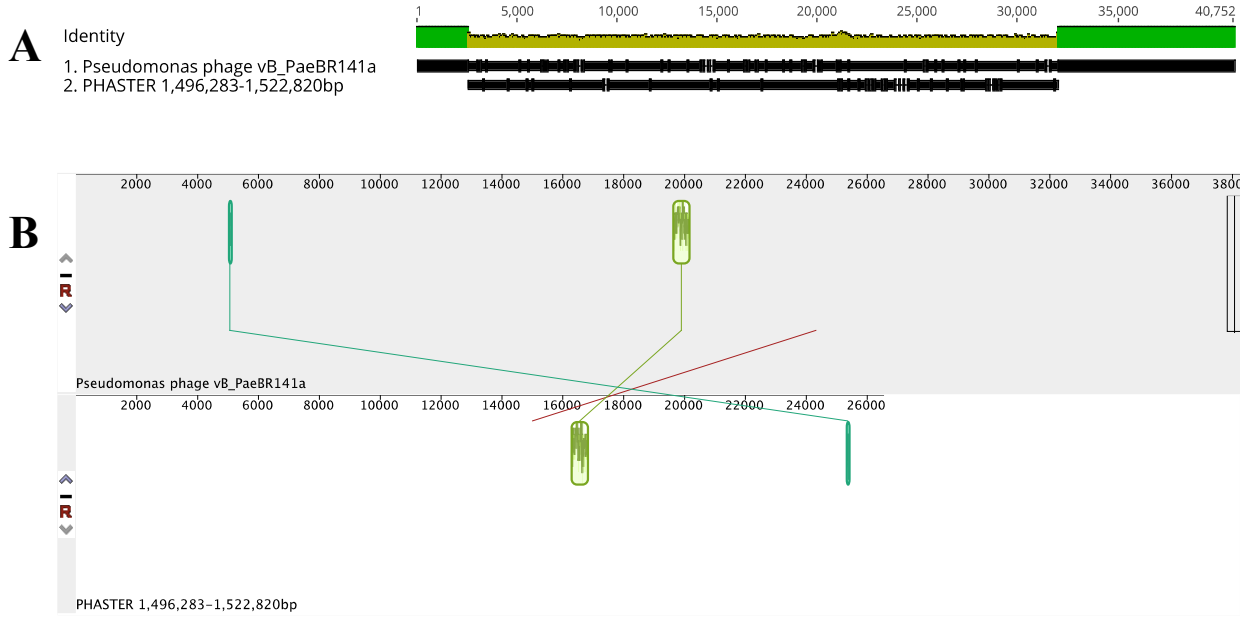

Figure 3.17 Bioinformatic analysis of PHASTER identified prophage region 1,496,283 - 1,522,820 in *M. album* BG8 using the multiple sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative *Pseudomonas phage vB_PaeBR141a.*

3.5 Discussion

3.5.1 Comparison of Putative Prophage Sequences in Methanotrophs

As part of PHASTERs programing it attempts to identify closes related phage relatives bases on the same algorithms it applied to bacterial sequences. Essentially, looking at the prevalence of identified phage genes in a sequence. The program then suggests a potential genealogical relative as well as an extensive list of phages which show homology to the putative prophage region (Arndt et al., 2016). This approach has one significant downfall which lead us to take a different approach. Essentially, it is heavily reliant on annotated known phage genes. Considering phage biology as a whole this is almost unrealistic, as many genes remain hypothetical, or simply unknown. Moreover, as PHIGARO lacks a similar build in measure in its interface to draw equal comparisons between the produced sequences of each software it made more sense to BLAST each produced sequence individually. Additionally, by BLASTing the entire putative phage sequence is compared against the entire length of databased phages means that unknown or hypothetical genes are not being excluded in terms of percent identity.

In regions were both PHASTER and PHIGRO identified putative prophages, the regions themselves usually aligned with moderate to high percent identities. Discrepancies occurred mainly on the edges, highlighting the fact that each program uses different algorithms to determine the cut offs of putative phage regions. This coupled with the fact that each software did uniquely identify prophages its counterpart did not, accentuates the point that no program should be used in isolation to analyze the extend of lysogeny in a bacterial host strain.

In terms of percent identity comparisons to phage relatives a wide range of results were seen some aligned with a percent identity as little as 4.12% to their closest BLAST host while others showed percent identity as high as 50.40%. These numbers have their own implications. Low percent identities can be indicative of a few things, simply put it could mean that the region was misidentified as a putative prophage. However, this isn't necessarily the case. Low percent identity could also correlate to an incomplete prophage or one that has been attenuated by its host. It has been shown that bacteria through subsequent divisions and time will select against genes which could cause the prophage to become active again. For example, by removing genes essential for lysis and "domesticating" a prophage region, a bacteria can prevent itself from

being lysed by preventing the induction of a harbored prophage (Bobay, Rocha, & Touchon, 2012; Howard-Varona et al., 2017). More excitingly though low percent identities could be indicative of a novel phage which has yet to be sequenced and characterized in NCBI.

In terms of the higher percent identities achieved through sequence analysis it is important to note that none of these sequences come close to reaching 100% identity with a known phage. This again could be due to bacterial modifications to the house phage genome, in attempts to attenuate these viruses (Bobay et al., 2012; Howard-Varona et al., 2017). However, as we were able to induce and visualize lysogenic phage in these strains it would be wrong to assume that all these predicted prophage regions are inactive. Therefore, in these cases we would suspect the differences in percent identity to be a result of differences in unannotated phage genes, and sequencing which would classify these phage as unique entities.

A final key talking point, is its interesting how in most cases the most closely identified relatives are in fact not phages of methanotrophic bacteria hosts, or even related strains. This implies that either phage have evolved over time infect methanotrophic hosts or simply have been in the environment all along waiting to be characterized. Overall, this emphasizes that a lot more remains to be uncovered in the realm of phage biology, including how these entities evolved, and the full extent of their presence in the environment.

3.5.2 Use of Mitomycin C to Induce Prophages in Methanotrophic Bacteria

Using MitC, we were able to successfully induce and visualize phage in three out of five tested methanotroph strains: *M. marinus* A45, *M. trichosporium* OB3b, and *M. album* BG8. Since its discovery as an antibiotic in the late 1950's,(Shiba, Terawaki, Taguchi, & Kawamata, 1959) MitC has been suspected of having inducing effects on established prophage regions, confirmed by its use as a potent inducer of phage in *E.coli* K-12 in 1959(Otsuji et al., 1959). It has become the standard in the field for first line inductions attempts(Otsuji et al., 1959). This in part is due to its highly effective mode of action, inhibiting the formation of new DNA in the bacterium (Shiba et al., 1959). This subsequently induces prophages via the cell's inhouse SOS response through the action of RecA, an enzyme essential to DNA repair and maintenance, on prophage repressor genes (Hare, Ferrell, Witkowski, & Grice, 2014; Maslowska, Makiela-Dzbenska, & Fijalkowska, 2019).

According to AUC analysis (Figure 3.7) and statistical tests, MitC at a concentration of 2μg/ml appeared to produce more reliable, impactful inductions than at 1μg/ml. Essentially, the smaller AUC observed with the higher drug concentration can be correlated to earlier and more effective cell lysis and release of phage virions. This leads to the hypothesis that, at the higher concentration, prophage induction is more rapid and more prevalent in the host population, accelerating the infection. This would be difficult to confirm in the context of methanotrophic organisms however due to the inability to consistently form lawns on plates.

It should also be noted that the addition of MitC to a culture didn't always result in the induction of a phage. For instance, despite PHASTER and PHIGARO detecting putative phage regions in both *Methylocystis sp.* Rockwell and *M.* denitrificans FJG1, and both strains showing decreases in OD in response to MitC addition, no phage particles could be identified through TEM of samples from the induced culture. Several factors could be contributing to such results. First and foremost, the MitC may have been killing the bacteria via the SOS response, instead of inducing a viable phage. Perhaps, for these organisms, a concentration of a 0.5μg/ml could be tested for induction of viable phages. Alternatively, different known inducers such as UV radiation or H_2O_2 *(aq)* could also be tested. Additionally, many phage-associated genes have yet to be fully elucidated (many remain annotated as 'hypothetical proteins'), hence many regions flagged as a potential prophages by computer software could be unrelated phage or have degraded over time, losing some of the genes required to re-enter the lytic life cycle (Bobay et al., 2012; Howard-Varona et al., 2017).

3.5.3 Analysis of Induced Bacteriophage

TEM analysis of lysate samples from all three induced strains revealed the presence of what could be classified, based on morphology, as a *Siphoviridae* phages (Figures 3.8 A-F). This order of phages, which make up 61% of known tailed bacteriophages, is commonly represented by Lambda, T1, and T5 phages (H. W. Ackermann, 2006; "Siphoviridae," 2012). DNA extraction and phage genome sequencing would be required to definitively place these phages in the *Siphoviridae* order; however, genomic analysis of the host DNA can provide clues as to which putative prophage regions may have been induced based on the properties of their most closely related phage relatives.

For instance, in *M. marinus* A45, two of the four putative prophage regions (Figure 3.1) had close relatives which showed similarity to *Siphoviridae* phages, while the other two regions showed more similarity to *Myoviridae*. Therefore, presuming the isolated DNA sequence is from one of the identified putative phage regions, the induce phage likely corresponds to one of the sequences identified at 549,816-572,143bp or 3,831,981-3,868,776 bp – most closely related to *Streptomyces* phage Toma (Figure 3.9) and *Pseudomonas* phage vB_PaesPM105 (Figure 3.12), respectively, two *Siphoviridae* phages. The other two remaining regions at 2,114,852-2,152,751bp and 2,709,082 – 2,152,751bp appeared to have homology closer to *Myoviridae* phages *Sphingomonas* phage PAU (Figure 3.10) and *Escherichia* phage vB_Ecom_G17 (Figure 3.11).

M. album BG8, only had one putative prophage region which sat at 1,496,283 -1,522,820 bp. It aligned closely with a *Siphoviridae* phage *Pseudomonas* phage vB_PaeBR141a (Figure 3.17), aligning with what we observed under TEM.

In the case of *M. trichosporium* OB3b, things get a bit more finicky. Any of the putative prophage regions could have resulted in the observed induction, except the one located at 838,186-885,310 bp. Regions 1,531,646 – 1,574,415 bp, and 1,661,356 -1,697,037 bp, aligned most closely with the *Siphoviridae* phages *Pseudomonas* phage JD024 (Figure 3.14) and *Pseudomonas* phage YMC12/01/R24 (Figure 3.16) respectively. While region 1,588,865 – 1,637,826 bp aligned with an uncultured *Caudovirale* phage which could meet the description of either a *Siphoviridae* or *Myoviridae* phage. The aforementioned excluded region of 838,186-885,310 bp aligned most closely with the *Myoviridae* virus *Cyanobacteria* phage AS-1 (Figure 3.13), which based on our TEM imaging is unlikely to be the induced region in this strain.

Region 1,531,646 – 1,574,415 bp (region 1,530,827 – 1,564,662bp for PHIGARO) is of particular interest as it was one of the aforementioned regions whereby the overlapping PHASTER and PHIGARO did not produce the same BLAST results in regard to which phage would show the greatest homology. For this region PHASTER aligned most closely to a *Bordetella* phage vB_BbrM PHB04, showing a percent identity of 54.6%, while the PHIGARO sequence aligned best with an *Arthrobacter* phage Jawnski, with an identity of 52.7%. According to BLAST results *Pseudomonas phage* JD024, where both sequences showed a percent identity of 49.1% was the closest relative with the highest coverage (83.03%) and percent identity shared between both regions. What is of particular interest to this region is the two relatives unique to

each sequence are both of *Myoviridae* origin. While the overlapping relative *Pseudomonas phage* JD024 is classified as a *Siphoviridae.* If we go one step further and compare these relatives to each other we then see that the *Bordetella* phage vB_BbrM PHB04 and *Arthrobacter* phage Jawnski share only 36.27% identity. With *Arthrobacter* phage Jawnski and *Pseudomonas phage* JD024 despite being of different orders sharing an even higher 41.55% identity. *Bordetella* phage vB_BbrM PHB04 and *Pseudomonas phage* JD024 showed an identity of 27.98%.

If this region was in fact the one induced by MitC induction, it generates some implications when considering the use of predictive prophage bioinformatic software, as neither software alone pulled a sequence which would match to the phage, we observed upon induction which was clearly a *Siphoviridae* (Figure 3.8). Therefore, in this instance based upon what was physiologically observed it would make more sense to take the overlapping result between the two sequencing even though it showed a lower percent identity according to BLAST. Thus, emphasizing the need to utilize more than one more prediction methodology when trying to predict the extent of lysogeny in bacterial hosts. It should also be noted that there is also the possibility that the sequences corresponding to the induced prophages were not recognized by the software's used, as each program does have its limitations.

3.5.4 Noted Experimental Limitations

Based on the cell density obtained in the induction experiments, we expect the titer of the resulting lysate to be relatively low. Specific titer numbers could not be confirmed however, due to the difficulty/impossibility of growing methanotrophs as bacterial lawns on plates. This highlights a substantial gap in the field of phage research, whereby the majority of protocols are biased towards bacterial strains which are easily culturable in vitro and on plates. With 99% of environmental bacteria predicted to be "unculturable"(Sharma, Ranjan, Kapardar, & Grover, 2005) – meaning that current or standard laboratory techniques are not sufficient enough to enable these microorganisms to grow in lab environments (Stewart, 2012) – new methodologies for the determination of standard phage measurements such as titer and bust size must be developed.

Another challenge encountered was the inability to reliably propagate the phages through subsequent amplifications. Although it remains uncertain why this was an issue, two theories

stand out as potential explanations. For one, the presence of a prophage in a host genome can sometime confer the host with resistance towards further phage infections (Susskind et al., 1974). To overcome this issue, most studies involving the induction of lysogenized phages use a second host that does not contain the prophage (different strain of the same species, or different species altogether) to serve as indicator strain to facilitate the enumeration of phages on plates (McDonald, Smith, Fogg, McCarthy, & Allison, 2010). Secondly, a combination of this low cell density and other environmental conditions could have signaled the phage to return to lysogeny as conditions were not desirable for a virulent life cycle at this point (Casjens & Hendrix, 2015; Howard-Varona et al., 2017).

As alluded to above this created difficulties in obtaining a large enough quantity of DNA for further analysis. For instance, there was not enough genetic material to clearly visualize the samples using gel electrophoresis, a methodology which is commonly used to analyze and separate out induced phage DNA by size of the genome.

3.6 Conclusion

In summary, three software programs PHASTER, PHIGARO and IslandViewer were used to identify methanotrophic bacteria strains which carry putative prophage regions. The analyses showed that the three algorithms are not always in agreement with regards to what constitutes a putative prophage region, and that when they identify a potential prophage in the same region of the host genome, the phage sequences identified never overlap perfectly.

Using MitC, a potent antibiotic and known inducer of lysogenized phages, we were able to induce and visualize three phages, from three out of five analyzed methanotrophic strains: *M. marinus* A45, *M. album* BG8, and *M. trichosporium* OB3b. TEM analysis allowed us to determine the dimension of the phages and that all three of them belonged to the *Caudovirale* order, specifically members of the *Siphoviridae* family, which aligns with information provided by the BLAST sequencing computationally of derived prophage regions which aligned them to their closest relatives.

This study highlights the fact that many standard phage protocols must be adapted, or new ones must be created to enable better characterization of phages of methanotrophic bacteria. This being said, this is the first report of the induction of prophages from this class of bacteria, which

lays the ground for a better understanding of the ecology and better development of technologies based on methanotrophic bacteria.

3.7 Acknowledgements

First and foremost, thanks to Dr. Marina G. Kalyuzhnaya, and David Collins, for providing the strain *M. marinus* A45, and assisting with TEM protocol development. Special thanks to Arlene Oatway, for her technical support and guidance in visualizing induced phage under TEM microscopy. Additional thanks to Liam Rieder, for providing a second set of hands in carrying out induction experiments.

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4 Identification and isolation of virulent phages of methanotrophic bacteria

4.1 Abstract

Methanotrophs are a unique subset of bacteria known for their capacity to metabolize methane from impure sources such as industrial waste streams and in turn convert them into valuable products such as isoprenoids for bio jet fuels, or polyhydroxybutyrate (PHB), a bioplastic precursor. These capabilities have put methanotrophs in the industrial spotlight, however there is much about these organism's innate biology, including their susceptibility to virulent bacteriophages, which needs to be elucidated prior to optimizing these strains for industrial scale fermentation.

The current knowledge of phage systems in methanotrophic bacteria is limited. Moreover, the field of phage research holds an inherent bias towards strains of bacteria which can easily be cultivated in laboratory environment and which form lawns on agar. Therefore, we sought to design and optimize a system which could not only isolate a novel environmental phage, but also be used with bacterial which are not suited to traditional culturing means, like methanotrophs. This system was first optimized using *Escherichia coli* ATCC 11303 and phage T4, and then later applied to environmental samples to try and bio prospect a virulent phage capable of infecting any of five strains of methanotrophic bacteria.

If successful this knowledge could be directly applied to industry both in attempts to attenuate phage infection in large batch fermentations, or more abstractly as part of the product recovery process of valuable biomolecules.

Key words: methanotrophs, bacteriophage, bioprospecting, product recovery

4.2 Introduction

Methanotrophs are a unique subset of bacteria characterized for their ability to metabolize single carbon compounds, such as methane or methanol as their predominate source of carbon and energy (Söhngen, 1906; Trotsenko & Murrell, 2008). These compounds can be converted into high value products such as ectoines, polyhydroxyl-alkanoates (PHAs) and isoprenoids (Strong et al., 2015). Because of this, methanotrophs have garnered the interest of bioindustry, suggesting they could be used as a platform for producing products which are normally derived from the oil and gas industry. However, prior to the industrialization of these microorganisms much about their overarching biology remains to be elucidated, particularly how they interact with other biological entities such as bacteriophages and the impact/role these viruses may play in a methanotroph based ecosystem.

Presently, only two studies describing virulent phages in methanotrophic strains have been published, the first identified a phage native to environments prone to methanotrophic growth across the USSR capable of infecting and lysing both *Methylosinus sporium* and *Methylosinus trichosporium* (Tyutikov et al., 1980). The second isolated phages from fish guts which are capable of infecting and lysing *Methylocystis sp.* (Tyutikov et al., 1983).

Considering the global phageome and its impact it can have one microbiology is a relatively recent train of thought. However, what the research is showing us is that bacteriophage as a microbial entity are having a much larger effect at the community level of ecosystems than originally thought. For instance, phages are believed to play a tremendous role in the horizontal gene transfer of key virulence factors such as antibiotic resistance genes and toxins across bacterial genomes (Canchaya et al., 2003). Phage presence in an environment can also impact its biodiversity, with the human phageome being tied quite closely to dysbiosis of gut microflora (Manrique et al., 2016).

In terms of industrial process, the presence of phage has traditionally been considered a detriment. For instance, in terms of the industrial fermentation of yogurt, the presence of phages can result in the loss of an entire fermentation batch, as they lyse the probiotic bacteria responsible for the products properties (Kilic et al., 1996). However, as we learn more about these organisms it becomes clear that they could provide tremendous value to bioindustry. For

instance, phage have been used as deterrent for antimicrobial growth in the beer industry (Kelly et al., 2012).

Therefore, by isolating and characterizing a phage capable of infecting methanotrophic bacteria we hope to achieve several goals. First and foremost, considering what little is known about the environmental phageome, and the impact phages can have on methanotrophic growth both in vivo and in vitro we hope that by new virulent phage capable of infecting methanotrophs we can begin to answer some of these questions. Secondly, once a virulent phage is found the eventual goal would be to incorporate into large scale bio processes, in particular we envision doing this as a means of product recovery for biomolecules such as PHAs, whereby the current methods of product recover are quite costly and involves the use of noxious chemicals such as chloroform, which can be difficult to dispose of and have detrimental environmental effects (Bedding et al., 1982; Koller et al., 2013).

4.3 Materials and Methods

4.3.1 Microorganisms

Escherichia coli ATCC 11303 and its phage T4 (ATCC 11303-T4) were acquired from the American Type Culture Collection (ATCC) depository. The strains of methanotrophic bacteria used in this study were : *Methylosinus trichosporium* OB3b (origin), *Methylocystis sp* WRRC1 (origin)*, Methylomonas denitrificans* FJG1 (origin), and *Methylocystis sp.* Rockwell (origin), and *Methylomicrobium album* BG8.

4.3.2 Media and Bacterial Cultures

Luria-Bertani (LB) medium was used for the growth of *E. coli.* In short, 1 L of medium was made by dissolving 25 g of LB, Miller (LB) preparation (Fisher Scientific) into 1000 ml of distilled H_2O. The medium was autoclaved to ensure sterility. Hard LB agar – used for colony isolation and T4 titer calculations – was made by combining 25 g LB preparation with 15 g agar technical (Fisher Scientific) with 1000 ml distilled H_2O and autoclaving to ensure sterility. The medium was allowed to cool to 60°C before being poured into plates. Soft agar was made by combining 1.25g LB preparation and 0.375 g agar technical with 50 ml distilled H_2O and

autoclaving for sterility. Soft agar was aliquoted immediately after autoclaving and stored at 50°C to ensure it would not harden prior to use.

E. coli glycerol stocks kept at -80°C were thawed and streaked onto LB agar plates, and grown at 37°C overnight to produce single colonies. A single colony was used to inoculate 10 ml of LB broth in a 30-mL shake flask to serve as pre-culture – incubated at 37°C and 250 rpm for approximately 12-18 h. The preculture was then used to create a working plate for experimental inoculations.

AMS medium was used for experiments performed with all strains of methanotrophs tested. The recipe and instructions for this medium can be found in Chapter 3 Section 3.3.4.

Methanotrophs were grown in AMS medium. A starter culture was created by inoculating 1-2% of methanotroph culture from stocks in 100ml fresh 1XAMS and allowing it to grow at 30°C and 150 rpm until cultures reached stationary phase. From here experimental cultures were generated by taking 1ml of the starter culture and using it to inoculate 100ml of fresh 1 X AMS, 20-24hours prior to experimentation. Once cultures were in early to mid-logarithmic growth the filtration experiments would commence.

Growth of *E. coli* and methanotrophs in liquid cultures was assessed by measuring optical density OD at 600 nm for *E. coli* and 540 nm for methanotrophs using a UV-Vis spectromphotometer (Biochrom Ultrospec 50 UV-Vis).

4.3.3 Phage T4 Growth and Titer

Phage T4 lysate was prepared by inoculating a desired volume of LB medium (this varied between experiments, but 20 ml were typically used for standard phage work and 250 ml were used for system optimization), with a 1% v/v inoculum of *E. coli* preculture. OD_{600} was monitored until cultures reached an OD_{600} of 0.1-0.3 (early log phase). At this point, cultures were infected with a multiplicity of infection of 0.01. Incubation was then performed at 37°C and XX rpm, with OD monitoring throughout. When lysis was observed and OD decreased to < 0.1, the lysate was harvested by filtering the media through a 0.2-μm syringe filter.

Phage T4 titer was assessed using the double-layer agar assay (Kropinski, Mazzocco, Waddell, Lingohr, & Johnson, 2009) and whole plate dilutions. A 10-fold dilution series of phage stock in LB was prepared down to a dilution of 10^{-9}. Dilutions 10^{-5}-10^{-9} were plated in

triplicate by adding 100 µl of overnight bacteria culture and 100 µl lysate to 3 ml of soft agar and briefly vortexing before pouring over hard agar plates. Plates were then incubated overnight at 37°C in an incubator (Ectotron Infors MT, Canada). The phage plaques were counted, and titer was reported as plaque forming units per ml (pfu/ml). It is important to note that this method could not be used for phages of methanotrophs due to the slow growth and sometimes impossibility to grow these strains as lawns on plates.

4.3.4 Environmental Samples Preparation

Environmental samples were provided from two tailings ponds sites: SWAN BML, MBL12 Platform 1-C-14, 10.5 m sample site from August 26/2016, and a second tailings site, which cannot be disclosed due to the nature of the samples. After harvesting, samples were stored at 4°C until they could be cleaned and processed for use in phage bioprospecting experiments.

Samples were mixed with phage buffer (Table 4.1) in a 50-ml falcon tube in a 1:2 ratio and left to settle overnight. They were then centrifuged at 1750 × g (IEC clinical centrifuge, OM428) for 20 min. The supernatant was then extracted and run through a 0.2-µm cellulose filter, and stored at 4°C. This process was repeated until the samples settled and no more phage buffer could be separated out.

Table 4.1: Recipe for 1 L phage buffer.

Phage Buffer
10 ml Tris (1M), pH 7.5
10 ml $MgSO_4$ (1M)
4g NaCl
980 ml ddH_2O
Plus, 1ml $CaCl_2$ per 100ml of phage buffer added immediately before use.

4.3.5 Bioprospecting Experiments

The protocol for bioprospecting phages from environmental samples was adapted from Ghugare et.al, and from resources on the PhagesDB online database (Ghugare et al., 2017; Russell & Hatfull, 2016) and is illustrated in Figure 4.1. 10-15 ml of early log phase cultures of a bacterium of interest were filtered across 0.2-µm or 0.45-µm cellulose filter using a Millipore filter apparatus and vacuum pump (Model No. 400-1901, Barnant Co., Barrington, IL). Liquid medium was removed until only a thin layer of culture was left on the filter membrane. A cleaned tailings sample was then filtered through the system, allowing it to be in contact with the bacterial culture layer as it moves through the filter. The membrane filter was then removed from the filtration system using tweezers and placed into 50 ml of fresh medium (AMS with methane replenished in the headspace). Culture growth was monitored using OD_{540} for methanotrophs over the next 72-96 h. Samples were considered to be infected if experimental cultures showed significantly lower OD than the unexposed control cultures. The same procedure was repeated to establish experimental controls, but instead of running environmental samples through the filtration system standards, autoclaved AMS or NMS medium was used.

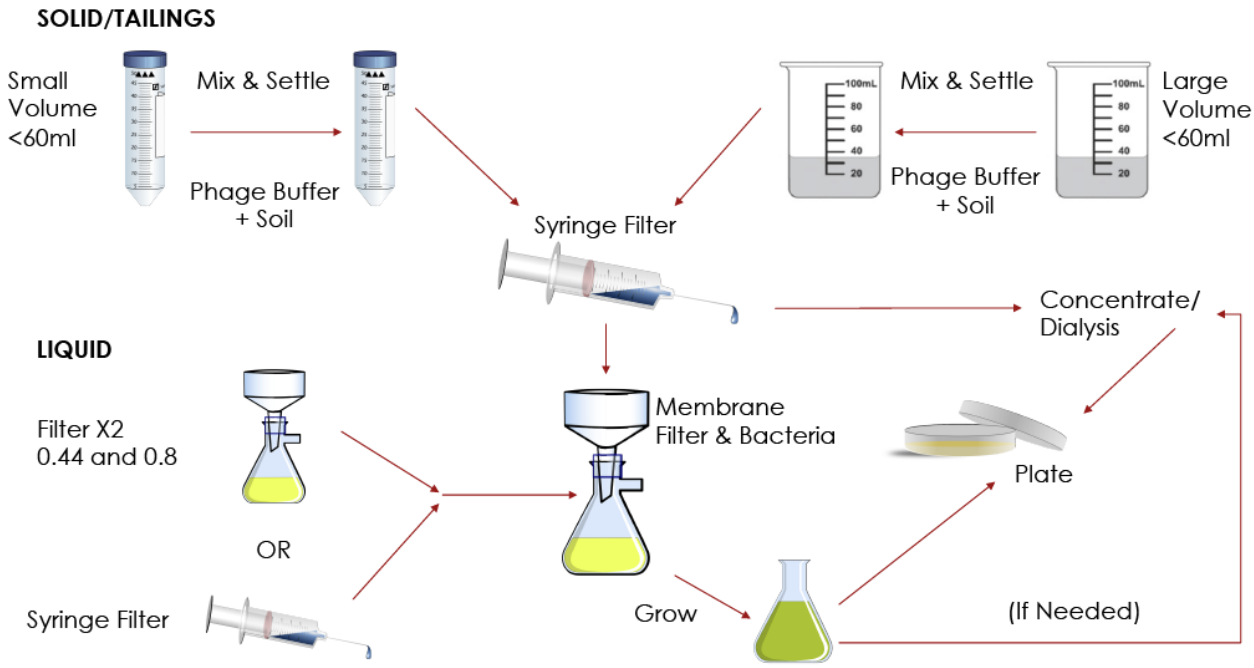

Figure 4.1: Protocol for bioprospecting phages of methanotrophs from environmental samples. Environmental samples were cleaned and filtered through a thin layer of bacteria placed on the filter membrane using a vacuum prior to experimentation.

The procedure was validated using cultures of *E. coli* as host and phage T4 stocks of varying titers (ranging from 6.5×10^{9} to 1.37×10^{10} pfu/ml) in replacement of the cleaned tailings samples to establish the limit of detections of phages present in a single sample. This particular bacterial host and phage were selected due to the well understood nature of lytic T4 infection in literature. The experimental plan is depicted in Figure 4.2. To begin, 250-ml of phage lysate was prepared during a single round of infection and then titered in accordance with the above protocol. The lysate was diluted down to 10 phages per 250 ml, 1 phage per 250 ml, and the remainder was set aside as a "pure" lysate positive control. An *E. coli* 11303 overnight culture was used to inoculate four 50-ml shake flasks of LB medium at 1%. The cultures were allowed to grow for 1-2 hours, until they reached and OD_{600} of 0.100-0.200. 10 ml of culture were filtered over a 0.2-µm cellulose filter as described above, leaving a thin layer of cells on the membrane. The phage T4 stocks of various titers were then filtered through the culture sitting on

the filter membrane. The filter was recovered and placed in 50 ml of fresh LB medium in a 200-ml shake flask, which was then returned to standard growth conditions. OD was monitored over the next several hours to verify if there was a reduction in OD.

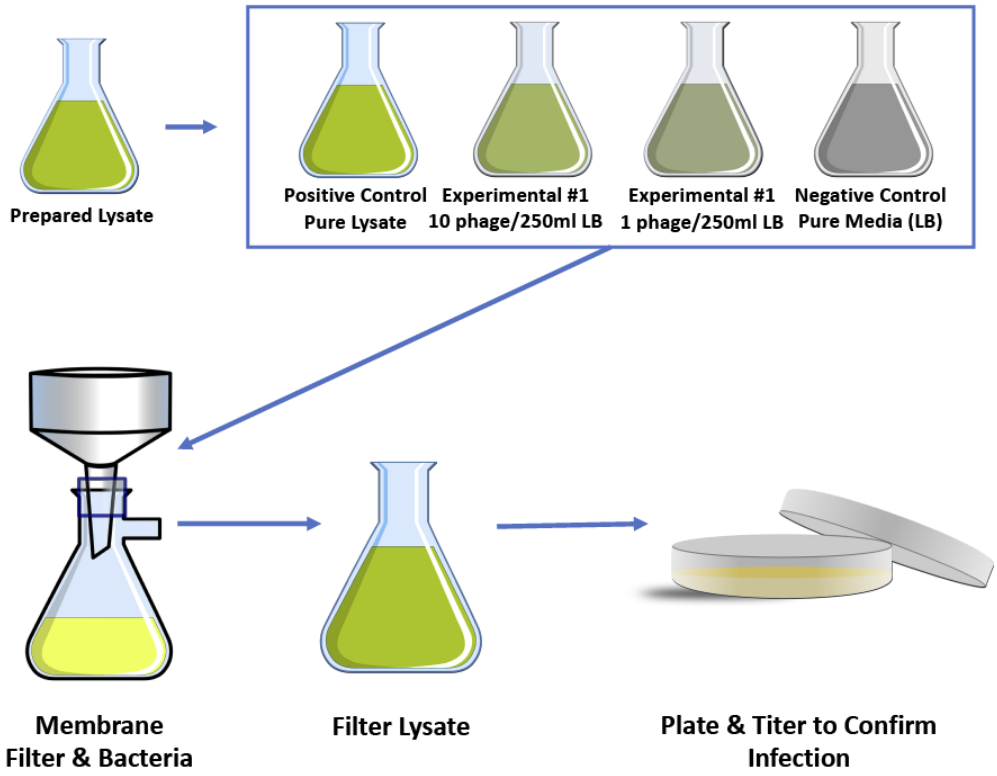

Figure 4.2: Protocol for bioprospecting bacteriophages from the environment for a methanotroph host. Environmental samples were purified and run over a thin layer of bacteria placed on the filter using a vacuum prior to experimentation. The preferential media for the strain of interest was run through the system using another filter coated in bacteria as a control.

4.3.6 Transmission Election Microscopy (TEM) Preparation

Cultures which showed evidence of a phage based on OD_{540}, where prepared for TEM imaging by filtering out the cell lysate and centrifuging it to remove cell debris and resuspending remaining phage in lambda diluent Lysate was then left overnight to settle and then visualized on copper coated grids using uracil acetate under TEM. For more details see Chapter 3 Methods section 3.3.7.

4.4 Results

4.4.1 Validation of phage bioprospecting protocol

Due to the particularities of methanotroph growth – many strains are not culturable on plates, exhibit slow growth, etc. – and since most classical methods for phage bioprospecting requires the growth of the host on plates as a lawn, a new system for the isolation of virulent phages of methanotrophs was proposed (see section 4.2). The system was tested using the well-characterized phage T4 and its easily culturable host *E. coli* ATCC 11303. In the method, host cells are deposited on a filter membrane through which the environmental sample is passed. The filter paper is then placed in LB medium and incubated to observe growth and potentially lysis of the culture. Figure 4.3 shows the results of experiments performed when 250 ml of LB broth containing varying amounts of phages (from 6.5×10^{9} to 1.37×10^{10} pfu) are passed through the filter paper with deposited *E. coli*.

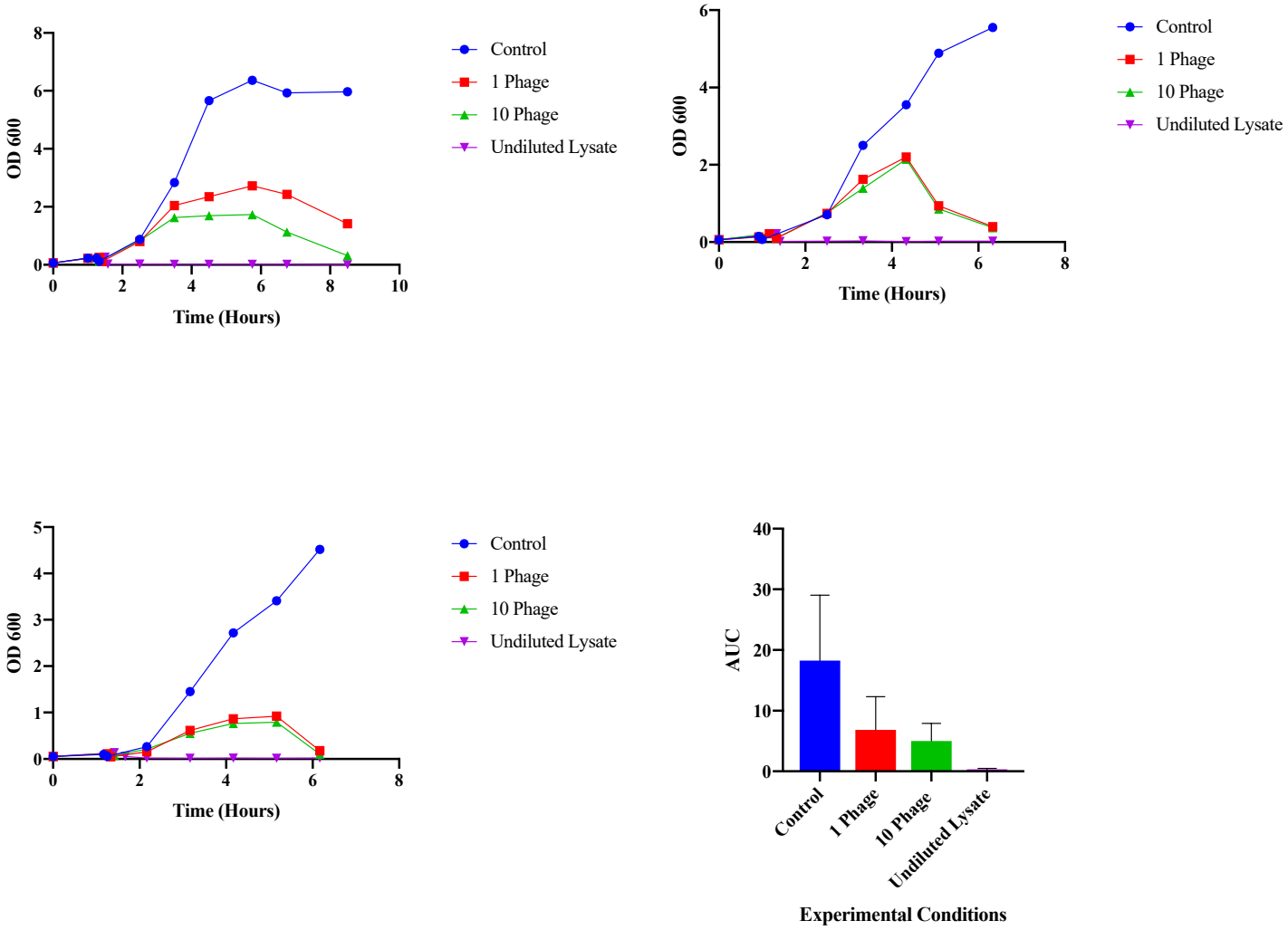

Figure 4.3: Validation of filtration system for phage bioprospecting using an established *Escherichia coli* ATCC 11303 and phage T4 infection model. Optical density (OD_{600}) measurements are reported for three different infection trials and their controls using the designed filtration system (A-C). For each control (no phage and undiluted lysate) and prepared T4 condition (1 and 10 phages per total volume), a 200 ml volume was passed through a layer of *E. coli* cells deposited onto a 0.25-µm membrane filter at a density between $1.59\text{-}1.79\times10^{5}$cells/cm^2. The titer of the lysate for each trial was as follows: 1.19×10^{10} pfu (A), 1.37×10^{10} pfu (B), 6.5×10^{9} pfu (C). Area under the curve analysis was performed to determine the significance between runs using a n=3. (D).

Generally, the control shows that the *E. coli* cells deposited on the membrane filters were able to initiate growth in LB media. In contrast, deposited hosts exposed to the undiluted high titer lysate (ranging from 6.5×10^{9} pfu to 1.37×10^{10} pfu) resulted in non-recoverable cell death. In

some instances, cell lysis could even be visually observed on the membrane itself. The experiments with both 1 phage and 10 phages per 250 ml of filtered phage stock led to typical infection curves once the filter was transferred and incubated in LB broth. The initial host growth was eventually superseded by the phage infection, leading to a crash in OD_{600} once the infection had successfully propagated through the culture.

Area under the curve was used to compare the impact of phage presence on culture growth. As seen in Figure 4.3D, all three conditions tested (1 phage per 250 ml, 10 phages per 250 ml and undiluted lysate) negatively impacted growth, with the undiluted lysate again having the most substantial impact. In fact, 1-way ANOVA analysis using Dunnett's test and a p-value of 0.05 found that both the 10 phage per 250ml preparation and the undiluted lysate experiments had statistically significant impacts on culture growth when compared against the control. The 1 phage per 250 ml preparation showed a decrease in AUC compared to the control, although this was not found to be statistically significant. The results show that, although 1 phage per 250 ml could be recovered using the protocol developed, the impact on the dynamics of the host culture are statistically significant when at least 10 phages per total volume are present in the preparation.

4.4.2 Isolation of Lytic Phage of *M. trichosporium* OB3b from Tailings Pond Samples

Preliminary experiments prospecting for phages of methanotrophs were performed on 250 ml of filtered mine tailings water (obtained from the SWAN-BML test site). Based on the results obtained with phage T4/*E. coli*, any sample containing no phage able to recognize the host of interest deposited on the filter membrane would lead to growth similar to the control once transferred to liquid medium. On the other hand, the presence of one or many phages targeting the host should demonstrate, at minimum, delayed growth with a slight decrease in OD_{540} and, at best, complete lysis of the culture.

Five strains were tested against the BML-Swan Hill tailings water samples: *Methyloscystis sp.*WRRC1, *Methylocystis sp.* Rockwell, *M. album* BG8, *M. denitrificans* FJG1, and *M. trichosporium* OB3b. However, *M. trichosporium* OB3b was the only strain which demonstrated a clear reduction in growth post-filtration of the tailings sample (Figure 4.4 A).

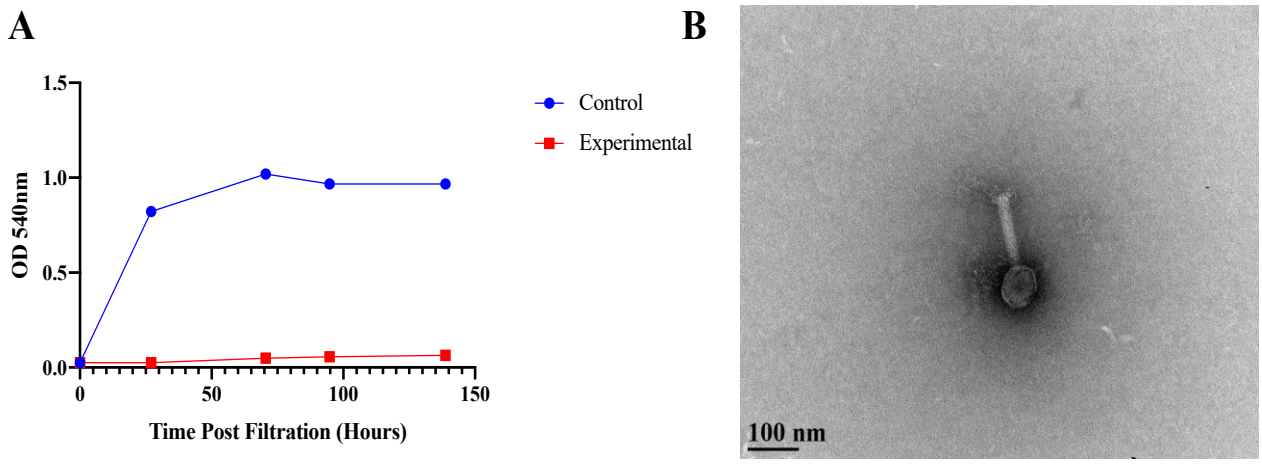

Figure 4.4: Isolation of a presumed lytic phage of *M. trichopsorium* OB3b from environmental tailings samples. Growth curve measured at OD_{540} comparing the growth trends of the control and the experimental cultures (A). TEM confirmation of phage, taken at 100,000× magnification (B).

Essentially, after being exposed to the tailings water, the bacterial cultures were unable to recover and maintained a low $OD_{540} < 0.100$. In addition, signs of poor growth – clumping and precipitation of cells – were noted. In juxtaposition, the control showed no symptoms of unhealthy or contaminated growth. Following these results, further examination of the resulting broth via TEM confirmed the presence of a phage (Figure 4.4 B), seemingly of the order *Caudovirale* based on head and tail properties. Further analysis of physiology suggests the family of origin to be *Myoviridae*, as noted by the slightly shorter, helical tail measuring on average 132.6 nm in length, and 23.6 nm in diameter, with standard deviations of 4.6 and 0.84 nm, respectively (Table 4.1). Moreover, the sheath is separated from the head by a short neck, which would not be present if the phage belonged to the *Siphoviradae* family. As expected, heads were binarily symmetrical, measuring on average 66.9 ± 2.8 nm (Table 4.2).

Table 4.2: Dimensions of *M. trichosporium* OB3b phage isolated from SWAN-BML Tailings Pond Samples

	Head Diameter (nm)	Tail Length (nm)	Tail Width (nm)
	66.6	136.2	24.7
	65.1	133.6	22.5
	66.9	128.1	23.1
	63.5	127.7	23.1
	71.8	139.2	23.9
	68	130.5	24.3
Average (nm)	66.9	132.6	23.6
SD (nm)	2.8	4.6	0.84

4.4.3 Isolation of A Lytic Phage of *M. album* BG8 from Tailings Pond Samples

In a further round of prospecting, 500 ml of pooled SWAN-BML tailing samples were tested against four methanotroph strains : *Methylocystis sp.* Rockwell, *M. denitrificans* FJG1, *M. trichosporium* OB3b, and *M. album* BG8. *Methyloscystis sp.* WRRC1 was eliminated due to inconsistent growth prior to the experiments and failure to reach mid-log phase at the time of testing. In this round of experimentation only *M. album* BG8 exhibited signs of infection, predominantly determined by the stunted growth of the host once placed in liquid medium following exposure to environmental samples; whilst the control continued to grow as expected (Figure 4.5 A).

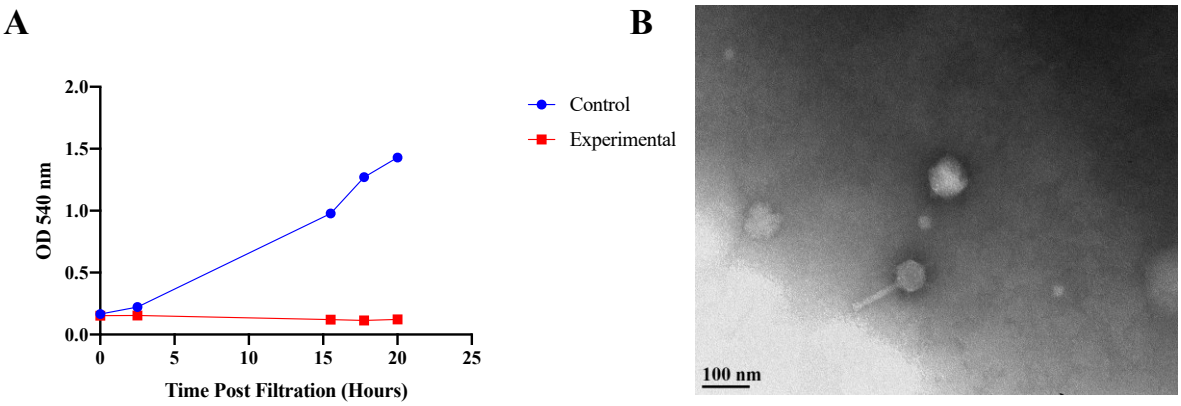

Figure 4.5: Isolation of a presumed lytic phage of *M. album BG8* from environmental tailings samples Growth curve measured at OD_{540} comparing the growth trends of the control and the experimental cultures (A). TEM confirmation of phage, taken at 100,000× magnification (B).

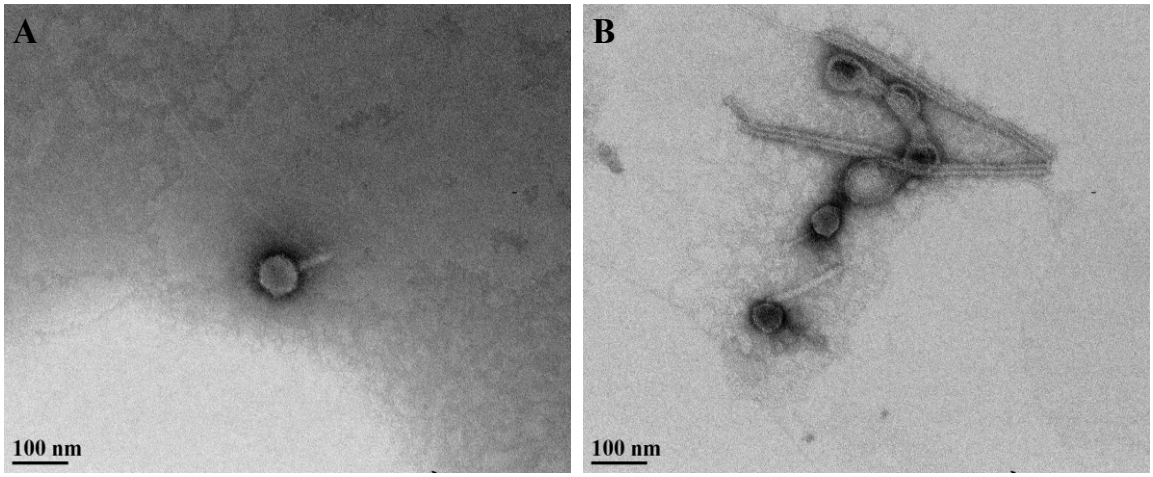

Figure 4.6: Visualization of presumed lytic phage of *M. album* BG8 showing a phage with a broken tail at 100,000 × magnification (A). Further imaging of the phage of *M. album* BG8 showing two phage particles with broken or bent tails next to cell debris. Image taken at 100,000 × magnification (B).

Again, analysis under TEM confirmed the presence of a phage (Figure 4.5B, Figure 4.6). However, in this case, due to poorer image quality, it is difficult to make clear assumptions on the phage physiology and family. However, based on the collection of images obtained, it is clear, that a *Caudovirale* was isolated. Heads were symmetrical measuring on average 62.4 ± 12.3 nm in diameter (Table 4.3). Tails appeared helical in nature and were found to be 105.7 nm in length with a standard deviation of 20.8nm, and 13.4 nm in diameter with a standard deviation of 1.3 nm. In one instance the phage tail was clearly broken (Figure 4.6 A), and its length measurement was excluded from the calculation of the mean. No clear distinction or "neck" could be seen between the head and sheath, leading to the assumption that the phage could be a *Siphoviridae*; however, considering the quality and degradation seen within these samples, genomic sequencing will be required to confirm these assumptions.

Table 4.3: Measurements of *M. album* BG8, phage from SWAN-BML Tailings Pond Samples

	Head Diameter (nm)	**Tail Length (nm)**	**Tail Width (nm)**
	70.1	101.5	14.9
	75.3	N/A	12.3
	54.4	128.2	12.4
	49.6	87.3	14
Average (nm)	62.4	105.7	13.4
SD (nm)	12.3	20.8	1.3

4.5 Discussion

4.5.1 Bioprospecting Phages of Bacteria Non-Culturable on Plates

Current standard methods for phage bioprospecting involve plating bacteria mixed with environmental samples in or on a soft agar overlay. If present in the environmental sample,, phages of the bacterium of interest will infect cells and form distinct plaques, which can then be extracted, amplified and sequenced (Cross et al., 2015). This process has two main limitations:

first, only a small volume of environmental sample can be tested against a single host, and second it is limited for use against bacterial strains which can be grown on plates. , This is significant since it greatly hinders the search for and investigation of phages of a vast number of bacteria, such as methanotrophs. It was thus important, for the present study and others, to develop a method enabling the isolation and retrieval of phages of such bacteria.

By combining and building on two protocols (Ghugare et al., 2017; Russell & Hatfull, 2016), we were able to demonstrate a process (Figure 4.1) capable of reliably detecting low quantities of phages in both a modeled system (*E. coli* and phage T4) and in environmental samples. The *E. coli*/phage T4 system demonstrated that our process could be used to isolate as few as 1 phage per sample volume and readily detect 10 phages per sample volume, causing culture lysis (Figure 4.3A-C) and statistically significant impacts on cell death rates as indicated by the area under the curve (AUC) (Figure 4.3D). This is not to say that the lower 1 phage per sample volume was unsuccessful as that condition still resulted in lysis of 50 ml cultures (Figure 4.3A-C); however, the impacts on overall culture growth dynamics were less pronounced (Figure 4.3D). Essentially, we can say that our system could be employed to put low or unknown quantities of phage in direct contact with a host of interest, creating an efficient way to test large volumes of environmental samples against a single host, even for bacteria that cannot grow on plates. This approach , which overlays a thin layer of bacteria on a cellulose filter and then subsequently uses it as an inoculum to a liquid culture, opens up the potential to discovery and subsequently to the study of phage in organisms not attuned to traditional methodologies.

In terms of the study at hand, this is essential as methanotrophs are often not amenable to growth as lawn on plates and have generally low growth yields when compared to heterotrophic bacteria, which would prevent us from using standard phage bioprospecting protocols. It should be noted that such issues are not confined to methanotrophs and in general there has been a bias in the field of phage research towards strains that grow well on plates. For example, it is estimated that about 95% of gut bacteria, including families like *Bacteroidaceae, Prevotellaceae, Ruminococcaceae, Lachnospiraceae*, are difficult to culture using traditional lab protocols, which has significantly limited our understanding and impact of the human gut phageome (Shkoporov & Hill, 2019). Therefore, we propose that our process could be employed as an initial step for bioprospecting phages for strains with unreliable growth, or instances where large volumes of samples need to be tested.

Tangential flow filtration for the concentration of environmental samples is perhaps a reasonable alternative, but the approach lacks specificity; and although the concentrate can be applied to different strains individually, the method is impractical for testing against multiple host strains (Alonso, Rodriguez, & Borrego, 1999). Moreover, it requires the laborious process of plating several test cultures, all requiring growth of the host as lawns. Also, if a phage of the strain of interest is in the sample but not in the aliquot used for plating, it could easily be missed. The system presented herein also provides a solution to missing low abundance phage or bioprospecting for phages for multiple bacterial targets at once as after each passage of the environmental sample on a filter with a deposited host, the same sample can be passaged through a new filter harboring a new target strain, and so on. Therefore, the original filtrate can be reused to test for the presence of phages of a completely different bacterium or multiple times with the same bacterium. This is possible based on two features: firstly, filters are selected such that they are small enough to trap the bacteria but not free phages, and secondly, the inherent nature of phage specificity means the phage will only attach and be retained upon encounter of its target host (Weinbauer, 2004). In other words, phages that cannot infect the host on the filter remain in the filtrate, which can be recycled for targeting to new hosts. Therefore, we propose that our methodology could be useful when bioprospecting for phage for a variety of hosts from a single environmental sample.

It should also be noted that the system was also able to isolate/induce a bacteriocin in *M. album* BG8 as observed under TEM (Appendix Figure A.6). Bacteriocins are antibacterial proteins produced by bacteria which defend against the growth of other bacteria (Cotter, Ross, & Hill, 2012). Often resembling phage tails, these high molecular weight proteinaceous structures can either take on F-type non-contractile structures (resembling *Siphoviridae*) or R-type contractile structures (resembling *Myoviridae*) (Michel-Briand & Baysse, 2002; Nakayama et al., 2000; Šmarda & Benada, 2005). Appendix Figure A.6 reveals a combination of what we assume to be native and contractile particles where the sheath appears shortened and the core is exposed. Based on TEM visualization, the isolated particles can be assumed to be R-type bacteriocins. It is possible that a few F-type particles are present in the sample; however, further analysis is required to confirm the origin of the bacteriocins in the sample. What triggered the bacteriocin release – as the particles were not observed with any other strain or replicate of *M. album* BG8 culture tested against the same sample – remains to be elucidated.

4.5.2 Isolation of Lytic Phage Capable of Infecting Methanotrophs

Two phages were isolated from tailings pond samples using the filtration system, as illustrated in Figures 4.4 – 4.6. All phage lysates were visualized using uranyl acetate staining under TEM. This methodology has known caveats including: (a) artifacts from positive and negative staining, (b) identification of short tailed phages can be limited as tails are hidden under positive staining in the head, and (c) beyond basic physiology, detailed information about the phage cannot be clearly discerned. However, due to simple experiment preparation, uranyl acetate staining can provide a good primary evaluation on the potential presence of a phage in an isolate (Ackermann, 2012).

The first tailed phage (Figure 4.4), likely of the *Caudovirale* order, was isolated using *M. trichosporium* OB3b as a target. Specifically, the phage could be characterized as a *Myoviridae* primarily in relation to its shorter helical tail, which showed clear detachment from the capsid by a short neck. This contrasts with the *Siphoviridae* order whereby capsid heads are directly attached to tails (Ackermann, 2006; "Myoviridae," 2012). One of the earliest characterized and hallmark examples of *Myoviridae* is phage T4, which was one of the original t-even phages isolated from environmental samples using *E.coli* as host(Abedon, 2000). However, despite being used quite ubiquitously in the field as a reference for *Myoviridae* phages, most information about the t-even set has been lost (Kutter et al., 1995). Since this discovery it is estimated that approximately 25% of all tailed phages fall into the *Myoviridae* classification (Ackermann, 2006).

The second isolated phage, shown in Figures 4.5-4.6, was harder to classify as it was often imaged with broken trails and a high amount of cell debris and non-specific uranyl acetate staining. As the phages are tailed, it can be assumed that they are of the *Caudovirale* order. However, the visual absence of a neck and the way the tails appear to connect directly to the capsid head lead us to conclude that these specimens are likely *Siphoviridae*. Lambda, T1, and T5 virions are the notable phages in this order. It is estimated that 61% of all phages can be classified as members of the *Siphoviridae* class (Ackermann, 2006; "Siphoviridae," 2012).

It needs to be emphasized that the present standard for classifying phages lies in genomic analysis of their DNA sequences (Adriaenssens & Brister, 2017). However, due to difficulty with

propagation of both phages, leading to low titers, sequences could not be obtained for sequencing. Therefore, more analysis is required before any assumptions about taxonomy can be made. The rationale behind why propagations were unsuccessful are discussed below.

4.5.3 Noted Experimental Limitations

Low titer and the impossibility to retrieve plaques from lawns were persistent problems complicating DNA recovery and in-depth genomic, morphological, and burst analyses of the isolated phages. Low phage counts could be a result of large amounts of cell lysis on the filter membrane itself, which would explain why the culture dynamics showed minimal recovery following experimental run through (Figures 4.4A-4.5A). If the phage infects too quickly and completely kills the predominant host, it can inadvertently prevent its own success as a virion (Erez et al., 2017) and progeny would be carried over with the filtrate, hence not contributing to the culture dynamics observed in the shake flasks after transfer of the filter and deposited host.

Another notable difficulty with this experiment was propagation of the phage following isolation. Often, after initial phage isolation, the phage showed low virulence when trying to infect subsequent cultures, meaning that in comparison to the control, no change in OD or no other symptoms of infection, such as 'clumping', were observed following exposure of the host cells to phage particles. TEM microscopy analysis showed that phage was still present in the cultures but not at high enough levels to result in significant population-wide lysis (data not shown). Several phenomena could explain these observations. For instance, if the isolated phage was not strictly virulent but actually temperate, the low culture density could be triggering the lysogenic life cycle (Casjens & Hendrix, 2015; Howard-Varona et al., 2017). If the phages were strictly virulent, the slow growth rate of methanotrophic bacteria themselves could be the limiting factor of viral propagation in culture (Erez et al., 2017). Other factors such as pseudo-lysogeny (Los & Wegrzyn, 2012) could also be involved.

4.6 Conclusions

In summary, we were successfully able to demonstrate a system capable of detecting low numbers of infectious phage in environmental samples. This was exhibited by comparison to the well characterized *E.coli*–phage T4 infection model, whereby we were able to theoretically show that as little as 10 phage per 250 mL of *E. coli* culture could have a statistical impact on culture growth and that 1 phage per 250 ml culture volume was still able to successfully lyse a 50 mL culture. With further refinement we believe that this system could be used to identify phages for bacteria that cannot be cultivated using traditional plate-based laboratory techniques, a noted bias in the field of phage research.

This system was put into practice using environmental tailings ponds samples where we successfully isolated two presumably lytic phages capable of infecting *M. trichosporium* OB3b and *M. album* BG8. In addition to these findings, a phage-related bacteriocin particles was induced in an experiment with *M. album* BG8. In theory, both phage and bacteriocin particles, once further classified, could have industrial implications. In particular, lytic phages and bacteriocins can be applied as a readily inducible, environmentally friendly, means of intracellular product recovery in methanotroph fermentations.

Nonetheless, these are the first reported isolated phages of methanotrophic bacteria since the early 1980's, and this study opens the door to the rapid isolation of new phages.

4.7 Acknowledgements

Special thanks to Arlene Oatway, for the support and guidance in imaging samples. As well as Dr. Tariq Siddique, for generously providing environmental samples. Additional thanks to Sara Almas and Liam Rieder who assisted in the collection of data for this chapter.

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5 Conclusions and Future Directions

The isolation and characterization of a total of five phages, three temperate and two virulent, capable of interacting with methanotrophic bacteria highlights the fact there is much to be elucidated about these organisms as they we scale up and commercialize methanotroph-based technologies. This section discusses the implications of these findings and future avenues of research.

5.1 Conclusion

Between the two approaches for phage isolation - induction of prophages using MitC and isolation of virulent phages using host cells deposited on filters - five different phages could be isolated and characterized under TEM. Two *Caudovirale* virulent phages were recovered from environmental tailings samples: a *Myoviridae* showing virulence against *M. trichosporium* OB3b, and a *Siphoviridae* capable of infecting *M. album* BG8. The remaining three phages were temperate and induced from the strains *M. marinus* A45, *M. trichosporium* OB3b, and *M. album* BG8. All three appeared to be *Caudovirales* of the *Siphoviridae* family. Physiological characterization and classification were made predominantly by TEM as low titer and difficulty propagating the phage made acquiring enough genetic material for sequencing a challenge.

Another major milestone of this project was the development and refinement of a protocol for the bioprospecting of novel phages from the environment. What is unique about this technique is that we know it can be applied to host bacteria that are typically unable to reliably grow on plates, making the majority of traditional phage techniques unsuitable. This is not only of interest to the fields of methanotrophs but has broader implications to the field of phage research as a whole.

Additionally, we were able to highlight the need to utilize more than one software when trying to identify putative prophage regions within host DNA. PHASTER although quite respected in the field comes with its own limitations. When comparing it to more recent software such as PHIGARO it becomes clear that no program on its own can yet detect with precision all prophage regions within a bacteria sequence. Therefore, our recommendation would be to get the most through picture of the extent of lysogeny within a host to cross reference multiple software.

Most importantly though, we demonstrated that phages have an impact on methanotroph systems, be it as virulent threats or as encoded prophage regions. We also showed that there is value in further analyzing the impact of phage systems on methanotrophic strains. This work has set the foundation for relevant analysis on how the fields of methanotrophy and phage biology relate, and what they could contribute to each other.

In terms of industry these results, provide a unique duality. For one they highlight the fact that phages be lysogenic or lytic could act as a threat to industrial scale batch fermentations of methanotrophs. By better understanding these processes we could take preventative action against them such as knocking out induction associated genes of putative prophages in industrial strains. Secondly, by fully understanding these systems we could theoretically "hijack" them, incorporating them into our system for the means of product recovery. This could be done by either inducing a prophage at the end of the production process, or by applying an isolated lytic phage at the end of fermentation to aide in cell lysis and product recovery. Better yet, we could monopolize on a methanotroph phage derived endolysin, using it to break open cells. This would prevent equipment contamination with phage, and the bacteria are less likely to develop resistance against an endolysin than a phage.

5.2 Future Directions

Several avenues of research could stem from the present work. The essential next step would be fully sequencing the genomes of the identified phages, annotate them and analyze them through BLAST an in order to confirm TEM assumptions about classification and functions. In regard to the chemically induced phages this would further allow us to characterize which prophage region was induced, helping validate the bioinformatic software used to identify these regions. Additionally, for each phage, several aspects of phage-host interactions need to be elucidated such as burst size, adsorption kinetics and host range. Until this fundamental information is established, industrial based methanotroph processes could run the risk of being subjected to both temperate or lytic phages.

Next, recovery rates of PHB, using an MitC-induced lysogen versus traditional chloroform extraction should be analyzed. If inducing a prophage appears to be as effective as traditional lysis techniques, then we could explore less expensive and more accessible means of inducing phages such as UV radiation, or hydrogen peroxide to aid in product recovery at the end of

fermentation. On this note, to achieve the same goal, further attempts should be made to propagate the environmentally isolated virulent phages. Should a high titer stock could be established, it could also be imposed as a regular means of product recovery.

Finally, it would be interesting to fully annotate and impact of the prophage regions on the host cells. Considering that several methanotrophs such as *M. trichosporium* OB3b, *Methylocystis sp.* Rockwell, and *M. marinus* A45 theoretically carry 1 or more prophages, it is possible that they confer some sort of evolutionary advantage to their hosts. Due to limitations in the field of both methanotroph and phage biology, most genes within these regions remain listed as hypothetical; elucidating their function could provide insights into methanotroph biology and some of their unique metabolic capabilities. On a related note, if these strains are industrialized, it may be worth exploring knocking out unessential prophage regions. This could help prevent the unintentional induction of a unwanted phages in a fermentation systems, as well as give us more control over which phages can be induced for product recovery.

Overall, much remains to be discovered at the interface of methanotroph and phage biology in terms of both classification and industrial applications. However, insight into this field has the potential to not only aid in the commercialization of methanotroph-based technology but also of other strains with similarly difficult traits.

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Appendix

Figure A.1 Bioinformatic analysis of PHASTER identified prophage region 2,819,754 - 2,872,113 bp in *M. capsultatus* Bath using the multiple sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative *Acidithiobacillus* phage AcaML1.

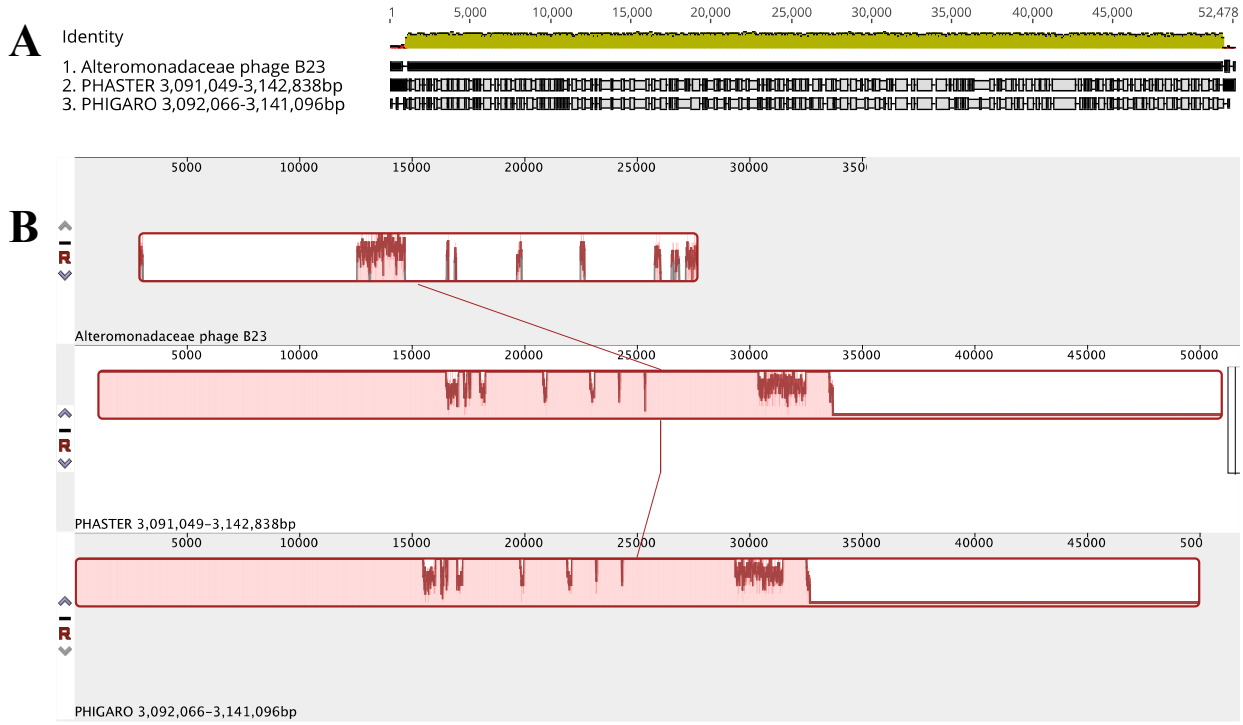

Figure A.2 Bioinformatic analysis of PHASTER identified prophage region 3,091,049 – 3,142,838 bp in *M. capsultatus* Bath using the multiple sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative *Alteromonadaceae* phage B23.

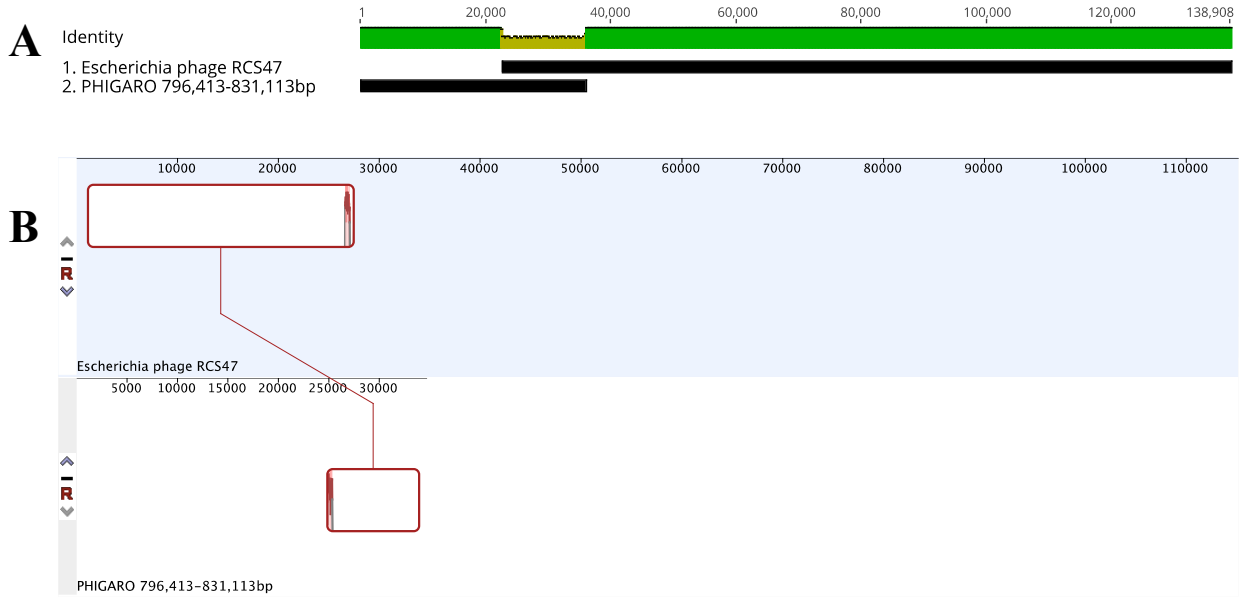

Figure A.3 Bioinformatic analysis of PHIGARO identified prophage region 796,413 – 831,113 bp in *M. denitrificans* FJG1 using the multiple sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative *Escherichia* phage RCS47

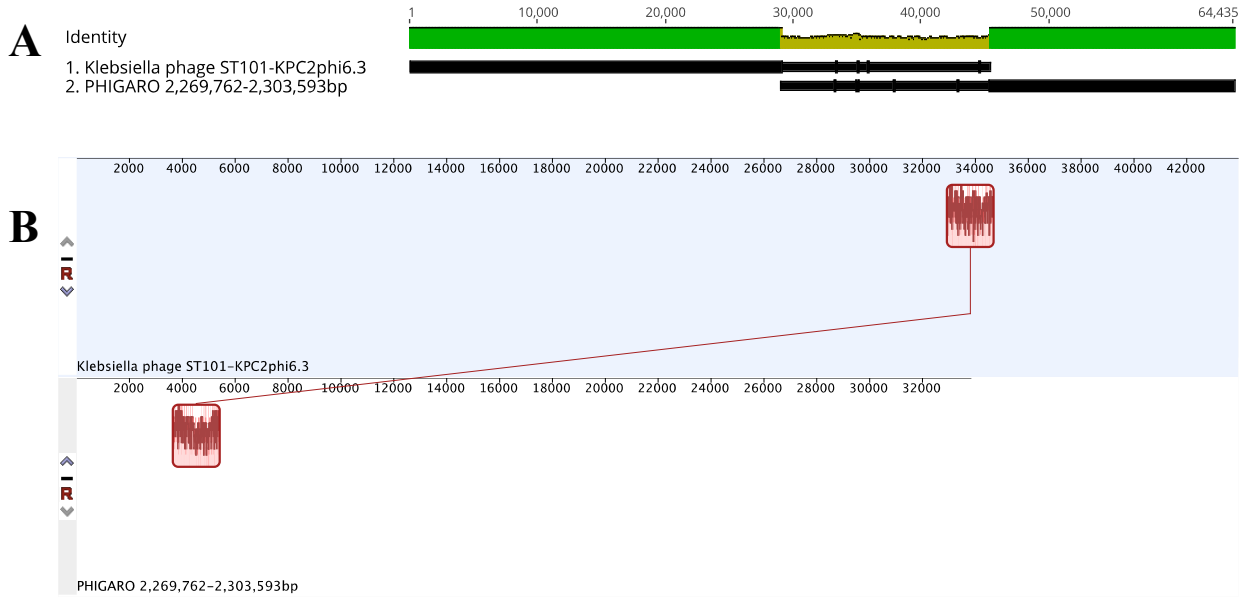

Figure A.4 Bioinformatic analysis of PHIGARO identified prophage region 2,269,762 – 2,303,593 bp in *M. denitrificans* FJG1 using the multiple sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative *Klebsiella* phage ST101-KPC2phi6.3

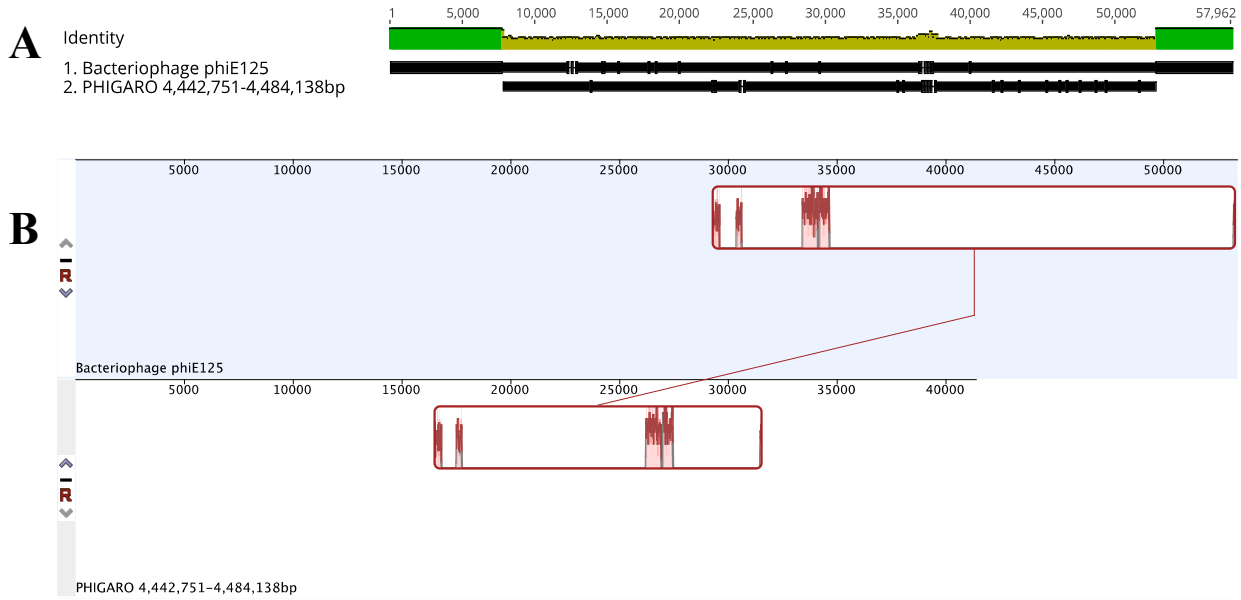

Figure A.5 Bioinformatic analysis of PHIGARO identified prophage region 4,442,751 – 4,484,138 bp in *M. denitrificans* FJG1 using the multiple sequence alignment tool (A) and MAUVE whole genome alignment plug-in (B) Geneious 11.1.5, against closest BLAST relative Bacteriophage phiE125

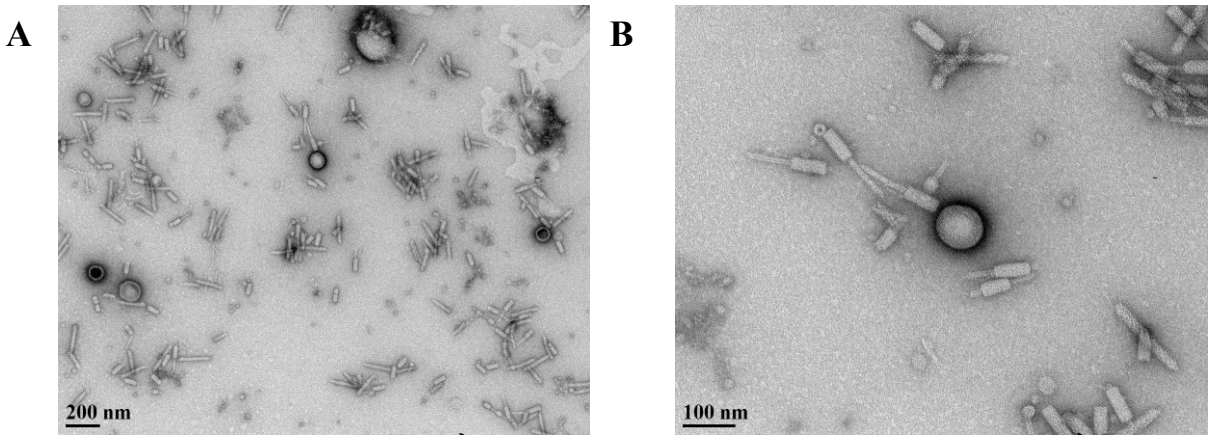

Figure A.6: Visualization of bacteriocins of *M. album* BG8 showing a phage with a broken tail at 10,000 × magnification (A). Further imaging of the bacteriocin of *M. album* BG8 taken at 100,000 × magnification (B)