Adaptation of *Methylomicrobium album* BG8 to growth at low pH

by

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Abstract

Methanotrophic bacteria are capable of converting single carbon sources such as methane or methanol, two common industrial waste products, into value-added compounds like bioplastics or biofuels. These microorganisms have significant potential for the mitigation of these low-value industrial by-products considered as waste in many industries, including energy and pulp and paper. The implementation of these bacteria has proven difficult outside of the laboratory as they grow slowly and demonstrate decreased production under industrial conditions. One potential solution to alleviate these issues is to apply adaptive evolution strategies to improve growth characteristics of the organism. In this study, the methanotrophic bacterium *Methylomicrobium album* BG8 was adapted to growth at low pH.

The growth of *M. album* BG8 was screened over a range of pH in both nitrate- and ammonium-based media. Since growth was not conducive at lower pH in ammonium-based medium, the study focused on adaptation in nitrate-based medium. Cells were adapted by sequential growth in media with decreasing pH, ranging from pH 6.86 (standard conditions) down to pH 3.80 when grown on methane and to pH 3.85 when grown on methanol. The growth of adapted cells was compared to that of unadapted cells at low and standard pH conditions. Adapted cells showed greatly improved performance at low pH and no alteration to performance at standard pH. Cell adaptation remained stable after passaging from low pH to standard pH and returned to low pH. Again, no loss or change in performance was observed upon passaging, suggesting the adaptation was

stable and at least not solely phenotypic. Transmission Electron Microscopy of cells showed minor differences in the cell shape and structure of M. *album* BG8 growing at low and standard pH in both adapted and unadapted cells. However, one significant difference was the loss of production of outer membrane vesicles (OMVs) in adapted cells. DNA analysis showed numerous mutations and Single nucleotide polymorphisms (SNPs) between adapted cells and the reference *M. album* BG8 genome of the parental strain. Adaptation in methane or methanol resulted in many of the same differences from the parental strain with a handful of different mutations for each carbon source. There were a number of mutations and SNPs located in genes related to membrane composition and functions, DNA/RNA synthesis and repair, and transposases – all of which can have a function in adapting cells to new environments. This work provides a template for adaptation of microorganisms to harsh environments and a starting point for adaption of *M. album* BG8 to growth in specific waste stream conditions, which could provide the potential to speed up industrial implementation of methanotrophs, decrease risks associated with moving from the lab to industry, and improve the performance of the industrial process.

Preface

As the primary author I was responsible for conceptualizing, designing, performing and analyzing experiments, as well as performing genomic analysis and writing the manuscript. Dr. Dominic Sauvageau and Dr. Lisa Stein were the supervisory authors who contributed in all stages from conceptualization, design, results analysis, advice and manuscript writing and editing. Arlene Oatway from the Biological Science Microscopy Unit performed TEM imaging of bacterial cells. Dr. Fabini Orata contributed to sending out samples for DNA sequencing, and genomic analysis. DNA sequencing was performed by the University of Washington PacBio Sequencing Services.

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1 Introduction

Methane is a potent greenhouse gas with 25 times the global warming potential of carbon dioxide over a 100 year period (Environment and Climate Change Canada, 2017). Methanol, on the other hand, is a toxic chemical that has adverse health effects on humans and animals, as well as negative effects in the environment (Ashurst & Nappe, 2019). Both of these substances are widely used and produced in different industries. For example, methane is produced by wastewater treatment and oil and gas plants, while methanol is a co-product from pulp and paper mills. Because of the low purity of these co-products, most industries must find a way to safely dispose of them, often resulting in significant costs.

Methanotrophs are a type of bacteria that have the ability to utilize single-carbon compounds, such as methane and methanol, as a sole source of carbon and energy. They can use this energy to produce different valuable products that can be used as bioplastics, biofuels, and other products. For example, methanotrophs have been used to produce the bio-polymer polyhydroxybutyrate (PHB), renewable diesel, ectoine, antaxanthin and more (Cantera *et al.*, 2017; Fei *et al.*, 2018; Khosravi-Darani *et al.*, 2013; Ye *et al.*, 2007).

Although some companies like Mango Materials and Calysta have industrial processes that already use them (Calysta, Inc., 2019; Mango Materials, 2019), the growth of methanotrophs outside the laboratory has generally proven difficult, as they grow slower and are not as robust as many other types of bacteria. Other challenges to their

industrial implementation involve mass transfer limitations of methane to reach the organism, mixing and hydrostatic pressure issues, and wall growth or flocculation amongst others (Gresham & Hong, 2015). This has made the scale up of methanotroph processes difficult. Another important problem is that industrial conditions are often far from ideal for growth of methanotrophs, with high temperature, acidity, and high concentrations of inhibitors being common. Low pH of potential feed streams is a common industrial condition often perceived as an obstacle to the implementation of bioprocesses.

There are different approaches to improving cell performance in specific environments, with the main ones relying on genetic engineering, directed evolution or adaptation. These methods each have their advantages and limitations. While genetic engineering can be used to directly confer specific traits to cells, a lot of genetic and functional information are required, which is not always available or possible. Directed evolution usually targets a specific gene or operon and requires a clear selection parameter to enable improvement of strains or function. On the other hand, adaptation can lead to beneficial traits through complex processes and mechanisms, and this with limited prior genetic knowledge. It is typically done through shock stress or through adaptive evolution (Elena & Lenski, 2003).

The objective of this study was to use adaptive evolution methods to improve growth of the methanotrophic strain *Methylomicrobium album* BG8 at low pH.

This goal was achieved through adaptation of *M. album* BG8 by passaging the cells into increasingly acidic conditions. Unadapted cells were first screened for growth at a range of pH to establish the starting conditions for adaptation. Cells were then adapted

by growing in sequentially lower pH until growth was negatively impacted and no further adaptation occurred. Adapted cells were then characterized to determine the effects of the adaptation process on growth performance, cell physical characteristics, stability of the adaptation, and genetics.

This work provides a framework for adaptive evolution in methanotrophs as a general method of improving growth, facilitating their implementation in industrial settings and improving their productivity.

2 Literature Review

2.1 Methanotrophs

2.1.1 Background

Methanotrophs, or methane-oxidizing bacteria, are bacteria capable of oxidizing single carbon compounds as an exclusive source of carbon and energy. While the majority of methanotrophs are Gram-negative Alpha- or Gamma-proteobacteria, there are other types of methanotrophs such as Verrucomicrobial or anaerobic methanotrophs, and some methanotrophs that have been reported as Gram-positive although never re-isolated (Dijkhuizen *et al.*, 1992; Hanson & Hanson, 1996). Typical proteobacterial aerobic methanotrophs grow abundantly in the environment, especially in places rich in oxygen and methane, although they can also grow under extreme hypoxia by reducing nitrogen oxides (Kits *et al.* 2015) or by fermenting single carbon substrates (Kalyuzhnaya *et al.* 2013). They can be found in rice paddies, forest soils, freshwater lakes, and many more places (Murrell, 2010). A large interest for these bacteria comes from their ability to consume methane, a potent greenhouse gas, as a source of energy for cell growth.

These bacteria use the enzyme methane monooxygenase (MMO) to catalyze the conversion of methane into methanol. There are two forms of MMO, particulate and soluble methane monooxygenase (pMMO and sMMO, respectively). pMMO is located within the intracellular membranes of methanotrophs, while sMMO is free within the cytoplasm (Murrell, 2010). pMMO contains copper and is found in the majority of methanotrophic bacteria, while sMMO has a dinuclear iron center and is less common. Moreover, sMMO is more thoroughly characterized due to easier purification compared to the particulate form (Murrell & Smith, 2010).

Following the initial oxidation of methane, methanol is oxidized by methanol dehydrogenases to formaldehyde, which can undergo a number of different pathways. It can be further oxidized to formic acid and finally carbon dioxide, or sent to the Ribulose monophosphate (RuMP) or Serine pathways for carbon assimilation and biomass synthesis (Hanson & Hanson, 1996). The carbon assimilation pathway used is dependent on the type of methanotroph. Type I methanotrophs, or Gammaproteobacteria, contain the RuMP pathway while Type II methanotrophs, or Alphaproteobacteria, contain the serine pathway. Other methanotrophs, specifically Verrucomicrobia, can also fix CO_2 via the Calvin-Benson-Bassham cycle (Smith *et al.*, 2010).

2.1.2 Morphology

The morphology of methanotrophs is dependent on the type of bacteria, as well as the specific strain. Organisms vary in size and dimension, and can be short or curved rods, vibrioid, cocci, or pear-shaped; they can be seen as single cells or grouped in pairs (Davies & Whittenbury, 1970; Hanson & Hanson, 1996; Whittenbury *et al.*, 1970). Cells can also form resting stage cysts or exospores (Hanson & Hanson, 1996). Gammaproteobacteria contain intracellular membranes that form vesicles shaped like discs organized throughout the entire cell. Alphaproteobacteria contain paired membranes that run along the edges of the bacterium and sometimes scatter throughout the middle of the cell (Davies & Whittenbury, 1970; Hanson & Hanson, 1996).

2.1.3 Products

One of the draws of methanotrophs in industrial biotechnology, aside from the fact that they can use methane as a substrate, is their ability to synthesize a number of valuable products such as bioremediation agents, single cell proteins, biofuels, health

supplements and other products (Strong *et al.*, 2015; Zhao *et al.*, 2015). One of the most interesting features of methanotrophs is that the Serine pathway in Alphaproteobacteria can lead to the polyhydroxybutyrate (PHB) cycle. PHB, the product of this cycle, is a bio-polymer that can be used in many products (Khosravi-Darani *et al.*, 2013)

2.1.4 Growth Conditions

Methanotrophs can be obligate, in that they only grow on methane, or facultative, in that they can grow on other multi-carbon compounds (Murrell, 2010). Methylotrophs, on the other hand, are capable of growing on other singe-carbon compounds, like methanol or methylamine (Hanson & Hanson, 1996). Growth of methanotrophs on methane is most common as it is the primary carbon source; growth on multi-carbon compounds often leads to heterotrophic bacteria outcompeting the methanotrophs and contaminating cultures (Dedysh & Dunfield, 2011). One drawback to methane as a carbon source is mass transfer limitations between the gas and liquid phase in the reaction. This can lead to lower cell density and slower growth rate (Tays *et al.*, 2018).

Growth of methanotrophs on methanol is also possible due to the enzyme methanol dehydrogenase (MDH) located in the periplasm of the cell (Smith *et al.*, 2010). There are two main types of MDH in methanotrophs – the calcium-requiring MxaF and the lanthanum-requiring XoxF. Growth on methanol reduces demands for energy input and oxygen consumption, as well as mass transfer limitations in liquid culture. However, the toxicity of methanol on methanotroph cells can negatively affect growth (Hanson & Hanson, 1996; Van Dijken & Harder, 1975). Batch growth of methanotrophs on methanol is difficult due to this toxicity, since higher amounts of carbon source are required to reach a high cell density. Cells can be adapted from growth on methane to higher concentrations

of methanol after a few passages, resulting in increased growth yield after a short lag phase (Best & Higgins, 1981; Whittenbury *et al.*, 1970). Methanotrophic cultures grown on methane typically reach a higher maximum optical density (OD) than those grown on methanol, and the lag phases and growth yields are also more consistent when grown on methane. However, a recent study showed that, at least in some Gammaproteobacteria, growth on methanol led to shorter lag phases compared to Alphaproteobacteria (Tays *et al.*, 2018). In the same study, methanol toxicity was not observed when *Methylomicrobium album* BG8, a Gammaproteobacterium, was grown at a 10 mM concentration of methanol; in fact, it grew faster and to higher OD than other methanotrophs on both methanol and methane. Toxic effects of methanol on the bacterium became apparent when the methanol concentration was increased to 20 mM (Tays *et al.*, 2018).

Methanotrophs also rely on using ammonium or nitrate for nitrogen assimilation. Ammonium has been shown to compete with methane for the binding sites on pMMO, sometimes leading to less efficient cell growth (Nyerges & Stein, 2009). In addition, oxidation of ammonia can also lead to toxic byproducts such as hydroxylamine and nitrite, which can further inhibit methane oxidation with varying degrees of severity, depending on each methanotroph (Nyerges & Stein, 2009).

Some Alphaproteobacteria reach higher maximum OD when grown on methane and using ammonium as nitrogen source, while Gammaproteobacteria showed minimal difference in maximum OD when either ammonium or nitrate were used as nitrogen source (Tays *et al.*, 2018).

Finally, it should be noted that many Alphaproteobacteria and some Gammaproteobacteria have the ability to fix dinitrogen (Trotsenko & Murrell, 2008).

2.1.5 Methylomicrobium album BG8

Methylomicrobium album BG8 – formerly called *Methylobacter albus, Methylomonas albus*, or *Methylomonas alba* – is a methylotrophic Type I Gammaproteobacterium originally isolated from mud, water and soil samples from around the world (Whittenbury *et al.*, 1970). *M. album* BG8 is neutrophilic, mesophilic and only contains pMMO; it thus responds to copper stimulation with increased MMO activity, cell growth density, and intracellular membrane abundance (Brantner *et al.*, 1997; Whittenbury *et al.*, 1970). It also has a completed, well-annotated genome sequence available (Kits *et al.* 2013).

M. album BG8 is one of the more promising strains of methanotrophs for industrial application because of its shorter lag phase, faster growth rate, higher growth yield and its ability to adapt and grow consistently across different conditions compared to most other methanotrophs (Tays *et al.*, 2018). *M. album* BG8 outperforms other methanotrophic strains in terms of growth yield and growth rate under most conditions but especially when grown on methanol, with no significant preference for either ammonium or nitrate as a nitrogen source (Tays *et al.*, 2018). It also performs better than other methanotrophic strains when grown on methane or methanol in an ammonium-based medium (Tays *et al.*, 2018). This is due to the fact that *M. album* BG8 has enzymes that allow for the reduction of nitrite and nitric oxide formed in ammonium-based media, preventing the toxic effects of nitrite on the cell (Kits *et al.*, 2015; Stein & Klotz, 2011). However, levels of ammonia that are too high can still lead to greater nitrite formation and

toxic effects on the cells, reducing viability (Nyerges *et al.*, 2010). *M. album* BG8 has been shown to grow slightly better when nitrate instead of ammonium is used as nitrogen source, as it was resistant to inhibition of growth in high nitrate concentrations (Nyerges *et al.*, 2010). In a continuous flow reactor operated under methane-limiting conditions, *M. album* BG8 was shown to outperform the Alphaproteobacterium *Methylosinus trichosporium* OB3b (Graham *et al.*, 1993).

2.2 Industrial Application

2.2.1 Application of Microorganisms

There is a lot of interest in using microorganisms, including methanotrophs, for use in industrial practices. In fact, industrial application of microorganisms for bioconversion/bioproduction schemes has been attempted in a number of species with varying degrees of success. Yeast is a very common microbe used in industry for a very long time, making food and beverage products, that has now been implemented into newer industrial applications, such as production of biofuels, with great success (Steensels *et al.*, 2014). *Escherichia coli* has also been used in a number of processes, enabled by its ability to be genetically engineered and to produce many different products (Theisen & Liao, 2016). Many other microbes have been implemented to produce compounds like biofuels (Kumar & Kumar, 2017), and products like enzymes for food application (Hellmuth & van den Brink, 2013) and Single Cell Protein (Ritala *et al.*, 2017).

Industrial processes like syngas fermentation have used different types of bacteria as microbial catalysts. In fact, many bacteria – *Clostridium aceticum, Acetobacterium*

woodii, Clostridium carboxidivorans and *Clostridium ljungdahlii* (Younesi *et al.*, 2005) – are capable of this fermentation, and, depending on the species, the process can be performed at a large range of temperatures, pH, and other properties (Munasinghe & Khanal, 2010).

Over the years, there have been a number of attempts to use methanotrophic bacteria to convert methane into products that can be sold commercially. One of the first attempts at this involved the production of Single-Cell Protein (SCP), which can be used as a valuable protein source in animal food products (Øverland *et al.*, 2010; Strong *et al.*, 2015). These efforts have continued over the years and are now the focus of commercialization by companies such as Calysta. Production of PHB has been another large area of focus, with companies like Mango Materials creating PHB from waste methane at an economically viable cost (Mango Materials, 2019). Other high-value products of interest from methanotrophs include ectoine, astaxanthin, and biofuels, which have all been implemented or investigated (Cantera at al., 2017; Fei *et al.*, 2018; Ye *et al.*, 2007).

2.2.2 Challenges and Limitations

Despite these successes and the ongoing interest in using microbes for bioconversion and bioproduction, many difficulties remain with scale up of laboratory experiments to commercial or even pilot plant scale, and with the implementation of processes under non-ideal conditions such as high temperature, low pH, higher concentrations of inhibitors, etc. (Crater & Lievense, 2018). While many microorganisms grow well when cultivated in controlled laboratory conditions, with the right amount of

nutrients and growth-supporting factors, when conditions change, even slightly, most show sub-optimal growth and low robustness.

Many industrial processes have to deal with gas to liquid mass transfer, which limits their efficiency. Unfortunately, typical solutions to this problem – increasing impeller speed, reducing bubble sizes, etc. – can cause other problems with the microbes themselves (Munasinghe & Khanal, 2010). Scale-up of laboratory practices can also lead to wall growth of bacteria or flocculation, which often reduce conversion efficiency (Gresham & Hong, 2015).

In methanotrophs, studies have shown a number of difficulties in predicting strains that will perform best in the laboratory as well as in more natural environments. Analysis of species suggests that *Methylobacter* should outperform *Methylosinus* and *Methylomonas* in laboratory settings (Beck *et al.*, 2013), but in practice *Methylosinus* and *Methylomonas* showed the best overall growth in the lab (Auman *et al.*, 2000). These difficulties also occur when methanotrophs are grown under slightly different nutrient conditions, as *Methylosinus* and *Methylomonas* were outcompeted by *Methylobacter* and *Methylosarcina* in experiments using low and high partial pressure of oxygen, respectively (Hernandez *et al.*, 2015; Oshkin *et al.*, 2015). The performance of methanotrophs when grown in pure cultures is also not a good predictor of growth in natural environments or in lab-made co-cultures, even with just two to three species involved (Yu *et al.*, 2016). Even when strains are grown under optimal conditions, methanotroph conversion of methane or methanol into products is still below the efficiency levels required for industrial practice (D. Park & Lee, 2013).

2.3 Effect of pH on Bacterial Growth

Each bacterium has a preferred range of pH, temperature, nutrient levels, etc. for growth. Most lab-culturable bacteria are mesophilic and neutrophilic, many growing ideally at temperatures in the vicinity of 30°C and pH 7. However, many others grow well in adverse conditions, such as very low concentrations of nutrients, high or low temperature, and low or high pH. Methanotrophs themselves encompass a range of strains that can be neutrophilic, acid-tolerant, or acidophilic (Murrell, 2010). Other neutrophilic bacteria like *E. coli* have the ability to grow moderately at low pH thanks to acid tolerance mechanisms. This being said, in general, low pH leads to reduced growth, metabolic strains, damages to the cell wall and even cell death (Lund *et al.*, 2014).

2.3.1 Bacteria and pH Tolerance

E. coli is one of the most extensively studied bacteria when it comes to growth at different pH. Despite being generally neutrophilic, a number of different *E. coli* strains can grow at lower pH, especially when treated with acid shock or through adaptation (Conner & Kotrola, 1995; Lin *et al.*, 1995; Presser *et al.*, 1998). For example, *E. coli* O157:H7 has been shown to survive when grown at pH 4 or above, depending on the acid treatment and temperature (Conner & Kotrola, 1995). *E. coli* M23 could grow to pH as low as pH 3.9 at 37°C, pH 3.6 when grown at 30°C, and between pH 3.8 and pH 4 at 10-25°C (Presser *et al.*, 1998). Another study showed that although *E. coli* did not grow below pH 4.4, it could survive at pH 2-2.5 (Lin *et al.*, 1995). The same study examined growth of *Salmonella typhimurium,* which grew at pH 4.0 and survived at pH 3.0, and of *Shigella*

flexneri which could not grow below pH 4.8 but survived between pH 2-2.5 (Lin *et al.*, 1995).

2.3.2 Neutrophilic Methanotrophs

Most strains of methanotrophs are neutrophilic and do not grow beyond a small range of pH (Murrell, 2010). In a previous study, *M. trichosporium* OB3b grew between pH 6-8.5, with optimal growth at pH 6.0-7.0; its growth rate decreased and lag time increased with pH increasing beyond these values due to decreasing MMO activity (Shah *et al.*, 1996). 100 different strains of methanotrophs of the groups *Methylosinus*, *Methylocystis*, *Methylomonas*, *Methylobacter*, and *Methylococcus* were classified and found to grow between pH 5.8 and 7.4 with their optimum growth rate and yield occurring at pH 6.6-6.8 (Whittenbury *et al.*, 1970). Included amongst these strains is *M. album* BG8, which can grow between pH 6.0 and 9.0, with best growth encountered near pH 7.0 (Bowman, 2006; Whittenbury *et al.*, 1970). In laboratory practice, *M. album* BG8 has been almost exclusively grown at pH 6.8 when used in a number of experiments (Han & Semrau, 2000; Kits *et al.*, 2015; Tays *et al.*, 2018). Many other groups of methanotrophs have been characterized as intolerant to extreme environments, such as the strains C*renothrix, Clonothrix, Methylosarcina, and Methylosoma* (Smith *et al.*, 2010). Other strains were labelled as tolerant to higher temperatures but were also neutrophilic (*Methylococcus, Methylocaldum, Methylothermus*), and finally a select few strains have been found to be either tolerant to acid stress (some *Methylocystis, Methylocella,* Methylocapsa) or thermoacidophilic (*Methylokorus, Acidimethylosilex, Methyloacida*) (Smith *et al.*, 2010).

2.3.3 Acid-Tolerant Methanotrophs

Some methanotrophs isolated from environments which have undesirable conditions like acidic wetlands, mining pits, forest soils, etc. have the capacity to tolerate mildly acidic or basic conditions (Conrad, 2009; Hanson & Hanson, 1996; Op den Camp *et al.*, 2009). Peat bogs, in which the pH is often below 4.5 – too low for most methanotrophs to grow in, are one particular environment from which samples had methane-oxidizing activity down to pH 3.3 (Kip *et al.*, 2011). It was determined that the surviving strain at this pH was *Methylocystis sp.* H2s, which was the dominating methanotroph strain despite its optimum pH being between 6 and 6.5 (Belova *et al.*, 2011; Kip *et al.*, 2011). Other Alphaproteobacteria, such as *Methylocella palustris* and *Methylocapsa acidophila*, display optimal growth between pH 5-5.5, and can grow in conditions as low as pH 4.2 (Trotsenko & Khmelenina, 2002), while some strains like *Methylovulum miyakonense* 83A5, with optimal growth at pH 6.5-7.0, cannot grow below pH 5.5 (Danilova & Dedysh, 2014). For a number of years, the only methanotrophs showing tolerance to low pH were Alphaproteobacteria, but recently *Methylomonas paludis* SH10 was the first Gammaproteobacterium found to be acid-tolerant. It was able to grow at pH 4.2-4.5, with optimal growth at pH 5.5-6.0 (Danilova *et al.*, 2013). Overall, most acid-tolerant methanotrophic species can grow between pH 4.2 and 5.0, but still grow better when grown at pH closer to 7.

2.3.4 Acidophilic Methanotrophs

Some methanotrophs can survive and even thrive in extreme environmental conditions, like high temperature and low pH. These strains, of which there are only a few, were found to be affiliated with the Verrucomicrobia phylum and were isolated from

geothermal environments (Op den Camp *et al.*, 2009; van Teeseling *et al.*, 2014). The first isolated strains were both thermophilic and acidophilic, can grow anywhere between pH 0.8 to 6.0 and grow optimally at pH 2-3.5 while also preferring temperatures between 55 to 60ºC (Op den Camp *et al.*, 2009). The next discovered group were more mesophilic, preferring growth between 35 to 45ºC. However, each of these strains were able to grow in pH as low as 0.5-0.6, with an optimum growth rate at pH 1-3 and only grew in pH as high as 5-6 (van Teeseling *et al.*, 2014). One of the downsides to using Verrucomicrobia in the laboratory or industrially is that they grow much slower than other strains of methanotrophs and to lower maximum cell densities (Op den Camp *et al.*, 2009; van Teeseling *et al.*, 2014).

2.4 Acid Tolerance Mechanisms in Bacteria

2.4.1 Effects of Acid Stress

Neutrophilic bacteria often have to deal with harsh conditions, even if only for short amounts of time, and have developed capabilities to protect themselves against such stresses. One such stress is acidic conditions, which can be experienced in nature, within the human body, or in industrial settings. The effects of acidification on cells depend not only on the cells themselves but also on the type of acid used. Strong acids will dissociate and hydrogen ions make their way into the cell through protein channels, weakened cell membrane areas, or transient water chains (Deamer, 1987; Foster, 2004). Weak acids can more freely pass through the inner membrane because they are often less dissociated and uncharged. They can then alter the pH gradient or bring external protons

back into the cell without using channels to expel protons, leading to more complicated acidification effects (Lund *et al.*, 2014).

Most bacteria are able to keep their internal pH relatively steady and near neutral over a large range of external pH in different media (Slonczewski *et al.*, 2009). When pH becomes too low for cells that have not been adapted, cell growth stagnates and eventually cell survival is no longer viable (Lund *et al.*, 2014). In Gram-negative bacteria, the porins in the outer membrane are large enough to allow protons to pass freely into the periplasm, meaning that shortly after external pH drops, the periplasm reaches the same or close to the same pH and remains there (Wilks & Slonczewski, 2007). The inner membrane provides a much stronger roadblock to proton movement, requiring larger changes in pH and stronger acids to cause a change in internal pH (Deamer, 1987; Foster, 2004; Gutknecht & Walter, 1981). When a strong acid causes a moderate change in external pH, *E. coli* has been shown to recover relatively quickly after an initial drop in internal pH (Wilks & Slonczewski, 2007). The rapid response to moderate pH changes in *E. coli* is likely due to buffering within the cell or changes in ion concentration gradients, as transcriptional responses take much longer (Lund *et al.*, 2014). As an additional response, some species have periplasmic or cytoplasmic chaperones that help prevent or repair protein degradation due to intracellular acid stress (Arnold *et al.*, 2001; Maurer *et al.*, 2005; Tucker *et al.*, 2002).

Acidification of the cell can lead to decreased enzyme activity, which can, in turn, affect many important cellular functions such as ATP production and metabolic pathways (Lund *et al.*, 2014). The optimal pH of enzymes that are used to respond to reductions in pH in the cytoplasm is lower than typical values, allowing them to work at full capacity

even under acidic conditions (Gale, 1946; Lund *et al.*, 2014). Decreased pH can also lead to protein unfolding within the cytoplasm and damage to cellular membranes through protein degradation. Moreover, DNA can also be damaged when exposed to low pH, with more damage occurring the longer the cells are left at low pH (Jeong *et al.*, 2008). When pH falls too low to be corrected via non-transcriptional methods, acid response mechanisms kick in to adjust the internal pH (Lund *et al.*, 2014).

2.4.2 Acid Response Mechanisms in Bacteria

When it comes to transcriptional regulation of internal pH, bacteria cope with different levels of acid stress though different mechanisms. The two main protective mechanisms are the acid tolerance response (ATR) and amino acid-dependent extreme acid resistance (XAR) (Bearson *et al.*, 1997; Lin *et al.*, 1995). ATR comes into effect when the level of acid stress is not likely to kill the cell. It improves the cell's ability to cope with extreme drops in pH, as low as pH 3 (Lund *et al.*, 2014). There are a number of different bacterial ATR responses and most bacteria possess at least one type of ATR mechanism (Bearson *et al.*, 1997; Lin *et al.*, 1995). When pH is below 2.5, XAR, present in some microorganisms, allows cells to survive even when they can no longer support growth (Foster, 2001). Typically, ATR mechanisms respond to pH change by maintaining pH homeostasis within the cells, and XAR mechanisms prevent the internal pH from falling to levels low enough to lead to cell death (Foster, 2001; Lund *et al.*, 2014).

The different strategies to combat acid stress include activating proton pumps to prevent proton build up, enabling reactions that consume protons or create ammonia, preventing or repairing the damage to the cell, and modifying the cell membrane (Lund *et al.*, 2014).

Some bacteria use a F_1F_0-ATPase located in the cell membrane which can prevent acidification through pumping out protons by consuming ATP, or by using the energy released from protons entering the cells to form ATP, which can provide energy for protection or reparation systems within the cell (Kobayashi *et al.*, 1986; Lund *et al.*, 2014).

Amino acid decarboxylases can consume protons in a decarboxylation reaction of the amino acids arginine, glutamate, lysine, and orthinine, all of which have optimal pH between 4 and 6 (Gale, 1946; Lund *et al.*, 2014). The decarboxylases also have an antiporter within the cell membrane that is activated when extracellular pH becomes too low. This allows desirable amino acids into the cell while exporting the products of the decarboxylation reaction (Lund *et al.*, 2014). These mechanisms are found in the Acid Response System's 2 and 3 (AR2/AR3) in *E. coli*; AR2 is the Glutamate-Dependent Acid Resistance system (GDAR) and AR3 is the Arginine-Dependent Acid Resistance system (ADAR) (Richard & Foster, 2004). The AR2 system contains the *gadB* gene encoding the glutamate decarboxylase which consumes a proton per reaction with glutamate, as well as *gadC* encoding the glutamate/γ-aminobutyrate antiporter (Hersh *et al.*, 1996; Richard & Foster, 2004). AR3 is similar to AR2, but uses the acid-inducible arginine decarboxylase AdiA, the arginine/agmatine antiporter AdiC and requires extracellular arginine (Richard & Foster, 2004). In *E. coli*, the GDAR system is the most commonly utilized system under normal acid stresses, as it outperforms ADAR and other amino acid-dependent systems (Diez-Gonzalez & Karaibrahimoglu, 2004).

Some other systems, such as the arginine deiminase or glutaminase and adenosine deaminases, lead to formation of ammonia, which can form ammonium ions with the additional cellular protons and increase intracellular pH (Martinelle & Häggström,

1997). Arginine deiminase, specifically, is activated at pH levels below 3.1 and converts arginine into orthinine, ammonia and carbon dioxide, and forms ATP which can then be used with other systems such as the F_1F_0-ATPase (Casiano-Colón & Marquis, 1988; Cunin *et al.*, 1986; Lund *et al.*, 2014).

As pH decreases, some bacteria will modify their internal cell membrane by converting unsaturated fatty acids (UFA) into cyclopropane fatty acids or by changing short chain saturated fatty acids into long chain UFAs in order to decrease proton permeability (Chang & Cronan, 1999; Fozo *et al.*, 2004; Kim *et al.*, 2005). Cyclopropane fatty acids in *E. coli* membranes have been shown to prevent proton movement into the cytoplasm, and *E. coli* will change its fatty acid content in order to adapt to the stress of an acidic environment (Brown *et al.*, 1997; Shabala & Ross, 2008).

Many species have the ability to utilize all or most of the known ATR and XAR mechanisms, while others possess only a select few tools relevant to the stress they typically experience (Lund *et al.*, 2014).

2.4.3 Acid Tolerance in Methanotrophs

To date, the acid response mechanisms of methanotrophs have not been extensively investigated. Some have been shown to alter their membrane composition to cope with acid stress: *Methylocella palustris* has a different fatty acid composition than most Alphaproteobacteria, and *Methylocapsa acidophila* has its intracellular membranes packed only to one side of the cell, differing from typical methanotrophs (Trotsenko & Khmelenina, 2002). It has also been shown that other acidophilic methanotrophs have increased amounts of 16:1 and 16:0 fatty acids than what is normally found in

methanotrophs (Dedysh *et al.*, 2000). This counters the response seen in other bacteria that have been found to shift away from short-chain fatty acids to more long-chained mono-unsaturated fatty acids with decreasing pH (Fozo & Quivey Jr., 2004). The Verrucomicrobial strain *Methylacidiphilum infernorum* V4 has been found to have a number of encoded genes that can help with acid tolerance, including glutamate and arginine decarboxylases, as well as glutamate/γ-aminobutyrate and arginine/agmatine antiporters (Hou *et al.*, 2008), similar to those described in the acid response mechanisms. This strain also possesses an agmatine deaminase that can hydrolyze agmatine and release ammonia, which can in turn combine with excess hydrogen ions and help increase pH (Hou *et al.*, 2008; Lund *et al.*, 2014).

2.5 Adaptation of Bacteria

2.5.1 Mechanisms of Adaptation

Adaptations typically occur through selection of genotypes that best prepare the cells to change their properties and allow them to better handle the conditions and stress of a new environment (Brooks *et al.*, 2011). Bacteria can also gain these advantages through phenotypic variations that help with the survival of cells under stressful conditions, and these phenotypes can be passed to further generations through epigenetic inheritance (Veening *et al.*, 2008).

One major cause of phenotypic and genotypic variation is mutations of the organism that allow it to better survive and thrive in its current growth conditions. Mutations in the genetic code often occur through single nucleotide polymorphisms

(SNPs – a mutation that alters the base in a single nucleotide position in the DNA sequence), small or large insertions or deletions, genomic duplications and transposable element insertions (Conrad *et al.*, 2011). These mutations can affect the phenotypic expression of the cell, but can also occur in non-coding regions or produce changes that do not affect the amino acid sequence of encoded proteins (Barrick *et al.*, 2009; Charusanti *et al.*, 2010; Conrad *et al.*, 2009; Herring *et al.*, 2006; Kishimoto *et al.*, 2010; Lee & Palsson, 2010).

Adaptation in microorganisms is typically due to small number of large adaptations (Elena & Lenski, 2003). Some populations of microorganisms have mutators, which result in an increased rate of mutation, much like selective pressure from being grown in a stressful environment (Barrick *et al.*, 2009; Gresham *et al.*, 2008; Kishimoto *et al.*, 2010). This higher mutation frequency isn't necessarily desirable since most mutations cause changes that are harmful to the organism, but there is also an increased chance of larger beneficial mutations that offset the loss of negative mutations (Taddei *et al.*, 1997). Adaptation frequency under a stressor begins with an initial rapid increase but eventually decreases as less beneficial mutations with smaller effects occur (Buckling, Craig Maclean, Brockhurst, & Colegrave, 2009). However, mutations still continue indefinitely even as their rate slows down because beneficial mutations, even with marginal effects, will continuously accumulate (Elena & Lenski, 2003).

Adaptation can be evaluated by testing fitness of the adapted versus the ancestral strain. This can be done in the conditions used for adaptation, the ancestral conditions or another set of conditions, and can be done by having the strains compete for resources

within the same experiment, or performing the experiments separately and comparing, for example, growth rate and other fitness characteristics (Elena & Lenski, 2003).

Adaptation of species greatly depends on the environment, if they are grown in one specific environment some will become specialists in this situation while performing poorly in other conditions. Adapted populations can also grow to be relatively good in a variety of environments, but not as good as a specialist in its preferred environment (Elena & Lenski, 2003). These differences come from different mechanisms of adaptation. Antagonistic pleiotropy (AP) produces mutations that improve performance in one environment while harming performance in a different environment, while mutation accumulation (MA) involves mutations to genes that are neutral in one environment, but that have a negative impact in another (Elena & Lenski, 2003). Independent adaptation on the other hand provides improvements in one environment, but no change in the other, which can be beneficial if the cells have to grow in both conditions (Elena & Lenski, 2003). This has been shown in *E. coli* adapted to better grow at different temperatures without affecting their fitness at previously normal temperatures (Bennett & Lenski, 1993). This was true for all but a few replicates, demonstrating that adaptations can vary between replicates of the same experiment.

2.5.2 Adaptive Evolution

Adaptation of cells to given environments or conditions can be carried out in a number of ways, including through shock stress and directed or adaptive evolution. Adaptive evolution involves cultivating organisms for many generations under conditions creating a specific selective pressure favoring a given trait. Microorganisms are amenable to this strategy because of their large population sizes and fast growth cycles allowing for

adaptation to occur more quickly; they also can be preserved easily and often have well characterized genomes enabling comparative analysis (Buckling *et al.*, 2009). Adaptive evolution can be used in many ways, such as monitoring how a species adapts to a given selective pressure over time and comparing this to other conditions or environments, or to adapt a species to specific conditions for potential applications in industrial contexts (Elena & Lenski, 2003).

Adapted evolution can also help us establish if evolutionary changes are the same when an ancestral host undergoes the same evolutionary selection in separate instances – essentially if the evolutionary changes can result from a single or various evolutionary paths (Elena & Lenski, 2003). Evolution experiments typically start with the ancestral strain of a population, which then undergo selective pressure, which will direct favourable adaptation to the stress. Samples can be saved for later analysis at different time points along the way for comparison with the ancestral strain or for the establishment of the evolutionary pathway. These comparisons can be genetic and/or phenotypic.

Such strategies have been used in many instances such as the Long Term Evolution Experiment in *E.coli* which has observed the dynamics of adaptation and the differences in evolving populations from their ancestor (Lenski, 2017). Other adaptive evolution experiments have been performed on *E.coli*, *Saccharomyces cerevisiae* and other bacteria to improve growth on non-optimal substrates, or improve tolerance to different inhibitors (Almario et al, 2013; Minty *et al.*, 2011). A few adaptive evolution experiments have been done on methanotrophic bacteria as well, adapting strains to different carbon sources and then evaluating the differences between populations, and

looking at mutations increasing or decreasing fitness (Agashe *et al.*, 2016; Chou *et al.*, 2011; Lee *et al.*, 2009).

2.5.3 Methods for Adaptive Evolution

Organisms can be grown and adapted in batch or continuous cultures (Arensdorf *et al.*, 2002; Elena & Lenski, 2003; Park *et al.*, 1991; Shah *et al.*, 1992). Two common methods include the use of chemostat and sequential growth. Chemostats function through a continuous addition of medium and removal of liquid culture at the same rate while matching the growth rate to the dilution rate in order to maintain reaction volume and not wash out cells or prevent growth (Novick & Szilard, 1950). The medium contains the nutrients which limit the rate of growth and achieves a steady state that can be controlled in order to evaluate growth and evolution of the organisms (Gresham & Hong, 2015). The chemostat provides a fairly simple view of the selection for adaptation because all cells are experiencing the same conditions at all times. However, chemostat operation can complicate cell adaptation when wall growth or flocculation, which lead to some cells experiencing different growth conditions, occurs (Gresham & Hong, 2015). Adaptation to a specific condition in chemostats can also lead to a decreased ability to react to other stressors. For example, *E. coli* adapted to glucose limitation grew poorly when presented with temperature or oxygen stresses (Gresham & Hong, 2015; Notley-McRobb *et al.*, 2002).

Sequential passaging refers to batch growth of a culture, followed by the inoculation of a subsequent batch using a fraction the previous batch as inoculum; this process is continued forward over multiple passages. When it comes to adaptation, the experimental method of sequential growth is simple, but the dynamics of adaptation are

more complex due to the changing conditions within the culture as nutrients are consumed and products are created. Moreover, selection in sequential growth can lead to the loss of certain genetic traits, which mostly occurs when a small portion of the population – which may not contain the adapted cell trait – is passaged onto the next culture (Elena & Lenski, 2003). However, this method also has its advantages because it allows for the use of stronger selective pressures and enables the double selection for faster growth rates (cells that have mutations to overcome the selective pressure and that grow faster in the imposed environment take over the culture) (Zelder & Hauer, 2000).

The experimental conditions for an adaptive evolution study are implemented such that an environmental stress is placed upon the cells that allows these adaptation strategies to select for the best prepared cells. The choice of adaptation growth method depends on the goal of the experiment: chemostats allow for better understanding of the mechanisms of adaptation, while sequential passages allow for faster selection of the best cells for a particular environment or given conditions (Gresham & Hong, 2015).

2.5.4 Adaptation for industrial applications

Adaptive evolution has been utilized to improve bacteria performance for industrial application in a number of different environments. Improving cell tolerance to different temperatures, acidities, inhibitor concentrations or toxic effects from substrates or products can greatly improve the industrial process trying to be implemented (Winkler & Kao, 2014). *E. coli* for example has been adapted to improve its isobutanol or ethanol tolerance, improve growth on glycerol or in minimal media, and produce homoethanol fermentation from xylose (Atsumi *et al.*, 2010; Cheng *et al.*, 2014; Conrad *et al.*, 2010, 2009; Goodarzi *et al.*, 2010; Minty *et al.*, 2011; Wang *et al.*, 2011). Similarly, industrial

strains of *Saccharomyces cerevisiae* have been adapted to different stresses including improved growth on thiamin, tolerance to hydrolysates, as well as improved tolerance to temperature and inhibitors (Almario *et al.*, 2013; Stambuk *et al.*, 2009; Wallace-Salinas & Gorwa-Grauslund, 2013).

2.6 Hypothesis and Objectives

2.6.1 Hypothesis

Adaptive evolution strategies can help improve the growth performance of *Methylomicrobium album* BG8 in non-ideal conditions, leading to a more robust bacterium for utilization in industrial settings. This study is a preliminary examination of the ability of *M. album* BG8 to grow in a harsh environment, specifically at low pH, with the goal of further adapting to other environments such as high temperatures or inhibitor concentrations, and working towards specific waste stream conditions that the strain could be grown in.

2.6.2 Objectives

The first objective was to determine the pH range in which *M. album* BG8 grew without any adaptation. The second was to implement adaptive evolution through sequential passaging to achieve growth in the lowest possible pH. Third, the bacteria were to be compared against unadapted strains for differences in growth and to test the stability of the adaptations. Finally, adapted cells were analyzed to determine the mechanism of adaptation through genome analysis.

3 Methods

3.1 Microorganisms

Methylomicrobium album BG8 (ATCC 33003) was received from the American Type Culture Collection. The master stock for the strain was received in liquid medium, and additional stocks were prepared from this culture to use for further work. Liquid cultures were preserved throughout experimentation and kept in 250-mL Wheaton bottles, or 30 mL of culture was transferred into 50-mL Falcon culture tubes for future analyses or culture inoculation. Cell banks of cultures grown on methanol were prepared in the medium of interest with 40% glycerol (Fisher Scientific, USA) and stored in -80°C, as they did not survive well in liquid cultures beyond 2-3 weeks.

3.2 Media

Bacterial liquid cultures were performed in Nitrate Mineral Salts (NMS) or Ammonium Mineral Salts (AMS) media (Whittenbury *et al.*, 1970). These media were prepared using distilled tap water, because methanotrophs often grow poorly in media that are too pure. Recipes for 10x NMS and 10x AMS solutions and Whittenbury trace solutions are shown in Tables 3.1 and 3.2. Phosphate buffers, hydrochloric acid (HCl) (Fisher Scientific, USA) and sodium hydroxide (NaOH) (Fisher Scientific, USA) were used to adjust pH of the media to desired levels. Phosphate stock buffer solution for NMS/AMS was prepared by dissolving 26 g potassium phosphate monobasic (KH_2PO_4) (Fisher

Scientific, USA) and 33 g sodium phosphate dibasic (Na_2HPO_4) (Fisher Scientific, USA) in double distilled water before autoclaving, producing a solution of pH ~6.8.

Phosphate buffers were prepared for pH adjustment by making 1 M solutions of KH_2PO_4 and Na_2HPO_4 each. Phosphate buffers at different pH were then prepared by combining the two phosphate solutions in different proportions. Specifically, pH 5.14 buffer was created using ~93 mL of KH_2PO_4 and ~7 mL of Na_2HPO_4, and pH 5.5 buffer was created using ~85 mL of KH_2PO_4 and ~15 mL of Na_2HPO_4. A pH 7.54 buffer was also made from ~10 mL of KH_2PO_4 and ~90 mL of Na_2HPO_4. The HCl and NaOH solutions used for pH adjustment were made by diluting 1 N HCl solution with double distilled water and making a 1 M solution of NaOH by mixing 40 g of pellets with 1 L double distilled water.

To make 1x NMS or 1x AMS solutions, 100 mL of the respective 10x solution were mixed with 900 mL distilled tap water, before autoclaving for 20-40 min at 121 °C and 2 atm. The sterile solution was then supplemented with 10 mL of phosphate buffer at a pH of interest which had previously been autoclaved or filter sterilized.

To make media for low pH adaptation, 1 L of 1x NMS or AMS at pH ~6.8 was prepared with 10 mL of phosphate stock buffer, then 0.1 M HCl or NaOH was added dropwise to the desired pH before autoclaving or filter sterilizing. The recipe was changed so that after adding phosphate stock buffer, additional 1.0 M KH_2PO_4 was added dropwise to reduce pH (instead of HCl, a stronger acid). This was done until the addition of the acidic phosphate buffer could no longer change the pH (~pH 4.5). At this point, the same procedure was used, except adding 5 mL of pH 5.14 buffer, which made the overall NMS

solution ~pH 5.8. 5-20 mL of KH_2PO_4 of phosphate buffer was then added to reach a pH of ~4.8, and finally 0.1 M HCl was added dropwise to reach the desired pH, before autoclaving/filter sterilizing the solution.

Table 3.1 10X Stock Solution: NMS/AMS

1. 10 g $MgSO4.7H_2O$
2. 10 g KNO_3 (NMS)/ 5 g NH_4Cl (AMS)
3. 2.28 g $CaCl_2.2H_2O$
4. 10 mL Whittenbury trace elements solution
5. 5 mL 0.1% Sodium Molybdate solution
6. 1 mL 3.5% FeEDTA solution
7. 0.5 mL 100 mM Copper Sulfate solution
- dissolve #1, 2 and 3 in 900 mL distilled water; then add the remaining components and bring volume up to 1000 mL, store at 4 °C - Note; Solution will often have orange precipitate settle to bottom, simply shake bottle before use

Table 3.2 Whittenbury trace elements for NMS/AMS

1. 0.5 g $FeSO_4.7H_2O$
2. 0.4 g $ZnSO_4.7H_2O$
3. 0.02 g $MnCl_2.4H_2O$
4. 0.05 g $CoCl_2.6H_2O$
5. 0.01 g $NiCl_2.6H_2O$
6. 0.015 g H_3BO_3 (boric Acid)
7. 0.25 g Na_2EDTA (disodium)
- In 1000 mL, store at 4 °C - Note: Solution may have orange precipitate at bottom, shake bottle before use

3.3 Growth and Measurements

3.3.1 Growth conditions

50 mL of sterile growth medium was placed in a 250-mL Wheaton bottle with a butyl-rubber septum cap in order to allow for extraction of samples while maintaining an air-tight environment. This ratio of liquid volume to headspace was used to ensure an appropriate amount of oxygen was available to the cultures. 2.5 mmol of methane (Praxair, Canada) or methanol (99.8%, Fisher Scientific, USA) were added as carbon source depending on the conditions tested. Methane was supplied to the cultures via injection using a 60-mL syringe with 0.22-μm filter and 20G syringe needle. This was done by first removing 40 mL of headspace from the bottle and injecting either 60 mL of 95% or 57 mL of 99.9% methane. Methanol was added by micro-pipetting 101.3 μL into each culture before screwing on the septum cap tightly. Cultures were inoculated with 1-4% v/v inocula using 1-mL syringes before incubation at 150 rpm and 30°C in incubator shakers (Ecotron Infors MT, Canada).

3.3.2 Optical Density

Cell concentration was determined throughout growth experiments using optical density (OD). It was measured in a 1-mL cuvette at a wavelength of 540 nm using a Ultrospec 50 UV/VIS spectrophotometer (Biochrom, UK). NMS or AMS media was used as a reference, depending on the experimental conditions tested.

3.3.3 Maximum OD_{540} and Growth Rate

Maximum OD_{540} was chosen as the point with the highest OD reached within each growth experiment.

Growth rate was calculated using data collected during growth that was plotted versus time on a semi-logarithmic scale (converting OD_{540} values into natural logarithmic functions). The slope (growth rate - μ) was calculated by performing a linear regression on as many data points as possible that were collected during the exponential phase of growth. In most cases this consisted of three to five data points, except for in a select few cases where only two points could be utilized. When possible, the same time points were chosen for each replicate.

3.3.4 pH Measurement

pH was measured using a pH meter and electrode (Denver Instruments Ultrabasic, Sartorius, Germany). Before use, the amount of 3 M KCl solution in the probe was verified. To begin, the probe cap was removed and the probe was rinsed with distilled water and then calibrated with colour-coded pH 4, 7, and 10 buffer calibration solutions (Fisher Scientific, USA). The probe was rinsed between each use and once calibrated was placed into a bottle or beaker of desired medium on top of a magnetic stirrer (Corning, USA) with stir bar and allowed to sit for 20-30 s until the probe measurement stabilized. After pH was measured, the probe was rinsed, and measurements were repeated until done. Upon completion, the probe was rinsed again, and placed back into a 3 M KCl solution.

The pH of solutions and media was measured prior to autoclaving, since the pH probe could not be sterilized (autoclaving did not alter media pH), as well as after growth to determine how pH changed during growth. Cultures had their pH measured right after reaching stationary phase, sometimes within a week, and sometimes after multiple weeks. Samples were measured with and without (supernatant only) cells present to verify if any differences were observable between these cases.

3.3.5 Concentration of Ammonium

The concentration of ammonium present in the media was measured to evaluate its accumulation in NMS medium at low pH. The protocol for NH_4^+ determination was based on the method described by Kandeler & Gerber (1988) with some modifications. Briefly, in this method any ammonium present will react with sodium salicylate (85 g/L, Sigma-Aldrich, Canada) when there is sodium dichloroisocyanurate (0.2 g/L, Sigma-Aldrich, Canada) present and form a varying blue-green coloured solution depending on the concentration of ammonium. The sodium nitroprusside solution (85 g/L sodium salicylate and 0.6 g/L sodium nitroprusside, Sigma-Aldrich, Canada) oxidizes easily and must be replaced if it turns brown as it is no longer viable. 100-fold diluted *M. album* BG8 samples in NMS, an AMS solution control, and NH_4^+ calibration solution (0.6607 g/L ammonium sulfate, Fisher Scientific, USA) were all added to separate wells of a 48-well plate. NH_4^+ calibration solution was initially 10 mM and was diluted with double distilled water to 10, 20, 50, 100, and 200 μM. A 2:1 aqueous solution mixture of sodium hydroxide (12g/L, Fisher Scientific, USA) and sodium nitroprusside (0.6 g/L, Sigma-Aldrich, Canada) was added to each well, and the solution was mixed again by tapping the sides of the 48-well plate instead of vortexing or placing the plate in a shaker. Tapping was done again after adding sodium dichloroisocyanurate solution to wells. It was found that incubating in the dark for 30 min at 30°C improved the performance of the assay. After incubation, the plate was measured in a plate reader against water at 600 nm using an Ultrospec 50 UV/VIS spectrophotometer (Biochrom, UK).

3.3.6 Statistics

Average values and standard deviations were calculated based on three replicates, except when indicated otherwise (cases when one or more samples did not grow). Standard deviation measurements were calculated for each measurement using the standard deviation of sample population method within Excel.

3.4 Adaptation

3.4.1 Screening

In order to screen for growth at different pH, *M. album* BG8 was grown in liquid cultures for two passages in the desired growth conditions for testing (AMS or NMS, methane or methanol). Once the second culture was 7 days old, a 1% inoculum was used to test for growth at different pH. This was first done by growing in AMS/Methane conditions using single bottles at pH ranging from 3 to 10. After this, the same experiments were performed with a narrower pH range (pH 5.5, 6, 6.8 and 8) and in triplicate for each condition – AMS/Methane, AMS/Methanol, NMS/Methane and NMS/Methanol. The results from this screening were used to determine the starting pH for adaptation studies in each condition.

3.4.2 Sequential batch adaptation

Sequential batch adaptation was performed as shown in Figure 3.1. Adaptation was started slowly at first by passaging cultures with 1% inoculum and allowing them to grow well into stationary phase. This was done for pH 6.5 in both AMS conditions (methane and methanol) and pH 5 in both NMS conditions (methane and methanol).

Once passaged to a lower pH, further passages were performed once cultures were in late log phase or in early stationary phase, usually every 2-4 days. In some cases growth would take up to 5-6 days and, in a few extreme cases, 14 days. Once cultures were ready to be passaged, 1 ml of grown liquid culture (corresponding to 2% inoculum) was transferred using a 1-mL syringe into 50 mL of media. The media was then fed with 2.5 mmol of methane or methanol. If cultures did not start to grow within 24-72 h of inoculation, another 1 mL inoculum was added to promote growth. If growth still did not occur within a few days, the passage was restarted from the previous culture (or older cultures if that failed as well) in a new set of bottles with fresh medium. The OD_{540} was measured at the time of inoculation, and recorded throughout the growth cycle, usually every 2-4 hours for periods at the end of lag phase, throughout log phase, and at early stationary phase.

Occasional plating of cultures onto LB broth (Fisher Scientific, USA) or Nutrient Broth (Merck, USA) with 15 g/L Agar (Becton Dickinson, USA) plates was done to test for contamination. If contamination was detected, the culture was restarted from the most recent uncontaminated liquid culture.

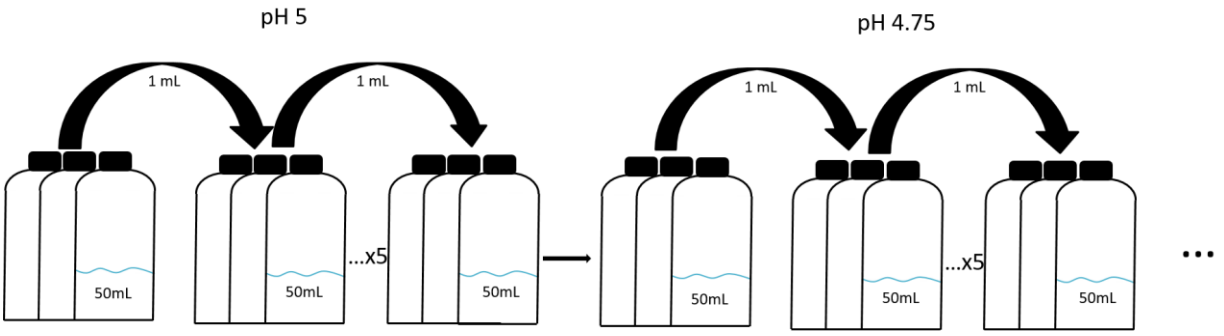

Figure 3.1 Sequential adaptation passages for adaptive evolution of bacteria. Cultures are grown in triplicate bottles at each pH in 50 mL of AMS or NMS media and fed with 2.5 mmol of methane or methanol as carbon source. Triplicate bottles are sequentially passaged with a 1 mL (2%) inoculum five times in each pH. After the fifth passage, one of the triplicate bottles is chosen to passage into triplicate bottles at the next, lower pH.

3.4.3 Comparison of adapted vs unadapted *M. album* BG8

After significant adaptation had occurred, adapted cells were compared to unadapted cells by looking at growth in NMS at pH 6.86 and pH 4.24 for cultures grown on methane, and in NMS at pH 6.86 and pH 4.05 for cultures grown on methanol. This was done by growing adapted and unadapted cells for 7 days in liquid culture then passaging 1% inocula from each of these into NMS at both pH of interest in parallel. Measures of OD_{540} were taken until stationary phase was reached.

Further analysis of adapted cells was performed by growing them on methane or methanol in NMS at pH 4.05 with 1% inoculum, passaging these cells in NMS at pH 6.86 for one growth cycle, and then inoculating from this culture into media at pH 6.86 and 4.05 NMS in parallel. Growth was again monitored by OD_{540} and once each culture had reached stationary phase, each culture (grown in pH 6.8 and 4.05) was passaged into NMS at pH 4.05, in which growth was compared again.

3.4.4 Stability of adaptation

Stability of adaptation was tested by passaging adapted cells grown on methane in NMS and adapted to pH 4.05 or grown on methanol in NMS and adapted to pH 3.95 into NMS at pH 6.86 for 5 sequential passages. The cultures were then transferred back to pH 4.05 or 3.95 for methane and methanol, respectively, to determine if growth remained the same in low pH after many generations grown at higher pH.

3.5 Genetic Analysis

3.5.1 DNA extraction and analysis

Genomic DNA was extracted from *M. album* BG8 samples taken at end of passages X, Y and Z using GeneJET Genomic DNA Purification kit (ThermoFisher Scientific, USA) and following the manufacturer's protocol. In order to harvest $2x10^9$ cells as recommended, 8-10 mL of cultures grown to an OD_{540} of ~1 were centrifuged in an Eppendorf 5424R microcentrifuge (Eppendorf, Canada) at 15,000×*g* for 2 min. Following extraction and concentration, DNA was run under gel electrophoresis (100V, 1h) to determine if the extract was of correct size or was degraded. DNA concentration was measured using fluorometric quantification (Qubit dsDNA broad range assay kit, Fisher Scientific, USA) and the ratio of absorbance at 260 and 280 nm (Nanodrop, Fisher Scientific, USA). Samples were further concentrated using a vacuum centrifuge (Savant SC110 SpeedVac, Fisher Scientific, USA). Finally, samples were cleaned up using Zymo DNA clean and concentrator kit (Zymo Research, USA) before being sent to the University of Washington PacBio sequencing services (Seattle, USA) for sequencing.

Sequencing was done using Single Molecule, Real Time (SMRT) technology (Pacific Biosciences, USA) with the Sequel System (Pacific Biosciences, USA).

Analysis of sequences was done with Geneious Version 11.1.5 (Kearse *et al.*, 2012) and extracted genomes were aligned with the known *M. album* BG8 genome (Kits *et al.*, 2013). Once genomes were aligned, they were compared and differences due to mutations or single nucleotide polymorphisms (SNPs) were noted and examined for amino acid changes and other potential consequences.

3.6 Morphology

Samples were concentrated in microcentrifuge at 15,000×*g* for 1 min. Supernatent was disposed of and another 1 mL of sample was added. This was repeated 4 times before resuspsending in another 1 mL of grown culture. 10 µL of sample was placed on copper grids with Formvar films for 2 min, then stained with 2% phosphotungstic acid (PTA) for 15 s. TEM images were taken on a Morgagni 268 Transmission Electron Microscope (FEI, Hillsboro, Oregon, USA) with Gatan Digital Camera. Images were taken up to 110,000 x magnification at 100 kV.

4 Results

This chapter provides experimental results for the adaptation of *M. album* BG8 to growth at low pH.

4.1 Screening

In order to test the ability of unadapted *M. album* BG8 to grow at different pH, cells were first grown in each combination of nitrogen (ammonium in AMS or nitrate in NMS) and carbon (methane or methanol) sources at optimal pH 6.8. These experiments were used to establish a reference for typical growth. Figure 4.1 and Table 4.1 show the growth curves and growth rates for each condition.

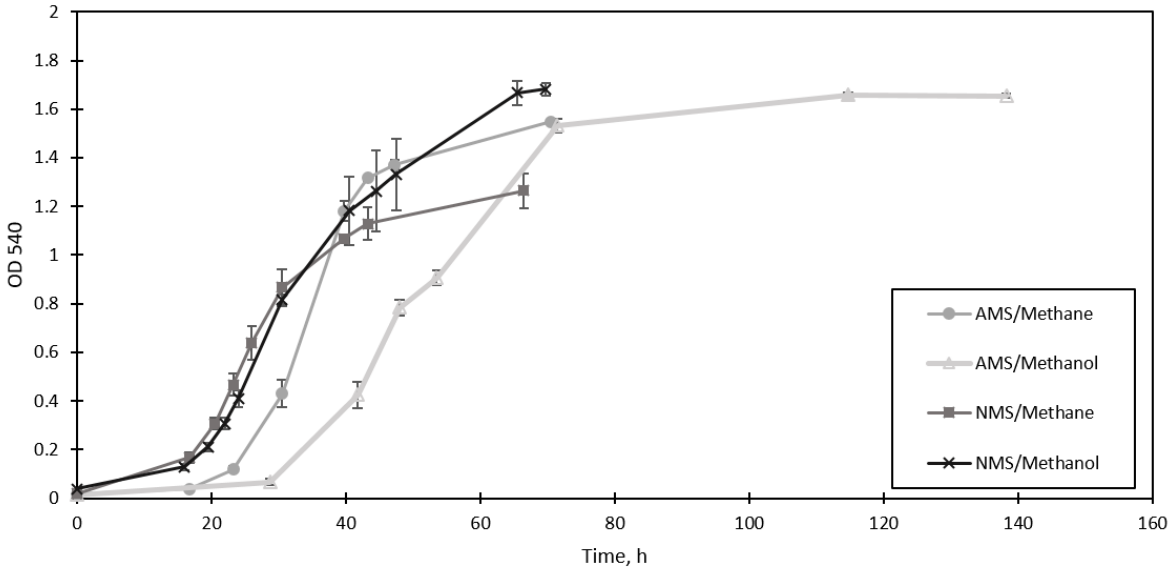

Figure 4.1 Growth curves for *M. album* BG8 grown in AMS or NMS medium at 30°C and pH 6.8 with methane or methanol as carbon source. Error bars indicate standard deviation from 3 replicates, except for NMS/Methanol which only had 2.

Table 4.1 Mean growth rate (μ) of *M. album* BG8 grown in AMS or NMS at 30°C and pH 6.8 with methane or methanol as a carbon source. Standard deviation is calculated from 3 replicates, except for NMS/Methanol which only had 2.

	AMS/Methane	AMS/Methanol	NMS/Methane	NMS/Methanol
Growth Rate (h^{-1})	0.175	0.135	0.151	0.139
St. Dev.	0.003	0.012	0.017	0.007

Cultures grown on methanol in AMS and NMS media performed very similarly, with maximum OD and growth rates being statistically equal. However, cultures grown on AMS displayed longer lag phases; it was observed that cultures grown on methanol typically have more variations in their lag phase – ranging from 12-48 h – regardless of the nitrogen source. Growth on methanol led to higher maximum OD (Figure 1, 1.66 in AMS, 1.68 in NMS) while growth on methane resulted in higher growth rates (Table 1, 0.175 h^{-1} in AMS and 0.151 h^{-1} in NMS). Cultures fed with methane and using AMS had a higher maximum OD (1.55) and higher growth rate (0.175 h^{-1}) than nitrate based medium cultures (1.26 and 0.151 h^{-1}, respectively), including having the best growth rate of all samples. NMS/Methane had the lowest maximum OD, reaching only 1.26 while the next lowest was AMS/Methane at 1.55.

Growth at different pH was initially performed in AMS/Methane conditions in single bottles to determine the appropriate conditions for more intensive testing. Table 4.2 shows the initial and final OD, final pH and duration of lag phase for growth at pH ranging from pH 3 to 10.

Table 4.2 Screening parameters for growth of unadapted *M. album* BG8 grown at 30 °C in AMS on methane at different pH.

Initial pH	3.0	4.0	5.0	6.0	6.8	8.0	9.0	10.0
Lag (h)	N/A	N/A	N/A	~72	~16	~16	~40	N/A
Final pH	3.01	4.12	5.29	4.36	4.5	6.14	6.51	8.39
Max OD	0.048	0.051	0.083	0.382	0.906	0.521	0.714	0.045

Here we can see that, based on the maximum OD, growth only occurred between pH 6.0 and 9.0. The culture grown at pH 6.8 grew to a lower density than expected when compared to Figure 4.1, but the growth rate remained the same as Figure 4.1. Density of growth was lower at pH 6.0, 8.0 and 9.0, and the lag phase was much longer at pH 6.0 and 9.0 than at pH 6.8.

Since no growth occurred below pH 6.0 in these initial screening tests, further investigations focused on growth on methane or methanol in AMS or NMS at pH 5.5, 6.0, 6.8, and 8.0. Figure 4.2 shows growth curves for these cultures.

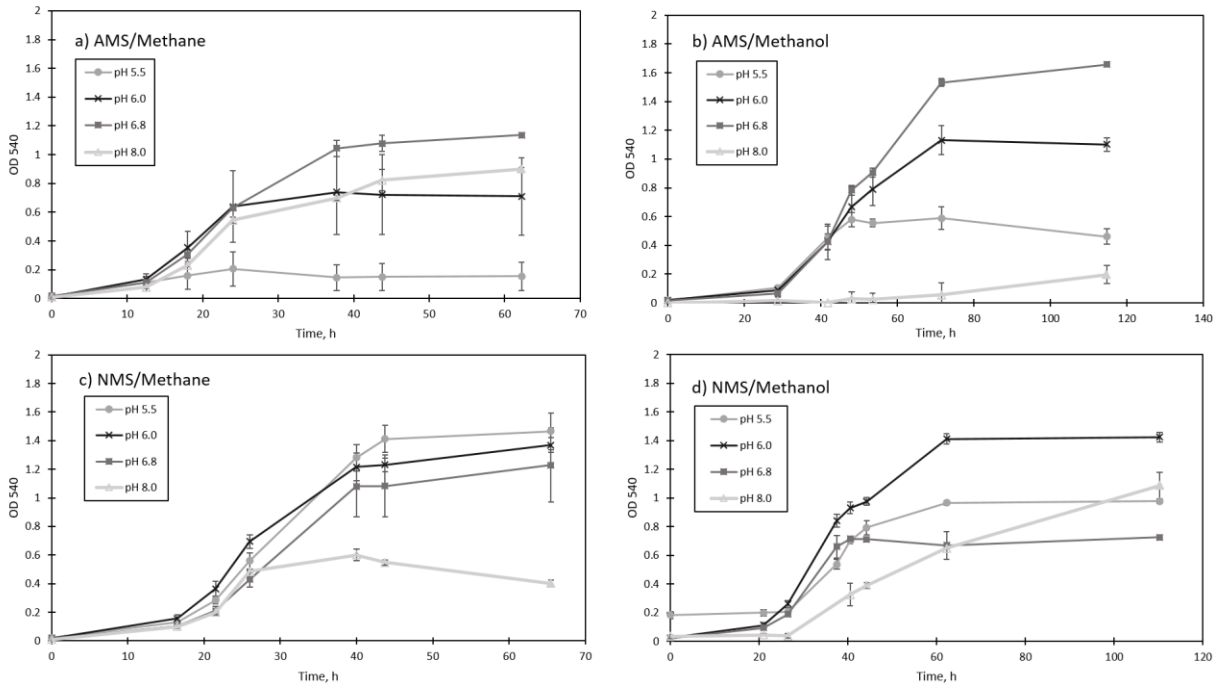

Figure 4.2 Screening of unadapted *M. album* BG8 growing on methane or methanol in AMS or NMS at pH 5.5, 6.0, 6.8, and 8.0. Error bars indicate standard deviation from 3 replicates, except for AMS/Methane which shows 2 replicates at pH 5.5 and 6.0.

As can be seen, growth was observed in all cases, although it was minimal for pH 5.5 on AMS/Methane and pH 8.0 on AMS/Methanol. In all other cases, the cultures display clear lag, exponential and stationary phases. It can also be observed that lag phase was similar for each growth condition regardless of pH, except for pH 8.0 on NMS/Methanol which had a lag phase that was ~10 hours longer than others.

Figure 4.3 shows the maximum OD obtained from each growth curve observed in Figure 4.2. Here it can be seen that growth on AMS in both conditions produced the highest OD at pH 6.8, but in both cases demonstrated significantly lower maximum OD at pH 6.0 and 5.5. Growth on methane led to an OD of 1.72 at pH 6.8 but of only 0.201 at pH 5.5, while methanol-grown cultures grew to 1.66 and 0.59 for the same respective

values of pH. Cultures in AMS/Methanol grew poorly at pH 8.0, only reaching an OD of 0.25, while cultures grown in AMS/Methane reached a max OD of 1.28 at this pH and performed similarly to other conditions at more acidic pH. Growth in NMS produced a higher maximum OD at pH 5.5 and 6.0 than at the normal pH 6.8. Growth on methane improved as pH decreased, growing to OD of 1.47, 1.37 and 1.23, at pH 5.5, 6.0, and 6.8, respectively, while the cultures using methanol grew best at pH 6.0 (OD of 1.42), but still higher at pH 5.5 (OD of 0.97) than at pH 6.8 (OD of 0.72).

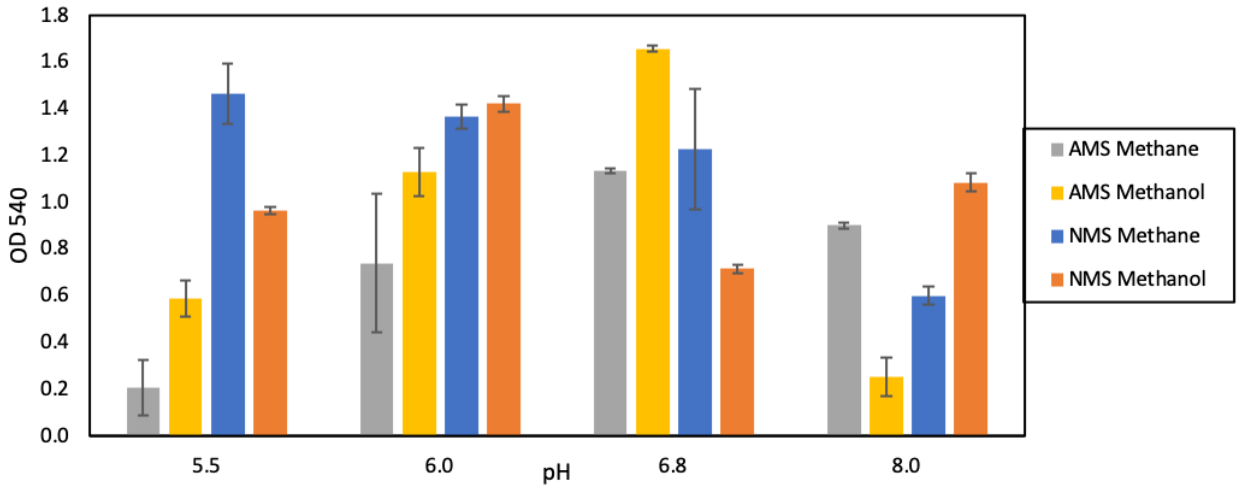

Figure 4.3 Maximum optical density of *M. album* BG8 growing on methane or methanol in AMS or NMS medium over pH range of pH 5.5 to pH 8. Error bars indicate standard deviation from 3 replicates, except for AMS/Methane which shows 2 replicates at pH 5.5 and 6.0.

Figure 4.4 shows the growth rate obtained from each condition observed in Figure 4.2. In this figure, both AMS conditions retain a high growth rate at pH 6.8, with growth rate rapidly dropping as pH decreases for growth on methane and steadily decreasing for methanol growth, from 0.149 h^{-1} to 0.056 h^{-1} and 0.131 h^{-1} to 0.092 h^{-1}, respectively. Each condition had differing growth rates at pH 8.0 as well, growing at 0.169 h^{-1} on methane and 0.039 h^{-1} on methanol. Growth in NMS methane conditions had slightly increased

growth rates at pH 6.0 and 5.5 compared to pH 6.8 while methanol conditions had statistically equal growth rate at pH 6.0 and a slower growth rate at pH 5.5 when compared to pH 6.8 (growth at pH 6.8, 6.0, and 5.5 was 0.147 h^{-1}, 0.157 h^{-1} and 0.154 h^{-1} on methane and 0.117 h^{-1}, 0.121 h^{-1}, 0.094 h^{-1} on methanol). Growth rate for cultures at pH 8.0 feeding on methane increased to 0.165 h^{-1}, and to 0.144 h^{-1} when grown on methanol.

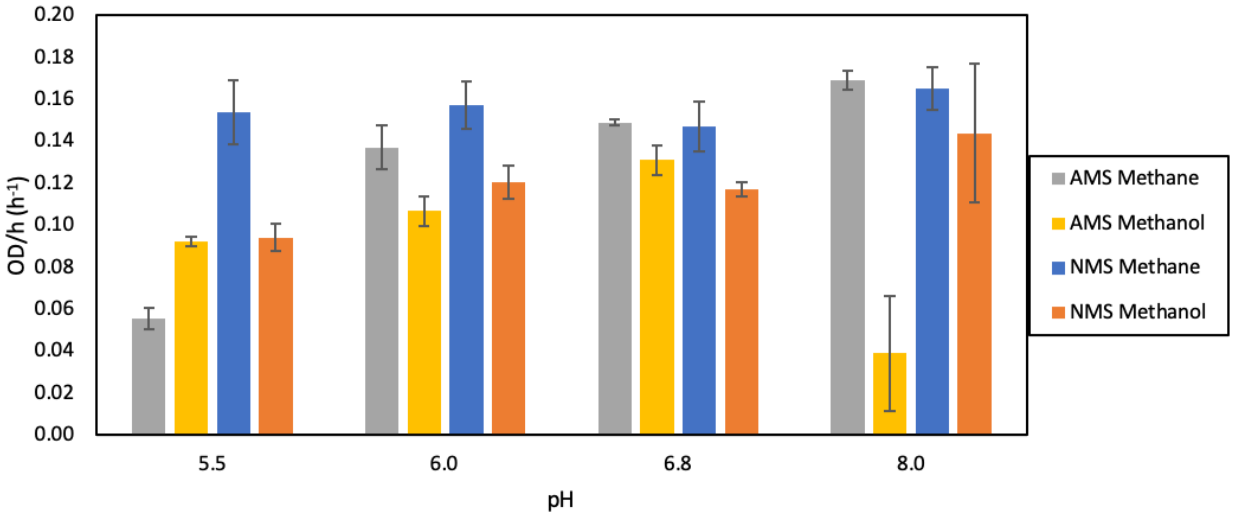

Figure 4.4 Growth rate (μ) of *M. album* BG8 growing on methane or methanol in AMS or NMS medium over pH range of pH 5.5 to pH 8. Error bars indicate standard deviation from 3 samples, except for AMS/Methane which shows 2 samples at pH 5.5 and 6.0.

4.2 Adaptive Evolution in Ammonium-Based Media

The initial step of adaptive evolution was performed by passaging cultures previously grown at pH 5.5 into the same medium at pH 5.0 to determine whether growth was still viable. Cultures were also passaged from pH 6.8 into pH 6.5 in single bottles to test if a smaller drop in pH affected growth as well. The maximum OD and the growth rate

of passages at pH 5.0 and 6.5 compared to pH 6.8 in AMS grown on methane and methanol are shown in Table 4.3.

Table 4.3 Growth rate and maximum OD of *M. album* BG8 grown in AMS on methane and methanol at pH 6.8, 6.5, and 5.0. Standard deviation was calculated from 3 replicates, except for pH 6.8 which only had 1 replicate.

	AMS/Methane			AMS/Methanol		
pH	6.8	6.5	5.0	6.8	6.5	5.0
Growth Rate (h^{-1})	0.175	0.037	0.076	0.135	0.035	0.051
St. Dev.	0.003	-	0.014	0.012	-	0.013
Max OD	1.547	.518	.282	1.657	0.490	0.300
St. Dev.	0.127	-	0.011	0.013	-	0.068

The maximum OD and growth rate of cultures grown in AMS was significantly decreased at pH 5.0. In fact, cultures did not grow beyond an average OD of 0.300 or present a growth rate even half the rate observed at pH 6.8 in AMS. Since growth during screening performed better at pH 6.0 than at pH 5.5, adaptation was attempted again from a higher pH, this time transferring cultures grown at pH 6.8 into AMS at pH 6.5 in single bottles. In this case growth only slightly improved, reaching an OD of 0.518 and 0.490 on methane and methanol, respectively. Growth rate was also much slower, indicating that growth at lower pH in AMS was not conducive to further growth and adaptation.

4.3 Adaptive Evolution in Nitrate-Based Media

The same initial approach was used with nitrate-based media. Figure 4.5 shows the growth curves for the passages corresponding to the first adaptation of cultures grown in NMS medium at pH 5.5 transferred to the same medium at pH 5.0.

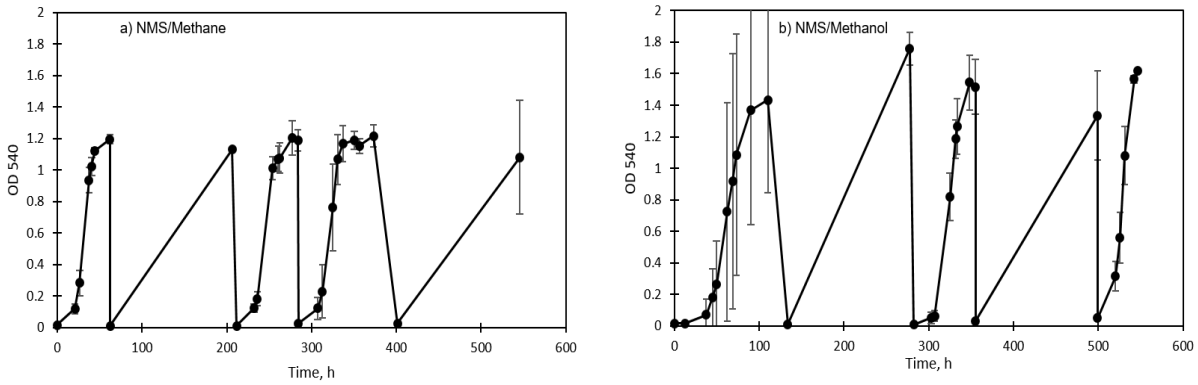

Figure 4.5 Adaptation of *M. album* BG8 to pH 5.0 (from pH 5.5) in NMS medium. The carbon source was a) methane, and b) methanol. Five sequential transfers are shown. Error bars indicate standard deviation from 3 replicates, except for AMS/Methane which shows 2 replicates at pH 5.5 and 6.0.

Transferred cultures grew well in NMS medium at pH 5.0, reaching similar maximum OD (~1.2 on methane and ~1.6 on methanol) while growth rate decreased slightly to 0.124 h^{-1} on methane and increased to 0.116 h^{-1} on methanol compared to those of cultures grown directly in NMS at pH 5.5 to 6.8 during the screening tests. Cultures grown in NMS/Methane (Figure 4.5a) responded quickly to the new medium, having a short lag phase and consistent, replicable growth. On the other hand, NMS/Methanol cultures had longer lag phases which varied between replicates; but after 3-4 passages the lag phase was reduced and replicates became consistent.

Sequential batches were then used to perform adaptation from this point forward. Cultures grown at pH 5.00 for 5 or more transfers were then passaged into the same medium at pH 4.75 for 5 or more sequential transfers before moving on to a lower pH. Figure 4.6 and Table 4.3 demonstrate the growth at each individual pH for 5 or more passages of adaptation while growing on NMS medium with methane as carbon source. Figure 4.7 and Table 4.4 show the same information when it was adapted in NMS with methanol as carbon source.

Figure 4.6 shows that samples grown on methane consistently grew to a maximum OD of 1.0-1.2, with some occasional passages reaching 1.4-1.5 (e.g. passage 1 at ~125 h in pH 4.24 (Figure 4.6f) and passage 8 at ~900 h at pH 3.95 (Figure 4.6i)). Growth rate responded differently, as seen in Table 4.3: while unadapted cells growing at pH 6.86 had a growth rate of 0.147 h^{-1}, the growth rate remained high (0.154 h^{-1}) for growth at pH 5.50. It then decreased slightly at pH 5.00 and 4.75 to 0.124 and 0.116 h^{-1}, respectively, before remaining consistently between 0.102 and 0.110 h^{-1} from pH 4.50 to 3.85. Once adaptation reached pH 3.85 and 3.80, both maximum OD and growth rate decreased (to between 0.8 and 1.0 and to 0.089-0.092 h^{-1}, respectively). It should be noted that the transfer to using HCl instead of just phosphate buffer to decrease the pH below pH 4.50 did not significantly affect the maximum OD (as shown in Figures 4.6 c) and d)) or the growth rate (Table 4.3).

Interestingly, Figure 4.7 illustrates that for cultures grown on methanol there was no drop in maximum OD as pH decreased throughout the whole adaptation study, even at the endpoint of pH 3.85 where cultures could not be viably transferred to a lower pH. Maximum OD remained between 1.4 and 1.8 over the whole range of pH adaptation. The

growth rates provided in Table 4.4 for NMS/Methanol show that, for unadapted *M. album* BG8 at pH 6.86, the growth rate was 0.139 h^{-1}; it then decreased to 0.094 h^{-1} at pH 5.50 and 0.116 h^{-1} at pH 5.00. From pH 4.75-3.85 growth rate had remarkable consistency, staying between 0.096 and 0.110 h^{-1}. Methanol growth showed little response to supplementation with HCl as shown by maximum OD (Figures 4.7 c) and d)) or growth rate (Table 4.5), similar to that of growth on methane.

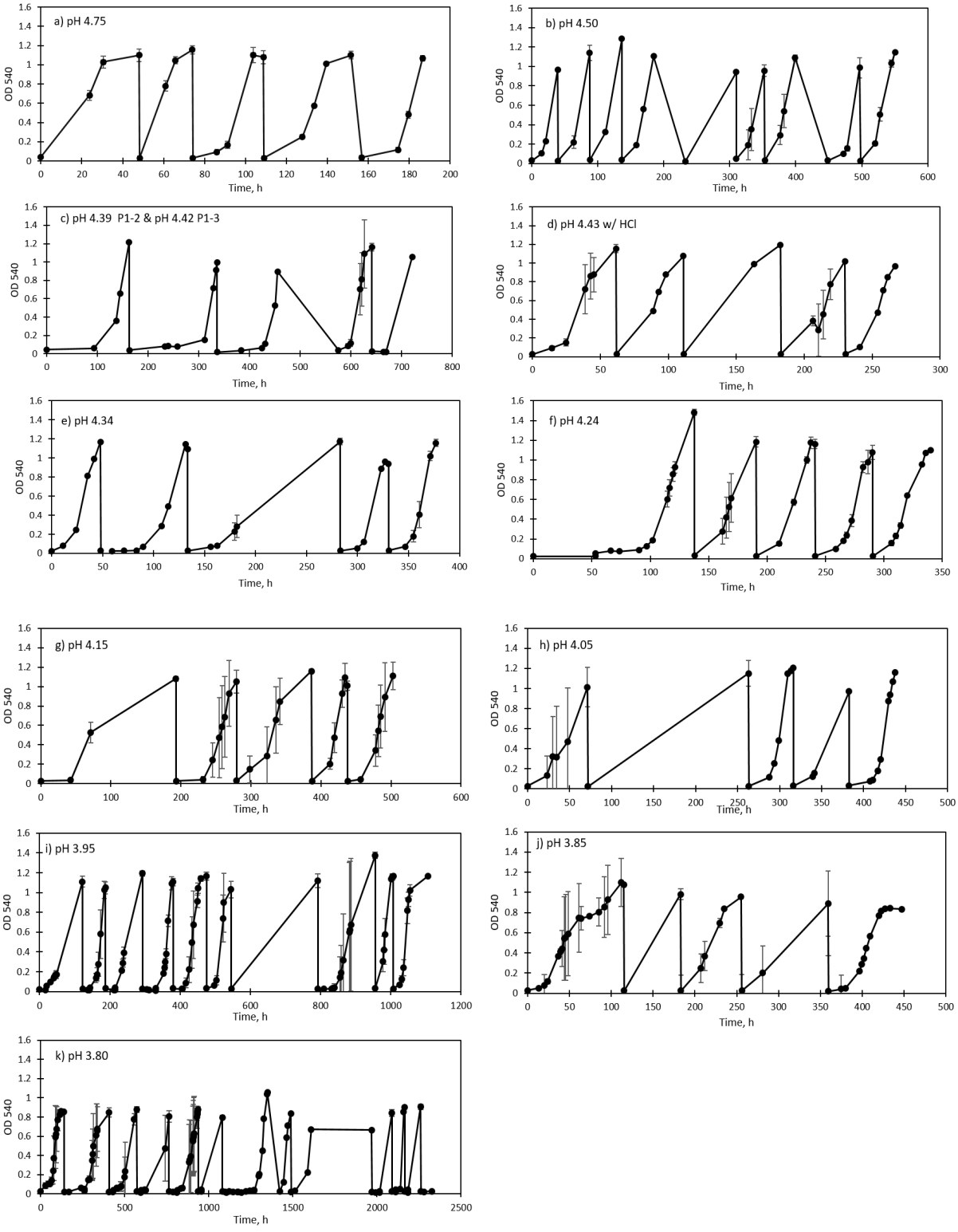

Figure 4.6 Sequential transfers of *M. album* BG8 for adaptation at lower pH in NMS/Methane conditions. a) pH 4.75, b) pH 4.50, c) pH 4.39 P1-2 & pH 4.42 P1-3, d) pH 4.43 with HCl, e) pH 4.34, f) pH 4.24, g) pH 4.15, h) pH 4.05, i) pH 3.95, j) pH 3.85, k) pH 3.80. Error bars indicate standard deviation for 3 or 2 replicates, passages with no error bars indicate when only 1 replicate grew.

Table 4.4 Growth rates (μ) of *M. album* BG8 grown in NMS/Methane conditions at each pH used for adaptation. Growth rates were calculated from the data found in Figure 4.6.

pH	**6.86**	**5.50**	**5.00**	**4.75**	**4.50**	**4.40**	**4.43 HCl**	**4.34**	**4.24**	**4.15**	**4.05**	**3.95**	**3.85**	**3.80**
μ	0.147	0.154	0.124	0.116	0.110	0.102	0.109	0.102	0.105	0.110	0.105	0.107	0.092	0.089
St.Dev	0.012	0.015	0.014	0.004	0.013	0.012	0.009	0.015	0.007	0.009	0.008	0.006	0.005	0.007
n	3	3	3	5	11	4	5	7	10	9	6	15	6	8

When looking at Figures 4.6 and 4.7 as a whole, it appears that the first few passages within a new pH would often have a longer lag phase, or require an additional 2% inoculum because it did not take off within 24-72 h. Once cells had adapted, lag phases decreased and no additional inoculum was needed. Growth rate and maximum OD remained similar between early and late passages, suggesting that adaptation most noticeably affected the length of the lag phases.

Another stand out from Figures 4.6 and 4.7 is that the standard deviation was smaller for cultures grown at higher pH. As pH decreased there was more often a difference in the lag phase between one or two of the replicates, resulting in large error bars for some passages. However, lag phase was the only substantial difference in growth, as growth rate and maximum OD were similar between each replicate. Often throughout adaptation only one or two of the three replicates would display growth. When only one replicate grew, no error bars are included in the Figures.

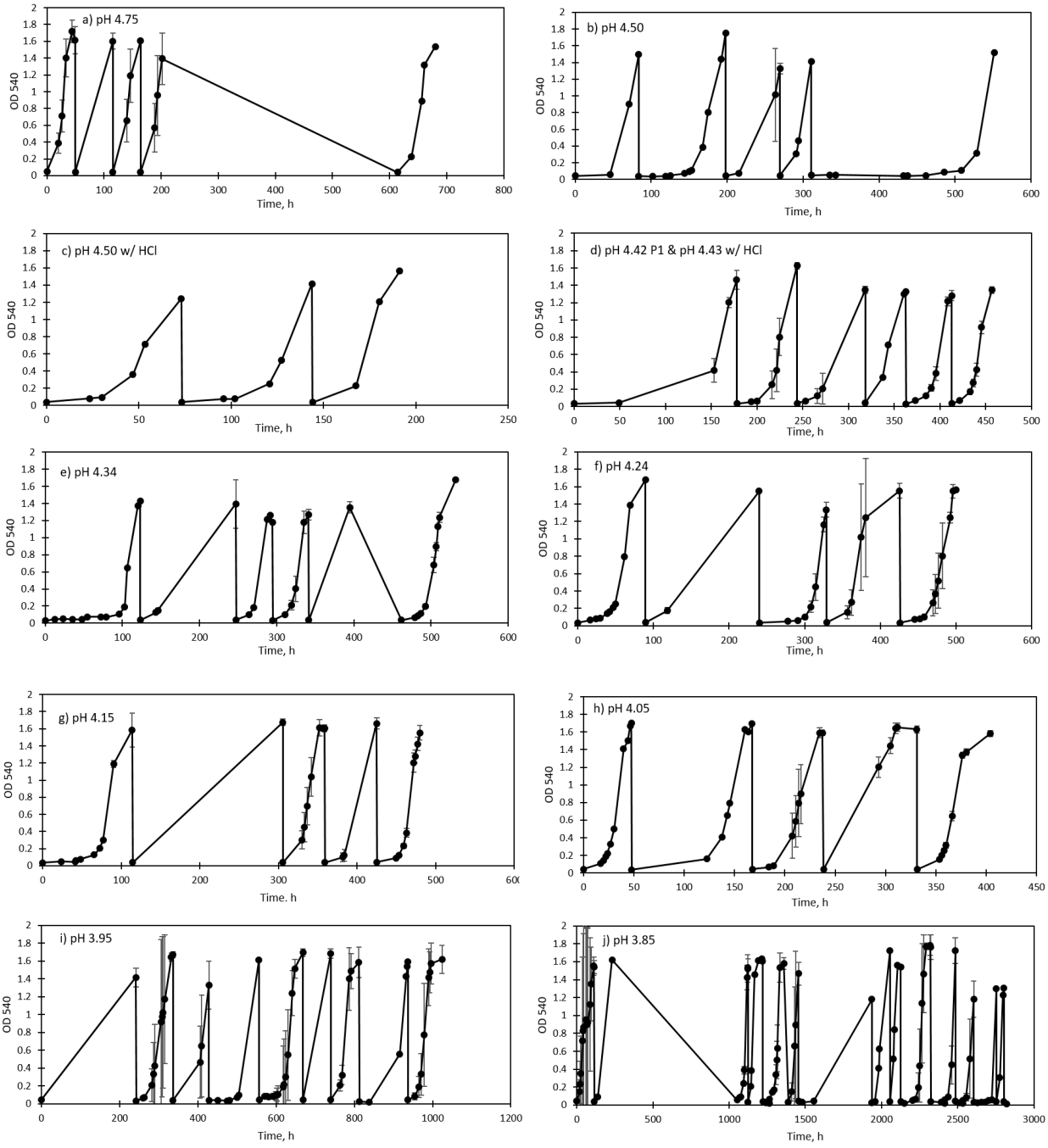

Figure 4.7 Sequential transfers of *M. album* BG8 for adaptation at lower pH in NMS/Methanol conditions. a) pH 4.75, b) pH 4.5, c) pH 4.50 with HCl, d) pH 4.42 P1 & pH 4.43 with HCl P1-5, e) pH 4.34, f) pH 4.24, g) pH 4.15, h) pH 4.05, i) pH 3.95, j) pH 3.85. Error bars indicate standard deviation for 3 or 2 replicates, passages with no error bars indicate when only 1 replicate grew.

Table 4.5 Growth rates (μ) of *M. album* BG8 grown in NMS/Methanol conditions at each pH used for adaptation. Growth rates were calculated from the data found in Figure 4.7.

pH	**6.86**	**5.50**	**5.00**	**4.75**	**4.50**	**4.50 HCl**	**4.43**	**4.34**	**4.24**	**4.15**	**4.05**	**3.95**	**3.85**
μ	0.139	0.094	0.116	0.105	0.098	0.101	0.100	0.101	0.102	0.108	0.110	0.096	0.096
St.Dev	0.007	0.007	0.011	0.011	0.010	0.010	0.006	0.002	0.004	0.009	0.008	0.003	0.009
n	3	3	3	6	3	4	9	8	6	9	7	10	5

Visual representations of the long-term adaptation experiments are shown by displaying the OD at the start and end of each passage along with a flow diagram of each pH passage for cultures grown on methane (Figure 4.8) and methanol (Figure 4.9). The flowcharts show growth at each pH, with replicate bottles labelled A, B, C. If any replicate did not grow within a pH cycle, it was marked with an X and not continued. If two or three replicates all made it through the passages at a given pH, the replicate with the best overall growth was selected for further passage to a new pH in triplicate – the other replicates were stored for potential future growth or analysis. In most cases, only one replicate survived all passages at a given pH. Adaptation did not continue below pH 3.80 when grown on methane and pH 3.85 when grown on methanol.

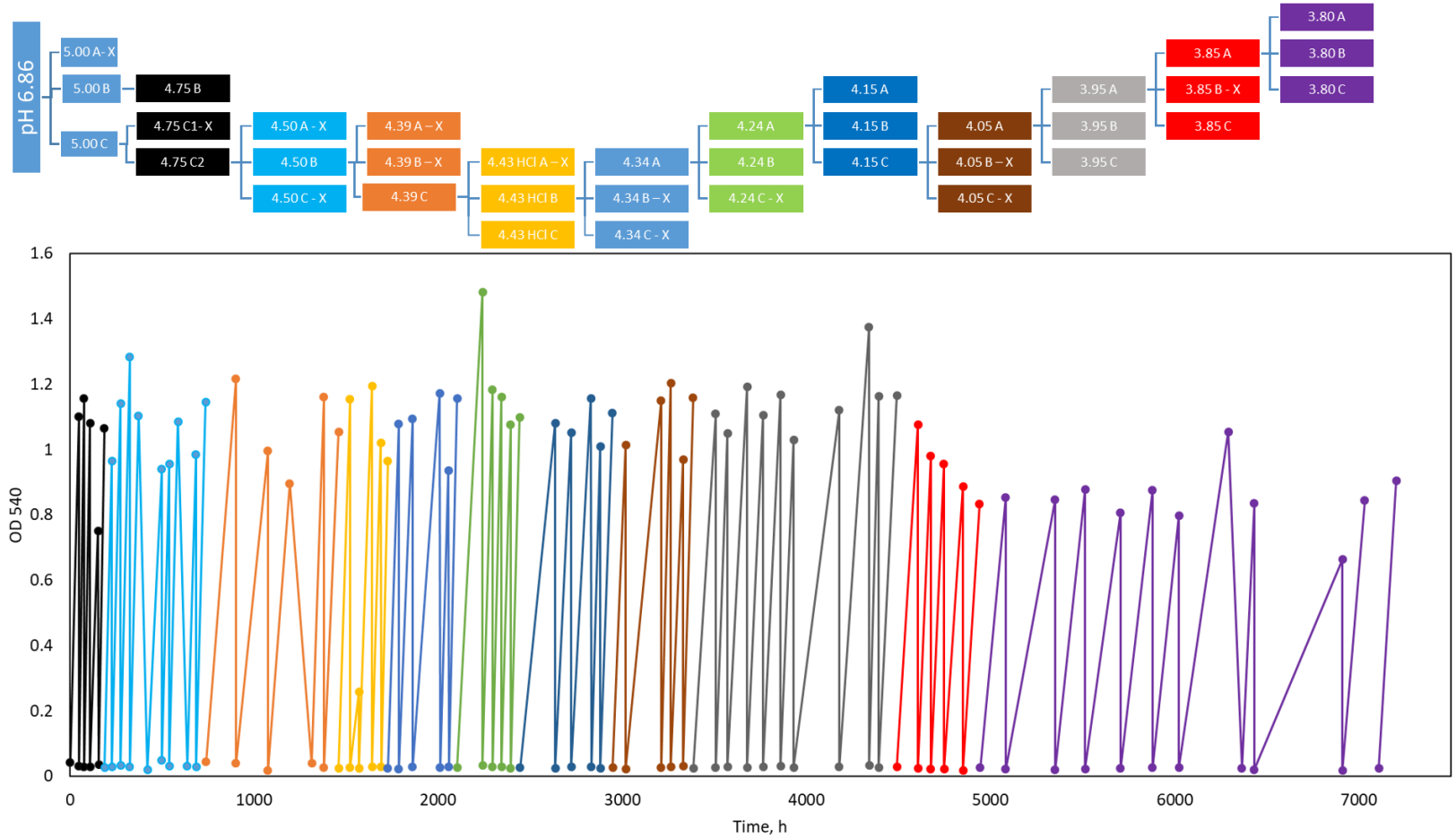

Figure 4.8 Long term adaptation of *M. album* BG8 grown on methane in NMS at lower pH. Adaptation was initiated at pH 6.86 and ended at pH 3.80. The flowchart (top) indicates the sequence of passages (5 passages per pH) performed for adaptation, while the graph shows the initial and final OD of each sequential passage. The colour scheme used indicates the round of passages in the flowchart and corresponding growth in the graph for a given pH. X indicates a group of passages in which the culture did not grow; no X means the replicate was carried forward or preserved for later use. 5 sequential passages were used at each pH, unless additional adaptation time was required. Error bars are not included to maintain visual clarity, but they can be found for each pH in Figure 4.6.

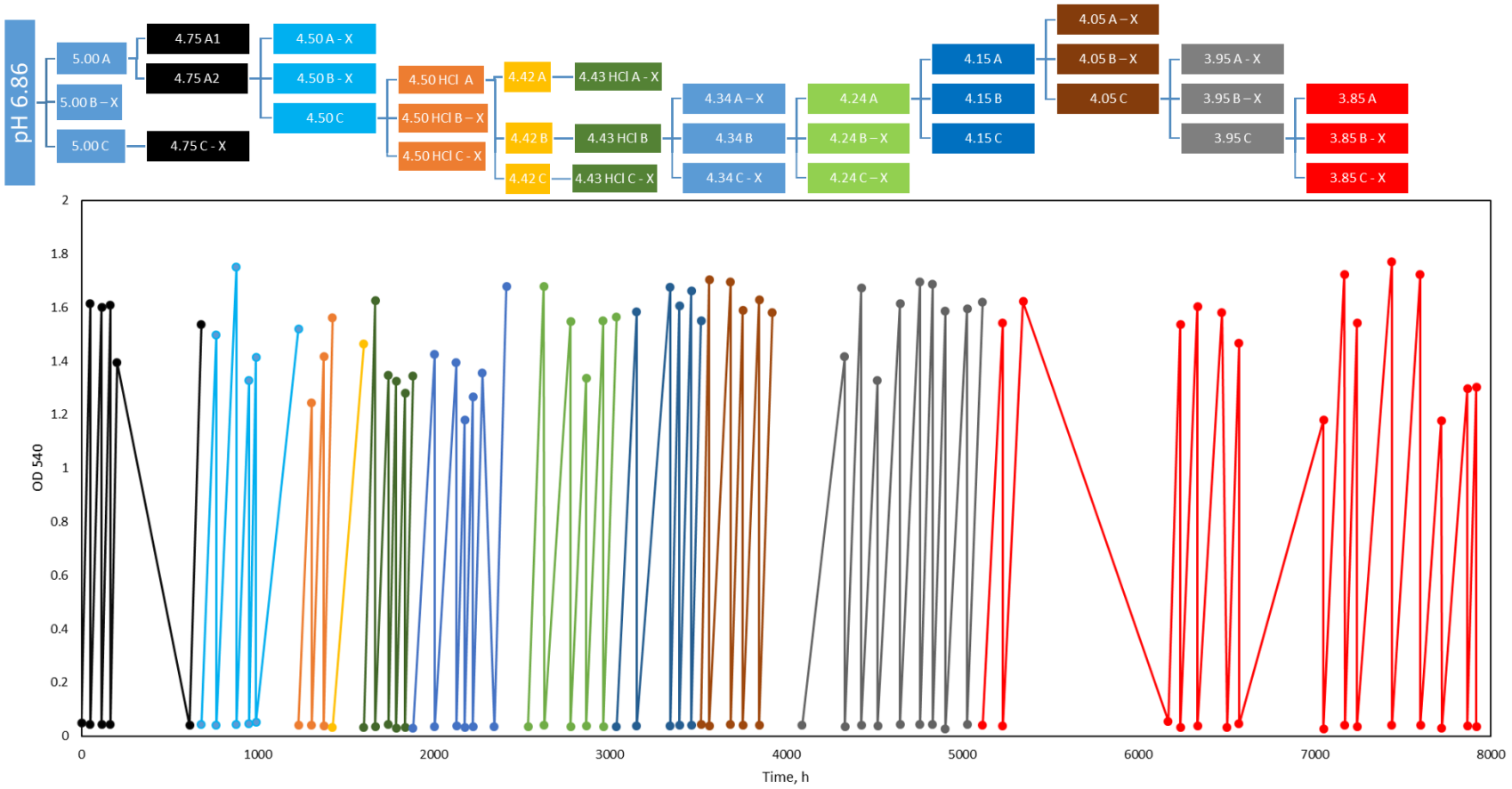

Figure 4.9 Long term adaptation at lower pH of *M. album* BG8 grown on methanol in NMS at lower pH. Adaptation was initiated at pH 6.86 and ended at pH 3.85. The flowchart (top) indicates the sequence of passages (5 passages per pH) performed for adaptation, while the graph shows the initial and final OD of each sequential passage. The colour scheme used indicates the round of passages in the flowchart and corresponding growth in the graph for a given pH. X indicates a group of passages in which the culture did not grow; no X means the replicate was carried forward or preserved for later use. 5 sequential passages were used at each pH, unless additional adaptation time was required. Error bars are not included to maintain visual clarity, but they can be found for each pH in Figure 4.6.

A few notable observations can be made when looking at the adaptation process as a whole. In Figure 4.8 the maximum OD was found to vary between passages at different pH values, but generally remained between ~0.9 and 1.2 for passages above pH 3.85. At and below pH 3.85, the maximum OD decreased to ~0.8-1.1. However, for the most part, maximum OD stayed fairly constant for passages at a given pH. The variations in maximum OD could be attributed to variations in culture adaptation and, occasionally, to some passages being inoculated with cultures in mid- to late log phase where the cultures were not fully grown. Another notable observation from this Figure is that the cultures adapted from pH 4.75 down to 4.05 in the same time it took to go from pH 3.95 to 3.80. Some passaged cultures could not grow with the 2% inoculum and needed to be restarted. When cultures could not grow after multiple attempts at passaging, cultures were re-initiated using samples from 2-3 earlier passages. Similarly, when contamination was found in a passage, samples from the last passage known to be uncontaminated were used to re-initiate growth. The larger gaps between passages, such as at 6500 h for growth on methane (Figure 4.8) and 4000 h or 5500 h for growth on methanol (Figure 4.9) were due to passages that failed to adapt or were found to be contaminated. Both cases would require the passage to be restarted from 2-3 passages prior.

Adaptation on methanol (Figure 4.9) provides many of the same observations as adaptation on methane (Figure 4.8). Firstly, differences in maximum OD between passages within a pH or between different pH were also observed. For example, for passages at pH 4.50 and pH 4.50 with HCl, the maximum OD varied from 1.2 to 1.6. Again, the range of maximum OD generally remained steady, ranging from 1.2 to 1.8.

One difference here from growth on methane is that there was no decrease in maximum OD was observed once pH reached 3.85, outside of a few passages at pH 3.85 between ~7000h and 8000h. Finally, it took the same time to adapt cultures from pH 4.75 to 4.05 as to adapt from pH 3.95 to 3.85. The large gaps in passages in methanol were solely due to failed passages from non-growth in passages, never from contamination issues.

In the case of growth on methane, it was determined during growth at pH 3.80 that cultures were contaminated. Testing was performed and contamination was found to go back to the first passage at pH 4.24, despite passages below this pH previously showing no contamination. To determine whether growth data collected below pH 4.24 was representative of methanotroph growth or was interfered by the contaminant, adapted cells grown on methanol to pH 3.85 were passaged into pH 3.85 with methane as carbon source. Growth was also restarted from the uncontaminated pH 4.24 culture and attempted to passage back down to a low pH for comparison to old cultures. Figure 4.10 shows growth for the new passages of *M. album* BG8 grown on methane from old cultures, as well as *M. album* BG8 adapted to low pH on methanol.

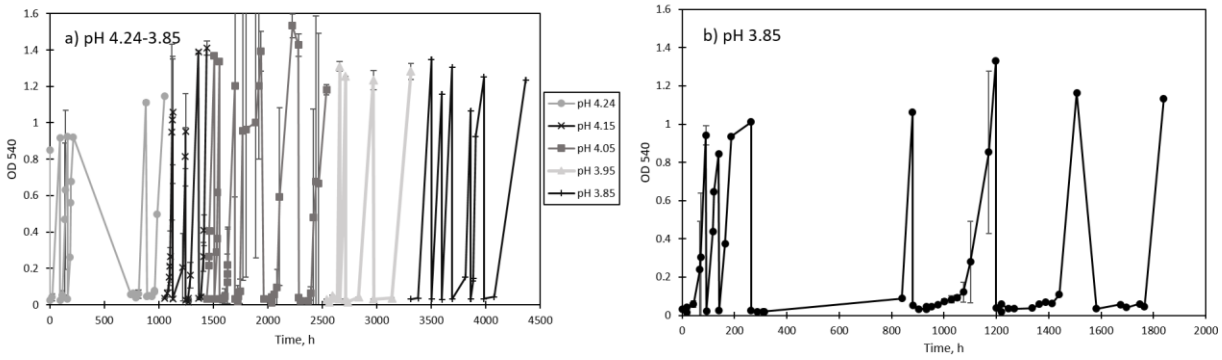

Figure 4.10 Sequential transfers of *M. album* BG8 grown on methane in NMS at a) pH 4.24 to 3.85 from uncontaminated methane culture, and b) pH 3.85 from uncontaminated methanol culture. Error bars indicate growth for 2 or 3 replicates, passages with no error bars indicate when only one replicate grew.

Once growth was efficiently established – after hurdles with long lag phases and supplemented inocula –, the maximum OD and growth rate reached similar values (between 0.92 and 1.41 for OD and 0.085 and 0.126 h^{-1} for growth rate in the culture adapted from pH 4.24 down to pH 3.85, and between 0.84 and 1.53 for OD and 0.071 and 0.098 h^{-1} for growth rate at pH 3.85 adapted from methanol growth) to those obtained from the original passages on methane at pH 4.24 to 3.85 (ranging from 0.97-1.48 and 0.089-0.110 h^{-1}, respectively).

4.4 Comparison of Growth of Unadapted and Adapted Cells

Once significant adaptation had occurred and growth in low pH was consistent, adapted cells were compared to unadapted cells by passaging each in low pH NMS medium and NMS at a normal pH 6.86. Results can be seen in Figures 4.11 and 4.12.

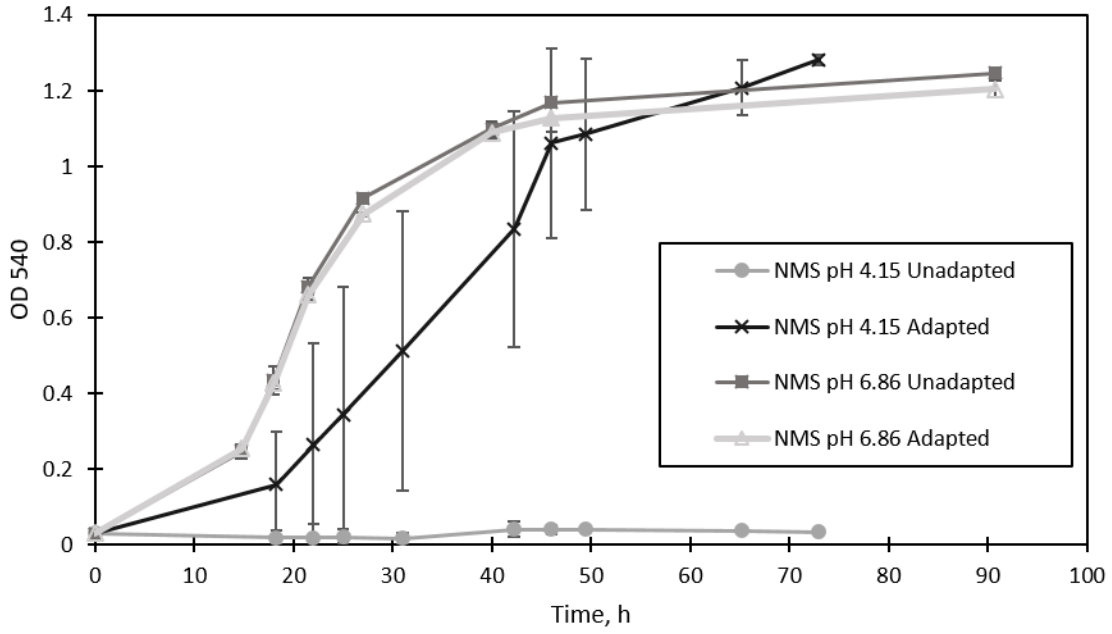

Figure 4.11 Growth curves of adapted and unadapted *M. album* BG8 grown on methane in NMS at pH 4.15 and 6.86 at 30°C. Error bars indicate standard deviation for 3 replicates.

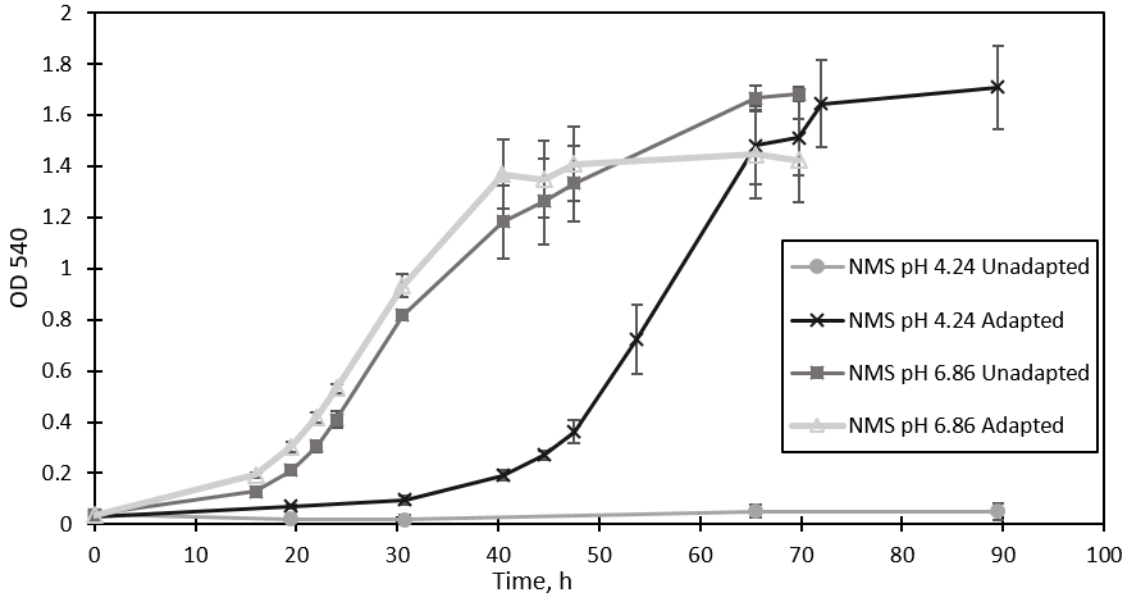

Figure 4.12 Growth curves of adapted and unadapted *M. album* BG8 grown on methanol in NMS at pH 4.15 and 6.86 at 30°C. Error bars indicate standard deviation for 3 replicates, except for pH 6.86 unadapted which only had two replicates.

Figure 4.11 demonstrates that both the unadapted and adapted cells grew similarly at pH 6.86, with growth rates of 0.151 and 0.142 h^{-1}, respectively. The unadapted cells reached a slightly higher OD, of 1.25 versus 1.20 for the adapted cells. It is also clear from these results that, unlike unadapted cells, adapted cells were able to grow at low pH, displaying a final OD of 1.28 and a growth rate at 0.117 h^{-1}. Cell growth in low pH did have a longer lag phase than either of the cultures grown at pH 6.86, and discrepancies in the length of lag phases between replicates explain the larger error bars observed for adapted cells growing at low pH.

Figure 4.12 shows similar results when cells were adapted and grown on methanol, but in this case both unadapted and adapted cells growing at pH 6.86 differed slightly in final OD, with unadapted cells reaching lower value of 1.42 (± 0.162) compared to 1.68 (± 0.025) for adapted cells. Growth rates also compared well to growth on methane, with unadapted cells growing slightly faster at 0.143 h^{-1} and adapted cells growing at 0.129 h^{-1}. Again, unadapted cells were not able to grow at low pH, and adapted cells grew well (maximum OD, 1.71, and growth rate, 0.107 h^{-1}) but only after a significantly longer lag phase than for growth at pH 6.86.

As a further comparison, adapted cells from a single pre-culture were used to inoculate new cultures at low and normal pH in parallel to compare performances. Figure 4.13 shows cells grown on methane at pH 4.05 passaged into NMS at pH 6.86 for one growth cycle, and from this passage into NMS at pH 6.86 and pH 4.05 in parallel. After each of these two passages completed one growth cycle, both were passaged in parallel into NMS at pH 4.05. As can be observed, cultures adapted and grown on methane showed no significant difference in growth-related parameters; in fact, they grew with extreme consistency between all passages. Growth rates were between 0.127 and 0.146 h^{-1} and maximum OD was between 1.09 and 1.15 for all passages.

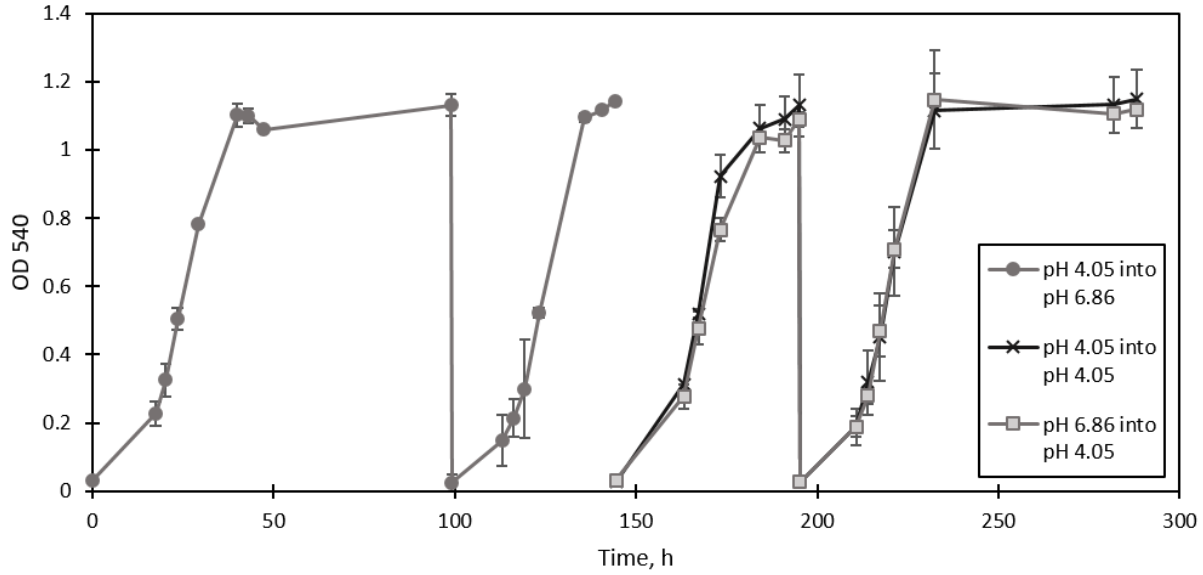

Figure 4.13 Comparison of growth of adapted *M. album* BG8 grown in NMS on methane. Cells were first grown at pH 4.05 and passaged into pH 6.86 for one growth cycle, then inoculated from this culture into pH 6.86 and pH 4.05 in parallel, after one growth cycle here both cultures were passaged in parallel back to pH 4.05. Error bars indicate standard deviation for 3 replicates, except for the first passage of pH 4.05 into 4.05 at 150h which only shows 2 replicates.

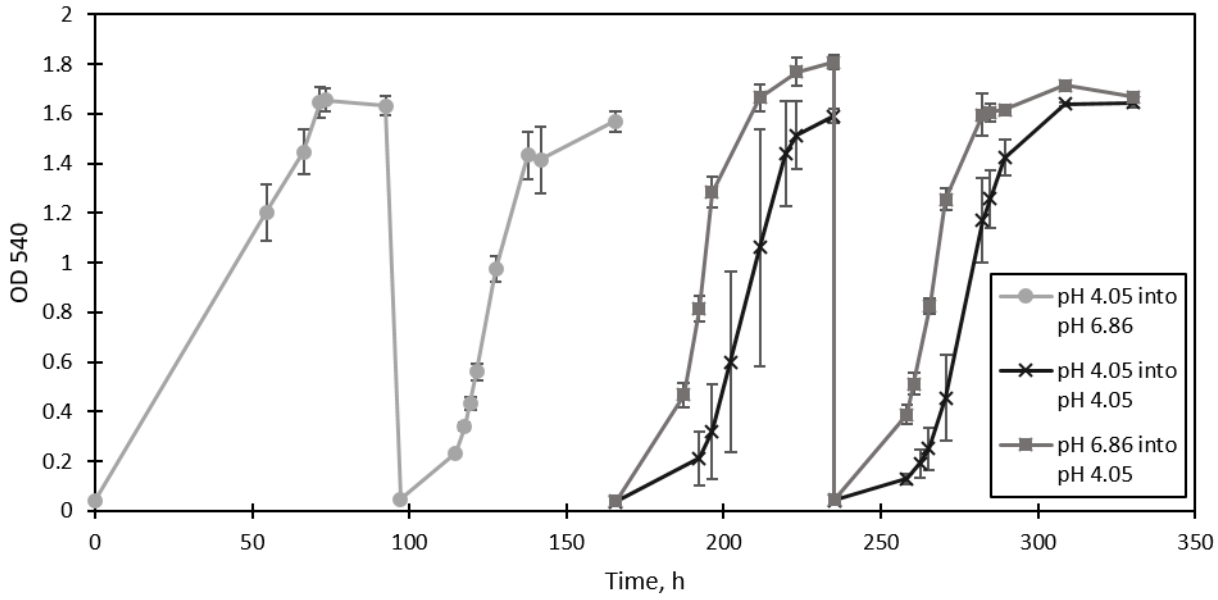

Figure 4.14 Comparison of growth of adapted *M. album* BG8 grown in NMS on methanol. Cells were first grown at pH 4.05 and passaged into pH 6.86 for one growth cycle, then inoculated from this culture into pH 6.86 and pH 4.05 in parallel, after one growth cycle here both cultures were passaged in parallel back to pH 4.05. Error bars indicate standard deviation for 3 replicates.

Figure 4.14 shows that growth parameters for cells adapted and grown on methanol differed for cultures at pH 6.86 versus pH 4.05. Cultures were passaged at 160 h from pH 6.86 into pH 6.86 and pH 4.05 in parallel. A higher maximum OD was reached in the former (1.81 vs 1.59) despite both conditions having similar growth rates (0.112 h^{-1} vs 0.120 h^{-1}, respectively). Interestingly, this maximum OD advantage carried into the next passage where both cultures grew at pH 4.05, with the culture passaged from pH 6.86 growing to an OD of 1.71 with a growth rate of 0.110 h^{-1} and the culture passaged from pH 4.05 grew to an OD of 1.64 with a growth rate of 0.113 h^{-1}.

4.5 Stability

To determine whether the adaptations observed were stable, cultures that had been adapted to low pH were passaged 5 times at pH 6.86 before being returned for more passages at low pH (pH 4.05 for methane-adapted cells and pH 3.95 for methanol-adapted cells). Results are shown below in Figure 4.15 and 4.16 for the methane- and methanol-adapted cells, respectively.

In Figure 4.15 it can be seen that when returned to pH 4.05 after 5 passages at pH 6.86, cells adapted to low pH in methane grew similarly to the original culture grown at pH 4.05. Growth rates before and after the passages were 0.105 and 0.122 h^{-1}, respectively, while both of these cultures reached a maximum OD of 1.13. Growth also did not change significantly throughout the passages at pH 6.86, with growth rates between 0.128 and .136 h^{-1}, and maximum OD of 1.02-1.14.

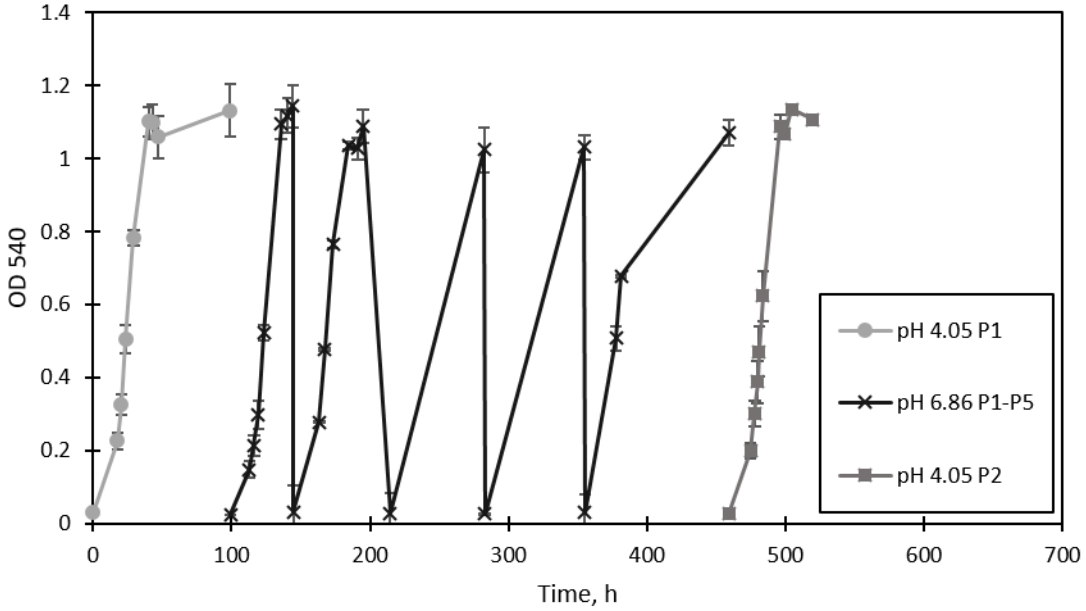

Figure 4.15 Growth of *M. album* BG8 in NMS grown on methane at 30ºC passaged from pH 4.05 (P1) into 5 sequential passages (P1-P5) at pH 6.86, and back into pH 4.05 (P2). Error bars indicate standard deviation for 3 replicates.

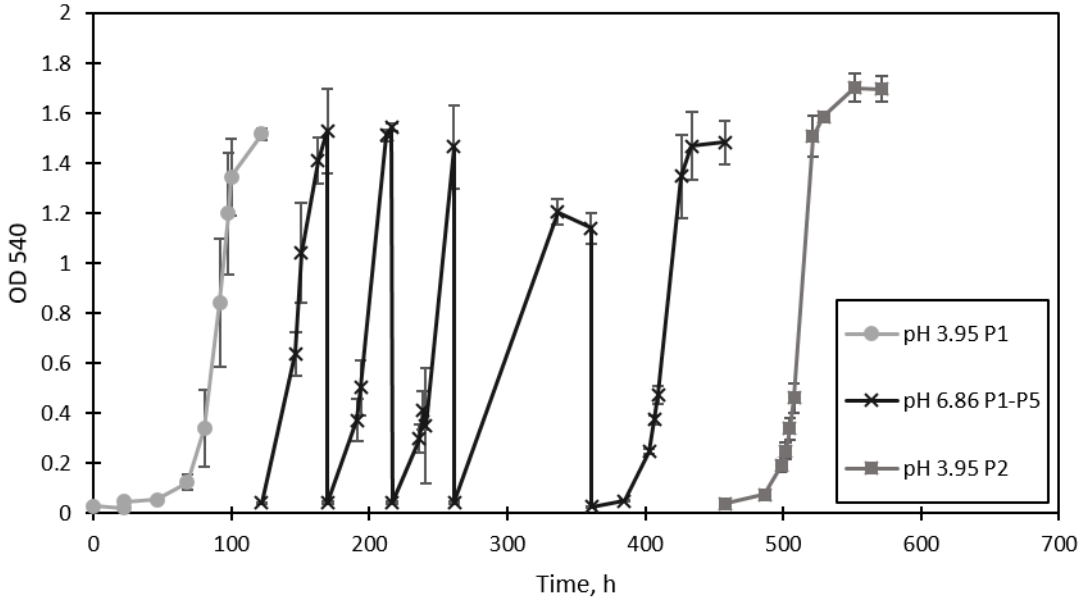

Figure 4.16 Growth of *M. album* BG8 in NMS grown on methanol at 30ºC passaged from pH 4.05 (P1) into 5 sequential passages (P1-P5) at pH 6.86, and back into pH 4.05 (P2). Error bars indicate standard deviation for 3 replicates, except for pH 6.86 passage 5 and pH 3.95 passage 2 which only had 2 replicates.

Figure 4.16 shows that cells adapted in methanol performed similarly to those adapted on methane. They retained their adaptation to low pH even after multiple sequential passages at pH 6.86. Growth rates before and after passaging at pH 6.86 were 0.092 and 0.103 h^{-1}, respectively, while maximum OD was 1.52 and 1.70, respectively. However, unlike cells adapted on methane, growth rate decreased when cultures were returned to pH 3.95 compared to the passages at pH 6.86. Cultures growing at pH 6.86 had growth rates between 0.111 and 0.119 h^{-1}. Maximum OD stayed between 1.46 and 1.70 for all passages except for passage 4 at pH 6.86, which was passaged before the culture fully reached stationary phase.

4.6 pH Measurement

The pH of NMS medium was measured before inoculation and after growth of cultures. The final pH and the change in pH were reported for each initial pH (Figure 4.17 a) and b), respectively) in order to highlight potential trends.

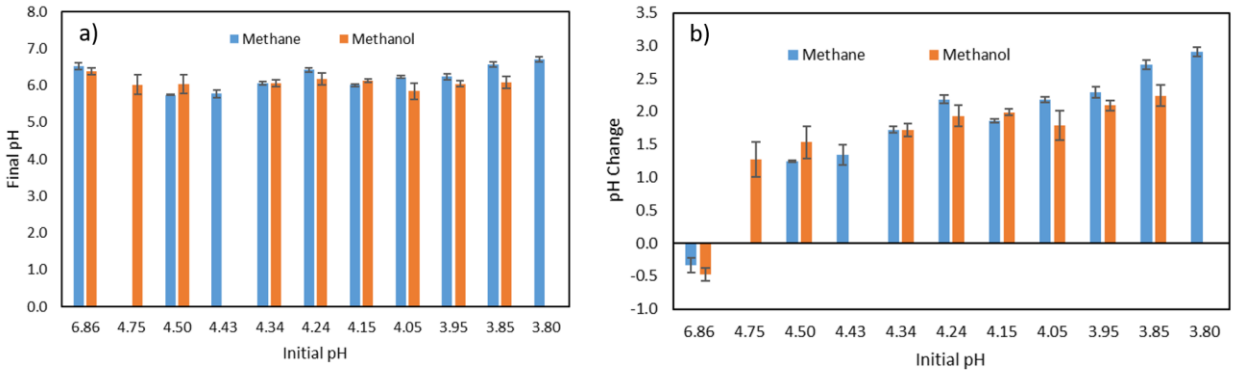

Figure 4.17 Final pH (a) and change in pH over the culture period (b) for each initial pH over the course of adaptation experiments in which *M. album* BG8 was grown in NMS medium on methane (blue) or methanol (red) at 30ºC. Each value is the mean of passages performed at the corresponding initial pH. Error bars indicate standard deviation for 3-7 passages at each pH.

The first thing of note in Figure 4.17 is that unadapted cells growing at pH 6.86 led to small decreases in pH over the course of the cultures, whereas all passages performed at lower initial pH with adapting cells led to increases in pH. Interestingly, there was also a difference in final pH when *M. album* BG8 was grown on methane or methanol. The final pH in methanol-grown cultures remained fairly consistent regardless of initial pH, ending between pH 6.00 and 6.17 for cultures initiated at pH 4.75-3.85. For cultures grown on methane, on the other hand, the final pH tended to increase as the initial pH decreased. For example, the final pH of a cultured started at an initial pH 4.5 was pH 5.74, and this gradually increased up to a final pH of 6.71 for cultures initiated at pH 3.80. There was also no significant pattern of final pH versus final OD in either growth condition.

Testing was done to establish whether the increase in pH when adapted cells were grown on methane or methanol was due to a release of ammonium. However, no ammonium was present in any of the samples tested for cells grown in NMS.

4.7 Genetic Characterization

DNA analysis was used to determine differences between the reference *M. album* BG8 genome sequence and the genome sequences of adapted cells. Tables 4.6 to 4.10 highlight the differences, mutations and SNPs, found between the cells adapted to low pH in methane or methanol and growing at pH 3.85 compared to the reference genome. All tables include mutations occurring in important coding regions – hypothetical proteins, repeat regions, and non-coding regions were not included in the analysis. Mutations

larger than 12 base pairs did not have the specific amino acid changes included in the tables.

Table 4.6 shows mutations of cells adapted and grown on methane at low pH compared to the reference genome. It displays 19 mutations of interest; while 25 other mutations found in different locations (non-coding, hypothetical protein, repeat regions) were not included in this analysis. Mutations ranged in length from 8 to 12,974 base pairs, and were distributed throughout the entire genome. 61.5% of the highlighted mutations were insertions and 38.5% were deletions.

Table 4.6 Mutations of interest in *M. album* BG8 adapted to methane at pH 3.85 compared to M. *album* BG8 reference genome.

Type of Mutation	Start Position	Length	IMG Reference Gene	Amino Acid Change	Function
Insertion	326,462	254	2508546663	> 5 amino acid change	WD40 repeat-containing protein
Deletion	517,080	51	2508546811	> 5 amino acid change	Serine/threonine protein kinase
Deletion	517,237	957	2508546812	> 5 amino acid change	Transposase IS5 family
Insertion	666,904	181	2508546918	> 5 amino acid change	Helicase family protein with metal-binding cysteine cluster
Deletion	1,782,881	351	2508547926	> 5 amino acid change	Transposase
	1,783,306	1,635	2508547927	> 5 amino acid change	Transposase
Insertion	2,173,171	1,185	2508548273	> 5 amino acid change	Carbamoylphosphate synthase large subunit
Insertion	2,571,781	30	2508548626	> 5 amino acid change	Porin
Deletion	2,571,890	12	2508548626	Ser/Ala/Thr/Gly	Porin
Insertion	2,628,177	12,974	2508548652	> 5 amino acid change	Non-ribosomal peptide
Deletion	2,629,940	1,763			synthase/amino acid adenylation enzyme
Insertion	2,914,335	8	2508548916	Glu/Gln +	Type I secretion protein TolC
Deletion	3,016,314	957	2508549022	> 5 amino acid change	Transposase IS5 family
Repeat Deletion	3,325,215	9	2508549300	Lys/His/Glu	Hybrid non-ribosomal peptide synthetase/type I polyketide synthase
Repeat Expansion	3,989,868	9	2508549931	Met/Gly/Gly	Chaperonin GroL
Deletion	4,014,110	957	2508549958	> 5 amino acid change	Transposase IS5 family
Deletion	4,246,250	568	2508540169	> 5 amino acid change	VCBS repeat-containing protein
Insertion	4,246,928	1,126	2508540169	> 5 amino acid change	VCBS repeat-containing protein
Deletion	4,281,503	957	2508550195	> 5 amino acid change	Transposase IS5 family

Table 4.7 shows the mutations found in growth on methanol compared to the reference genome. It displays 22 mutations of interest, with 19 other mutations found in

different locations that were not included in this analysis. Mutations ranged in length from 8 to 12,857 base pairs and were distributed throughout the entire genome. For the included mutations, 50.0% were insertions and 50.0% were deletions, different from results obtained for adaptation in methane. Between Tables 4.6 and 4.7 there were 6 mutations that were the exact same between methane and methanol growth, and another 8 mutations that occurred in the same gene but at a different position or with a different mutation length.

Table 4.7 Mutations of interest in *M. album* BG8 adapted to methanol at pH 3.85 compared to M. *album* BG8 reference genome.

Type of Mutation	Start Position	Length	IMG Reference Gene	Amino Acid Add/Loss	Function
Deletion	517,084	46	2508546811	> 5 amino acid change	Serine/threonine protein kinase
Deletion	517,237	957	2508546812	> 5 amino acid change	Transposase IS5 family
Insertion	901,919	30	2508547136	Tandem Repeat	TIGR03118 family protein
Insertion	1,784,549	10,510	2508547927	> 5 amino acid change	Transposase
Insertion	1,808,914	219	2508547947	> 5 amino acid change	tRNA-Gly
Insertion	2,299,625	1,185	2508548387	> 5 amino acid change	Esterase/Lipase
Deletion	2,571,896	12	2508548626	Thr/Gly/Ser/Ala	Porin
Insertion	2,628,076	12,857	2508548652	> 5 amino acid change	Non-ribosomal peptide
Deletion	2,629,790	1,646		> 5 amino acid change	synthase/amino acid adenylation enzyme
Insertion	2,914,342	8	2508548916	Ser/Asn +	Type I secretion protein TolC
Deletion	3,016,314	957	2508549022	> 5 amino acid change	Transposase IS5 family
Insertion	3,071,047	1,184	2508549068	> 5 amino acid change	Penicillin amidase
Repeat Deletion	3,325,222	9	2508549300	Lys/His/Glu	Hybrid non-ribosomal peptide synthetase/type I polyketide synthase
Deletion	3,620,423	1,144	2508549561	> 5 amino acid change	ATPase involved in chromosome pairing
Insertion	3,622,587	6,807	2508549563	> 5 amino acid change	Beta chain of methionyl-tRNA
Deletion	3,622,587	983		> 5 amino acid change	Synthetase CDS
Insertion	3,913,512	857	2508549858	> 5 amino acid change	S-adenosyl-methyltransferase MraW CDS
Repeat Expansion	3,989,868	9	2508549931	Met/Gly/Gly	Chaperonin GroL
Deletion	4,014,110	957	2508549958	> 5 amino acid change	Transposase IS5 family
Deletion	4,246,250	568	2508540169	> 5 amino acid change	VCBS repeat-containing protein
Insertion	4,247,010	1,125	2508540169	> 5 amino acid change	VCBS repeat-containing protein
Deletion	4,281,503	957	2508550195	> 5 amino acid change	Transposase IS5 family

Table 4.8 shows SNPs and point mutations in *M. album* BG8 compared to the reference genome that were found to be similar between adaptation to low pH on methane or methanol. It shows 47 SNPs and point mutations common to both *M. album* BG8 genomes of cells adapted to growth at pH 3.85 on methane or methanol. Of these, 3 were point insertions, 7 point deletions, and 37 had the nucleotide switched. These insertions and deletions caused a frameshift, which may or may not affect the amino acid at the position at which the change occurred (see position 2,747,292 vs 2,913,121), but eventually led to significant amino acid shifts (indicated by the +) continuing through the rest of the gene length. Of the nucleotide changes, 19 were silent mutations, 10 were conservative missense mutations, 7 were non-conservative missense mutations, and 1 was a nonsense mutation.

Table 4.8 Common SNPs and point mutations in *M. album* BG8 adapted to low pH when grown in NMS on either methane or methanol at pH 3.85 compared to *M. album* BG8 reference genome.

Position in Ref	Change	Gene	Amino Acid Change	Function
523,074	T-C	2508546818	Leu-Pro	Glycosyl transferase
523,137	A-G	2508546818	His-Arg	Glycosyl transferase
523,147	A-G	2508546818	Leu-Leu	Glycosyl transferase
523,258	T-C	2508546818	Gly-Gly	Glycosyl transferase
523,306	A-G	2508546818	Glu-Glu	Glycosyl transferase
523,546	C-T	2508546818	Asp-Asp	Glycosyl transferase
523,555	A-G	2508546818	Leu-Leu	Glycosyl transferase
569,545	G-C	2508546840	Leu-Leu	Malto-oligosyltrehalose synthase
1,030,576	.-G	2508547263	Ala-Gly +	Drug resistance transporter, EmrB/QacA subfamily
1,077,488	A-T	2508547309	Tyr-Phe	Choline dehydrogenase-like flavoprotein
1,077,492	A-.	2508547309	Lys-Asn +	Choline dehydrogenase-like flavoprotein
1,211,201	A-G	2508547433	Leu-Leu	Ketol-acid reductoisomerase
1,392,148	C-T	2508547595	Asp-Asp	Transposase
1,392,950	C-A	2508547595	Pro-Gln	Transposase
1,392,998	T-C	2508547595	Val-Ala	Transposase
1,393,073	C-A	2508547595	Pro-His	Transposase
1,641,433	A-G	2508547812	Asp-Gly	DNA-binding protein H-NS

1,942,714	A-G	2508548073	Asn-Ser	Flagellar basal body-associated protein
2,153,431	A-G	2508548256	Leu-Leu	DNA/RNA helicase, superfamily II, SNF2 family
2,277,970	G-A	2508548366	Asp-Asn	Succinate dehydrogenase, hydrophobic membrane anchor protein
2,353,695	T-C	2508548443	Pro-Pro	Putative thymidine phosphorylase
2,359,509	T-G	2508548448	Val-Gly	Putative permease
2,397,605	C-G	2508548479	Thr-Thr	Tandem-95 repeat protein
2,399,335	.-T	2508548479	Leu-Phe +	Tandem-95 repeat protein
2,477,863	G-.	2508548543	Arg-Arg +	Transposase IS5 family
2,477,913	G-.	2508548543	Gly-Ala +	Transposase IS5 family
2,647,577	A-G	2508548670	Glu-Gly	Cytochrome c
2,747,292	G-.	2508548773	Gly-Gly +	RHS repeat-associated core domain protein
2,913,121	A-.	2508548914	Asn-Thr +	Type I secretion membrane fusion protein, HlyD family
3,019,029	A-C	2508549025	Glu-Ala	Tfp pilus assembly protein PilN
3,535,118	C-T	2508549490	His-Tyr	Transposase
3,535,239	A-G	2508549490	Val-Val	Transposase
3,535,245	T-C	2508549490	Ala-Ala	Transposase
3,535,314	G-A	2508549490	Gln-Gln	Transposase
3,535,326	A-G	2508549490	Ala-Ala	Transposase
3,535,601	T-C	2508549490	Pro-Pro	Transposase
3,535,619	A-G	2508549490	Pro-Pro	Transposase
3,535,658	T-C	2508549490	Phe-Phe	Transposase
3,535,737	C-G	2508549490	Thr-Arg	Transposase
3,535,751	T-C	2508549490	His-His	Transposase
3,563,785	G-A	2508549518	Ser-Asn	Phosphatidylserine decarboxylase precursor
4,099,669	.-T	2508550031	Arg-Arg +	16S RNA G1207 methylase RsmC
4,195,163	T-C	2508550124	Val-Ala	Outer membrane protein
4,245,718	C-.	2508550169	Gly-Gly +	VCBS repeat-containing protein
4,384,652	G-.	2508550287	Gly-Val +	23S rRNA
4,387,600	C-T	2508550290	Gln-Stop	16S rRNA
4,431,432	T-C	2508550334	Leu-Pro	Transglutimase

Table 4.9 shows SNPs and point mutations specific to *M. album* BG8 adapted to low pH on methane compared to the reference genome. It shows 2 differential SNPs, as well as 2 deletions and 3 insertions leading to frameshifts as described for Table 4.8. The 1 nucleotide change was a conservative missense mutation. Overall, methane growth had 54 SNPs and point mutations found in important coding genes, while 13 other SNPs were found in hypothetical proteins, non-coding regions and repeat regions. The 53 genes included in Tables 4.8 and 4.9 have 38 nucleotides changes, 6 insertions and 9 deletions.

Table 4.9 SNPs and point mutations specific to *M. album* BG8 adapted to low pH on methane at pH 3.85 compared to *M. album* BG8 reference genome.

Position in Ref	Change	Gene	Amino Acid Change	Function
1,835,041	A-G	2508547976	Asn-Ser	Response regulator with CheY-like receiver, AAA-type ATPase, and DNA-binding domains
1,998,398	.-T	2508548130	Gly-Gly +	Flagellar hook-basal body protein CDS
2,225,425	C-.	2508548317	Tyr-Tyr +	Transposase IS5 family
2,421,428	.-T	2508548497	Ala-Cys +	Preprotein translocase, SecA subunit
2,522,227	.-A	2508548579	Gln-Thr +	ATPase involved in DNA repair
4,102,624	T-.	2508550034	Phe-Phe +	Transcription-repair coupling factor Mfd

Table 4.10 shows SNPs and point mutations specific to *M. album* BG8 adapted to low pH on methanol compared to the reference genome. It shows the 10 differential SNPs and point mutations, 8 of which were frameshifts due to 6 insertions and 2 deletions. The remaining 2 nucleotide changes were both conservative missense mutations. In addition to the 57 SNPs and point mutations included for methanol adaptation in Tables 4.8 and 4.10, of which 40 are nucleotide changes, 6 are insertions and 11 are deletions, there are 31 others not included.

Table 4.10 SNPs and point mutations specific to *M. album* BG8 adapted to low pH on methanol at pH 3.85 compared to *M. album* BG8 reference genome.

Position in Ref	Change	Gene	Amino Acid Change	Function
825,698	.-G	2508547068	Leu-Phe +	Helicase, type I site-specific restriction-modification system restriction subunit
1,823,423	.-T	2508547964	Val-Val +	Aminodeoxychorismate lyase
2,159,251	T-C	2508548261	Leu-Pro	UDP-N-acetylmuramyl pentapeptide phosphotransferase/UDP-N-acetylglucosamine-1-phosphate transferase
2,225,426	C-.	2508548317	His-Thr +	Transposase IS5 family
2,572,361	G-.	2508548626	Ser-Ser +	Porin
2,590,020	.-A	2508548636	Glu-Glu +	Adenosine deaminase
3,031,614	G-A	2508549037	Gly-Asp	Protein-export chaperone SecB
3,152,728	.-A	2508549129	Asn-Lys +	Efflux transporter, outer membrane factor lipoprotein, NodT family
3,355,027	.-A	2508549332	Gln-Gln +	Oxygen-independent coproporphyrinogen III oxidase
3,964,442	.-A	2508549913	Glu-Glu +	Glucose-6-phosphate 1-dehydrogenase

4.8 Morphology

TEM images of *M. album* BG8 are shown in Figures 4.18 and 4.19. Figure 4.18 shows images of unadapted *M. album* BG8 cells grown on methane in NMS at pH 6.86 and adapted cells grown at pH 3.85 and pH 6.86. Figure 4.19 shows the same for cells adapted to low pH on methanol. In Figure 4.18 cells grown at pH 6.86 without adaptation look healthy, showing good texture, and no dark or transparent cells. There is also a good distribution of circular and oblong cells, with many circular cells being the same size, and some of the oblong cells starting to divide. One interesting feature is that the medium seemed to be abundant in Outer Membrane Vesicles (OMVs), which locate themselves primarily in areas where cells were grouped together. Upon adaptation to low pH, some cells began to show less texture. More cells appeared darker or even black or seemed to be transparent and lighter in colour. Cells also appeared to be generally smaller. Moreover, the media from adapted cell cultures at pH 3.85 no longer contained OMVs. When adapted cells were returned to pH 6.86, texture remained noticeable, although

perhaps not as much as originally seen in unadapted cells, but dark or transparent cells were not observed. Also, interestingly, adapted cells at pH 6.86 still did not produce OMVs.

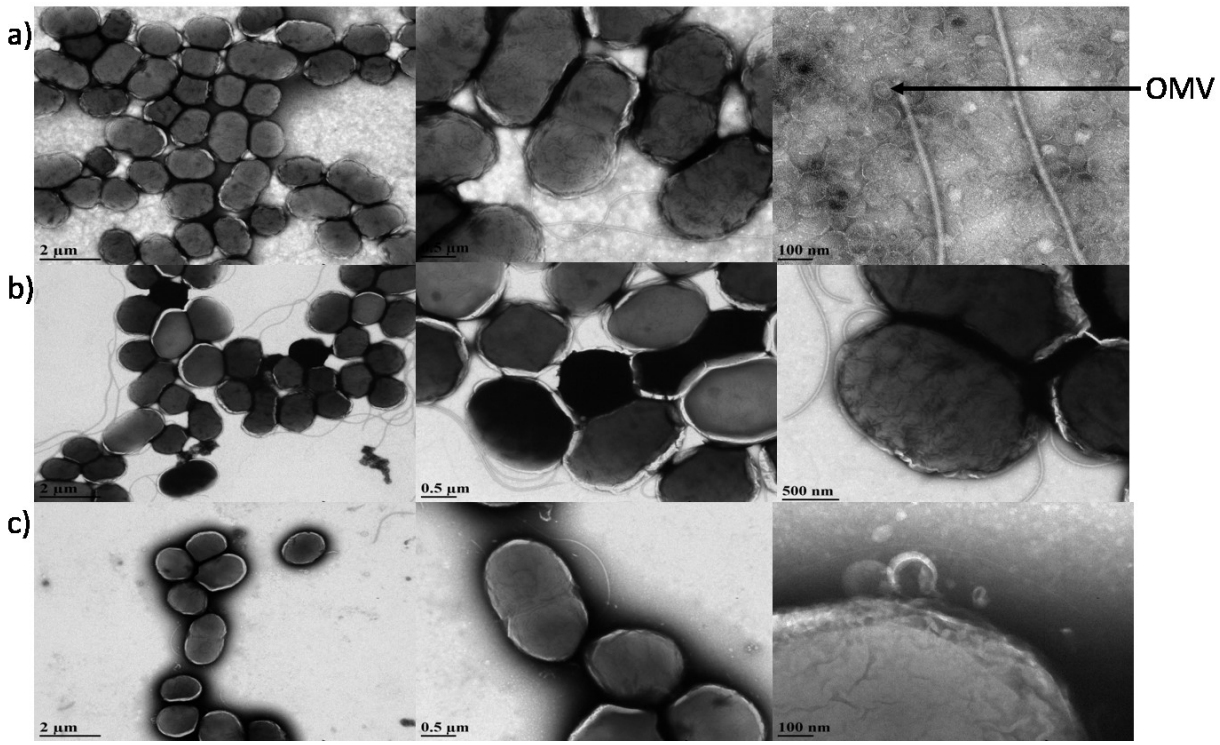

Figure 4.18 TEM images of *M. album* BG8 cells in NMS grown on methane. Cell conditions are a) Unadapted pH 6.86 b) Adapted pH 3.85 c) Adapted pH 6.86.

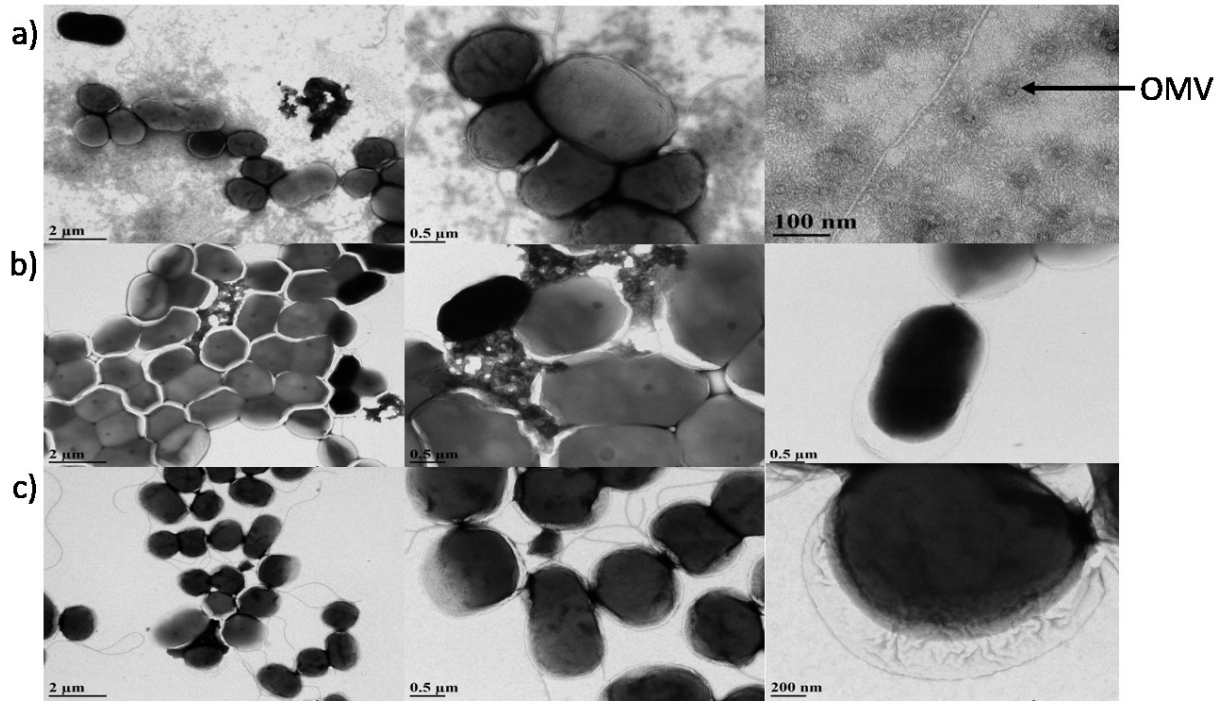

Figure 4.19 TEM images of *M. album* BG8 cells in NMS grown on methanol. Cell conditions are a) Unadapted pH 6.86 b) Adapted pH 3.85 c) Adapted pH 6.86.

Cells grown on methanol in Figure 4.19 immediately appear to be more transparent, even for unadapted cells at pH 6.86. Darker cells still showed texture, and few cells were completely black. These unadapted cells also produced OMVs similar to those produced when grown on methane. Few of the adapted cells grown at pH 3.85 showed any texture; most were transparent and light in colour, while the rest were completely dark. Again, similar to adaptation to low pH in methane, OMVs were no longer observed when adapted cells were grown in the low pH medium. Another notable aspect at this pH is that the inner membranes of some cells seemed to be retracting from the outer membrane. When adapted cells were returned to pH 6.86 transparency decreased

and texture returned, while OMVs were still not present in the medium. At this pH some cells still showed retraction of the inner membrane seen at pH 3.85.

5 Discussion

5.1 Screening of Growth Conditions

The first notable observation from the screening of growth in different carbon and nitrogen sources (Figure 4.1) is that *M. album* BG8 grows to its highest density on methanol in either nitrogen conditions. A potential cause of this could be that the direct uptake of methanol by the cells, skipping the MMO step in the carbon assimilation pathway, reduces the need for additional oxygen or reducing equivalents used in that step, rendering the process of biomass production more energetically efficient (Best & Higgins, 1981; Hanson & Hanson, 1996; Van Dijken & Harder, 1975).

It can also be seen that growth rate on AMS/Methane is significantly faster than for the other conditions, and that this conditions also led to a greater maximum OD than NMS/Methane (Figure 4.1). This agrees with previous studies that have shown that *M. album* BG8 copes well with ammonium co-metabolism inhibition and nitrite or hydroxylamine toxicity to perform better in AMS conditions than in NMS (Nyerges *et al.*, 2010; Tays *et al.*, 2018).

Preliminary pH screening tests in AMS/Methane were performed and established that the parental strain to be utilized in adaptive evolution studies could only grow at pH ranging between pH 6.0-9.0 (Table 4.2). Even though, in these specific tests, growth at pH 6.8 (considered the reference condition) performed poorly compared to previous passages – suggesting that the culture used may not have been ideal –, comparison of growth at different pH was still considered valid and a range of pH, from pH 5.5 to 8.0, for more elaborate screening could be determined.

In addition, cells did not grow well at pH 5.5 in AMS/Methane – OD did not exceed 0.21, which was attained before the start of exponential phase in optimally growing cells in this medium (pH 6.0-6.8). Cells performed slightly better in pH 5.5 in AMS/Methanol conditions, growing to a maximum OD just below 0.60 – which was still lower than OD of 1.13 and 1.66 obtained at pH 6.0 and 6.8. NMS/Methane conditions showed similar growth rate, lag phase, and maximum OD for cells grown at pH 5.5, 6.0, and 6.8. Growth rate and maximum OD were greatest at pH 6.0 in NMS/Methanol conditions, while pH 5.5 led to maximum OD lower than that obtained at pH 6 but higher than at pH 6.8; all three pH had similar growth rate and lag phase. However, the pH 6.8 bottles grew to less than half of the typical max OD of ~1.8 for an NMS/Methanol condition, as shown in Figure 4.1. From these experiments, the best conditions for growth were found to be pH 6.8 for AMS/Methane, pH 6.8 for AMS/Methanol, pH 5.5 or 6.0 for NMS/Methane, and pH 6.0 for NMS/Methanol (Figures 4.2-4.4), based on a combination of maximum OD and growth rate.

Comparing all of the conditions to each other we can see that, while AMS led to higher OD in reference conditions, these values decreased considerably at lower pH (Figure 4.3). On the other hand, results were better (higher maximum OD, no significant decrease in growth rate) in NMS at lower pH (Figures 4.3 and 4.4). This suggests that *M. album* BG8 encounters little detrimental effects from growth at pH 5.5; or perhaps, based on the increase in maximum OD, even has a preference for slightly acidic environments. It should be noted that, as commonly encountered with methanotrophs, growth was occasionally inconsistent between different replicates performed in the same conditions but at different times. This can have multiple potential causes, from the water used to

make media (which cannot be too pure nor have too many impurities) to inconsistent agitation and temperature due to manipulations in incubators. However, cultures that were initiated at the same time showed reliable consistency between replicates.

Methanol toxicity is known to affect methanotrophs to different degrees. The levels of growth of *M. album* BG8 on methanol observed throughout this study are consistent with the little inhibition due to methanol toxicity previously reported for this strain (Tays *et al.*, 2018). However, the same previous study showed growth began to be inhibited when the concentration of methanol was 20 mM, with a growth rate of 0.055 h^{-1}, (compared to 0.144 h^{-1} for 10 mM methanol) (Tays *et al.*, 2018). This conflicts with the work shown here, in which cultures were fed methanol at a concentration of 50 mM and displayed no signs of toxicity; the growth rate observed was 0.139 h^{-1} in AMS/Methanol which compares well to the 10 mM condition reported above. However, growth on methanol in this study did still regularly display a longer lag phases and more variation in their duration. This suggests methanol toxicity may still have had effects on cultures, but once the cells overcame these within lag phase, consistent growth rates and maximum OD were achievable.

5.2 Adaptive Evolution in Ammonium-Based Media

Attempted growth in AMS medium at pH 5.0 led to very slow growth rates and low maximum OD. This was not surprising as the pattern displayed in the screening passages suggested growth in AMS was reaching its limits as pH went below 6.0 (Figures 4.2-4.4). This slower, lower growth is likely due to the uptake of ammonium, the only nitrogen

source in this medium, being limited at pH lower than 6.0 because passive diffusion into the cells becomes limited. *M. album* BG8 does encode for the ammonium transporter AmtB, which has been shown in purified form to lose 80% of activity at pH below 5.5 (Wacker *et al.*, 2014). Another potential factor at play could be that *M. album* BG8 may oxidize ammonia into nitrite via hydroxylamine, reactive molecules that can generate nitrous acid, nitrosonium (NO+), and other possible cytotoxins at acidic pH.

After multiple attempts to improve growth (which included restarting cultures and increasing the concentration of carbon source by adding additional inoculum) in AMS at pH 5.5 and 5.0 failed, growth was attempted at pH 6.5, as a potentially better starting point for the adaptation study. However, at this condition, growth was still significantly lower than that at pH 6.0 during screening tests (Table 4.3, Figures 4.2-4.4). Again, multiple attempts were made to improve growth by adding inocula from cultures fully grown at pH 6.8 but cells never grew to a growth rate or maximum OD conducive to attempting further adaptation studies. One possible reason for this change in the performance in AMS could be small differences in the preparation of media using phosphate buffers and HCl to adjust pH. However, this only further supports the fact that adaptability in AMS was poor. These reasons, along with the success of adaptation in NMS, led to the discontinuation of adaptive growth in AMS.

5.3 Adaptive Evolution in Nitrate-Based Media

Adaptive evolution of *M. album* BG8 in NMS was initiated by transferring cultures formerly grown at pH 5.5 into the same medium at pH 5.0 and passaging them five times

(Figure 4.5). Each culture was allowed to grow well into stationary phase before being passaged by transferring the equivalent of a 2% inoculum into fresh medium. In the cases when the culture would not start to grow, an additional 2% inoculum (equivalent to a total 4% inoculum) was added to support growth. For example, after three successful passages in NMS/Methane at pH 5.0 using a 2% inoculum, growth struggled to take off and an additional 2% inoculum was added, enabling the culture to grow (Figure 4.5). This strategy, consisting of increasing the inoculum to 4% when cultures were not readily growing, was used henceforth when required. In all cases, the initial adaptation to pH 5.0 did not impede on the health of the cultures as they grew to similar OD and had similar growth rates than cultures grown at pH 5.5, and this using either methane or methanol.

After 5 sequential passages on both methane and methanol were successful, cells were passaged into pH 4.75 with their respective carbon source. From this point on, cells were passaged from late log phase or early stationary phase, to facilitate and accelerate the onset of growth in the new passages, while also allowing the assessment of maximum OD and growth rate.

Adaptive evolution was then further pursued by transferring the fifth passage at pH 5.0 into the same medium of interest at pH 4.75 for five more passages, and so on with decreasing pH. Figures 4.6 and 4.7 show the passages for adaptation at each pH for growth on methane and methanol, respectively. It rapidly became apparent that the lag phase was longer and more replicate failure could be observed (Figures 4.8 and 4.9). among the first 2-3 passages at a new pH. It bears repeating that when a replicate could not readily grow, an additional 2% inoculum would be added, and if this failed, the passage would be restarted from the previous passage. Additional inoculum would not

be added beyond a total of 4%, as 2% inocula increased pH by ~0.10-0.15, so further addition would increase the initial pH of a medium to a level that would be greater than the previous pH condition, therefore not helping with adaption to the new lower pH. In most cases, after 2-3 passages, cultures became more consistent, primarily when considering decreased lag phase and fewer failed passages. In cases when additional 2% inocula were still required after 5 passages, growth in the same pH would be continued for additional passages. With growth that did not require the 2% addition, one or more replicates would often still fail to grow, with the odds of this increasing as pH decreased.

For both cultures growing on methane and methanol, once adaptation cultures reached pH 3.95 the length of lag phases increased significantly, additional inocula were needed more often, and more replicates failed – with often only one replicate growing; this resulted in more failed passages. Cells were not sufficiently adapted to the new pH after 5 passages and required further adaptation passages at that pH. And again, this continued into pH 3.85, which methanol cultures were unable to overcome, while methane cultures adapted quickly here but then stagnated at pH 3.80 growth.

Throughout the adaptation process some interesting differences between growth on methane and methanol could be observed (Tables 4.4 and 4.5). Growth rate remained similar for growth on methane between pH 6.86 and pH 5.50. It then dropped slightly at pH 5.00 and again at pH 4.75 where it maintained a relatively constant level from here to 3.95, beyond which growth rates at pH 3.85 and 3.80 were significantly reduced. Methanol growth was slightly different in that a drop in the growth rate was noticed immediately upon moving from pH 6.86 to pH 5.50; the growth rates then remained steady

until a further decrease was observed at the final pH of 3.85. The significant drop in growth rate at pH 3.95 or 3.85 is another indication that the cells were reaching their limit for growth in this low pH.

Looking at the complete adaptation maps (Figure 4.8 and 4.9) highlights many important observations. For one, the cultures took the same amount of time to adapt from pH 4.75 to 4.05 as they did adapting from pH 3.95 to 3.80, demonstrating the cells required more time to adapt at the lower pH. A few reasons for this slow in adaptation are that passages have longer lag phases because cells need to increase extracellular pH, that there are more failed passages that need to be restarted at lower pH, and cells need more than just five passages within a pH before fully adapting and being passaged to a lower pH. It is also interesting to note that growth rate and maximum OD did not vary between early and late passages within a pH step. This suggests that pH adaptation most noticeably affected the length of the lag phases, where cells needed to adjust to the new growth conditions. Eventually, pH becomes too low for cell growth to be viable due to the energetic strains of these low pH conditions, causing the adaptation process to reach its limit and slow down or stop altogether.

Cells were unable to be passaged beyond pH 3.80 on methane and pH 3.85 on methanol (Figures 4.8 and 4.9). This suggests that the mechanisms involved in the adaptation to this low pH can no longer expel protons from the cytoplasm or periplasm, meaning membranes and proteins within membranes are being acidified and do not function properly (Lund *et al.*, 2014). This range of pH (3.80-3.85) as a limit to adaptation is consistent with studies performed with other neutrophilic bacteria, such as *E. coli* or *Salmonella typhimurium,* which have been shown to lose their ability to grow at pH

between 3.6 and 4.4 (Conner & Kotrola, 1995; Lin *et al.*, 1995; Presser *et al.*, 1998). Acidophilic bacteria have the ability to use other mechanisms to promote growth at lower pH, such as forming a Donnan potential or using different proton transport systems (Mirete *et al.*, 2017). One of these acidophilic mechanisms that *M .album* BG8 does encode for is the Arginine decarboxylase (AdiA), also found in other neutrophilic bacteria like *E. coli* which can function below pH 3.8 (Richard & Foster, 2004). However, this mechanism is primarily used for survival at very low pH, versus growth at moderately low pH (Lund *et al.*, 2014).

Re-adaptation of cultures was necessary after cultures adapted in methane at pH 3.80 were found to be contaminated. Cultures were restarted from uncontaminated cell stocks in order to evaluate if data was indicative of methanotroph growth. This was done in two ways to verify the adaptation. Cultures already adapted to low pH on methanol were grown in NMS/Methane at pH 3.85. At the same time, uncontaminated methane-grown cell stocks adapted to pH 4.24, were adapted from this pH down to pH 3.95. In both cases growth was shown to perform similarly to the contaminated cultures, suggesting that data from previous growth was representative of methanotrophic growth. This experiment also demonstrated that the adaptation could be repeated successfully, with similar or different mutations leading to the same endpoint. This is notable as many other experiments have shown populations grown in the same conditions separately that have adaptations which diverge from one another (Goho & Bell, 2000; R E Lenski & Travisano, 1994). However, other studies have shown results that support the work shown here, with multiple attempts leading to the same or similar growth outcomes (Agashe *et al.*, 2016; Kram *et al.*, 2017).

5.4 Adapted vs Unadapted

An essential aspect of the characterization of adaption lies in the establishment of a new phenotype, the extent of the resulting trait, and the evaluation of the cause of this behavior. For example, it was important to determine whether the adaption to growth at low pH impeded on the growth at conditions near normal pH. In this study, cells that had been adapted by growth on methane or methanol showed no loss of their ability to grow in the reference conditions (pH 6.86), with adapted cells growing very similarly to unadapted cells at pH 6.86 (Figure 4.11 for comparison of growth and adaptation in methane and Figure 4.12 for methanol). Unsurprisingly, unadapted cells did not grow at low pH with either methane or methanol, while adapted cells did grow. Interestingly, adapted growth at low pH even performed closely to growth at pH 6.86, with similar growth rate and maximum OD, but having a longer lag phase, especially when growing on methanol.

Adapted cells were further tested in their ability to alternatively grow from low pH to high pH and back (Figures 4.13 and 4.14). In this case, cells adapted through growth on methane again showed little to no change between passages at different pH, as well as when passaged at the same time into two different pH, or when passaged from two different pH each into the same pH. Methanol growth did indicate a slight growth advantage when passaged into pH 6.86 versus pH 4.05, and maintained this advantage into a passage at pH 4.05, this could be due to the passage in pH 6.86 resulting in healthier cells moving forward.

This data suggests that independent adaptation, rather than antagonistic pleiotropy or mutation accumulation, was the mechanism involved since performance at pH 6.86 was not negatively affected throughout adaptation to low pH (Elena & Lenski, 2003).

The stability of adaptations was maintained when adapted cells were passaged into pH 6.86 for ~40 generations, and this for both adaptation with methane or methanol (Figures 4.15 and 4.16, respectively). This suggests that adaptations were not just phenotypic, and are in fact maintained by the cells regardless of the current growth conditions (Smits *et al.*, 2006). One interesting feature for methane growth (Figure 4.15) was that growth rates in pH 4.05 after growth at pH 6.86 (0.122 h^{-1}) were actually slightly faster than what was reported in initial adaptation at pH 4.05 (Table 4.4 - 0.105 h^{-1}). There was also a small decrease in the growth rate of adapted pH 6.86 cells during methane (0.128-0.136 h^{-1}) and methanol (0.111-0.119 h^{-1}) growth when compared to unadapted cells grown at pH 6.86 (methane – 0.147 h^{-1}, methanol – 0.139 h^{-1}). However, overall growth performed similarly at low pH before and after passages at pH 6.86, as well as during the passages at pH 6.86, which further supports the theory that the method of adaptation was through independent adaptation, since growth in normal conditions was not affected (Elena & Lenski, 2003).

5.5 Consequences of Adaptation and Growth at Lower pH

Throughout experimentation it was determined that culture pH changed during growth. For example, growth on methane or methanol at pH 6.86 or above led to decreases in pH to ~6.50, while growth initiated at or below pH 4.75 saw differences when

cultures were grown on methane or methanol (Figure 4.17). In the latter case, growth on methane resulted in increasing pH over the length of the cultures; with the final pH having greater values as the initial pH decreased, (increasing by nearly 1.3 from inoculum pH of 4.50-3.80 (Figure 4.17)). This differed from growth on methanol, for which the final pH hovered around ~6.10, with a minimum of 5.84 and maximum of 6.39 (Figure 4.17). These increases in pH are consistent with the fact that many bacteria can alter the extracellular pH through metabolic reactions that create products such as lactic acid – for decreasing pH – or ammonia – for increasing pH (Ratzke & Gore, 2018). Most microbes change the medium in order to bring it closer the optimum pH for growth, but in some other cases pH is shifted away from optimum (Ratzke & Gore, 2018). One potential reason for the difference in the final pH of cells grown on methane versus methanol is that methanotrophs have been shown to produce different metabolites based on the carbon sources, which could alter the metabolites leading to increases in pH of the medium (Fu *et al.*, 2019; Tays *et al.*, 2018).

The increase in pH of the growth medium with growth could have resulted from the production of ammonia by the cells. In this case deiminase and deaminase systems produce ammonia which is protonated to form ammonium and raise the pH; however these functions are normally present at pH below 3.1 (Casiano-Colón & Marquis, 1988; Martinelle & Häggström, 1997). An ammonium determination assay was performed, and minimal levels of ammonium were found in growth media prior to growth, during mid growth, or after growth, suggesting that this was not the reason for increased medium pH.

Another consequence of the adaptation at low pH was the apparition of mutations and SNPs in the bacterial genome. Genome sequences of the reference *M. album* BG8

and the adapted cells were aligned using Geneious version 11.1.5 (Kearse *et al.*, 2012) and compared to each other. One limitation with this analysis is that the parental strain used for this work likely already had differences in its genome from the available sequenced reference genome. A better method would have required sequencing the parental strain from the start of adaptation along with the adapted strains. Despite this limitation, it is possible the observed mutations in the adapted cells are consistent with adaptation at low pH. Additionally, by comparing the mutations that occur in the two adapted cell lines (methane- and methanol-grown), it is possible to identify mutations that are unique to one of them (and thus highly unlikely to originate from the parental strain). The genetic analysis thus helps understand the potential modifications and mechanisms leading to better growth at low pH in adapted cells.

Many of the mutations found in Tables 4.6 and 4.7 are large insertions or deletions, which would cause remarkable differences in gene structure and folding. This is interesting in that insertions of this size (181-12,974 base pairs) surely affect the activity of the cells, despite their ability to continue growing with modifications which could, in some cases, be considered deleterious mutations. In a select few cases (e.g. Table 4.6 position 2,571,890 or 3,989,868) there is simply a small repeat insertion or deletion that is unlikely to greatly affect protein folding.

Much like the large insertions and deletions in the genome reported above, many of the changes observed at the single base pair level (Tables 4.8-4.10) were insertions or deletions of a base pair causing a shift in the amino acid sequence of the coded protein over the remainder of the gene length – essentially part of the protein would have a completely different amino acid sequence compared to the original protein. This in theory

could cause consequential changes in the genes, affecting length, structure, folding and function of the resulting protein. Considering the adapted cells retained the ability to grow, it appears the changes encountered here did not lead to deleterious mutations.

It is particularly notable that 47 of the SNPs and point mutations in both methane and methanol conditions were the exact same, as seen in Table 4.8. While it is likely that many of these SNPs were present in the genome of the parental strain compared to the reference genome, at least some of these changes could impart the adapted cells with traits that are important for growth and adaptation to low pH. It is likely some combination of both. However, considering that SNPs and point mutations occur more frequently than larger mutations, it is interesting that the changes were all at the exact same base pair locations and had the same base pair change. When it comes to SNPs and point mutations that were different between methane and methanol growth (Tables 4.9 and 4.10), most changes were in completely different genes, except for one deletion that occurred on the same gene for both conditions but one base pair location apart. All the SNPs that occurred were conservative missense mutations that likely would not cause a significant change in gene function or performance.

The reported 40 SNPs resulting in nucleotide shifts (Tables 4.8-4.10) were mostly nucleotide changes that did not alter the amino acid sequence of the specific protein, or that conferred conservative missense mutations that likely did not greatly affect the gene function. Of the select few SNPs that were non-conservative missense mutations or nonsense mutations, it is extremely interesting to see a nonsense mutation inserting a stop codon on the 16S rRNA gene and also notable to see changes in the Tfp pilus

assembly protein PilN, as well as Cytochrome c. These alterations could lead to protein folding and membrane function changes.

A number of studies on adaptive evolution of *E. coli* focused on identifying the mutations associated with adaptation to specific conditions (Barrick *et al.*, 2009; Charusanti *et al.*, 2010; T. M. Conrad *et al.*, 2009; Herring *et al.*, 2006; Kishimoto *et al.*, 2010; D.-H. Lee & Palsson, 2010). From these studies 61% of mutations found were single nucleotide changes, 29% were deletions, and 10% insertions or insertion movements (Conrad *et al.*, 2011). In this study, out of 223 total mutations found within the two genomes, 62% were found to be due to single nucleotide changes, comparing extremely well with the numbers previously discovered. However, larger mutations differed significantly from literature, with 13% coming from deletions and 25% coming from insertions.

By looking at the genetic location of many of the mutations and SNPs we see some interesting features. Many of the changes occurred in genes associated with membrane functions, whether it be specific membrane proteins, porins or membrane transporters. This is important because the membrane is a significant barrier to acidification of the cell, and many acid tolerance mechanisms occur or are initiated in the cell membrane (Lund *et al.*, 2014). Of the 105 mutations found in Tables 4.6-4.10, 25 are genes of membrane-bound proteins or proteins associated with membrane functions.

There are also surprising mutations occurring in DNA/RNA related genes, such as DNA-binding proteins, DNA/RNA helicase, 23S/16S rRNA and others – 9 of which have been discovered here. These are important as changes in cellular DNA and RNA are

crucial to DNA replication and cell survival, and DNA can be damaged by acidic conditions (Lund *et al.*, 2014). Some of these mutations may be positive, such as those in the 23S rRNA gene, which has been shown to carry mutations improving cell growth (Long *et al.*, 2010). In this case, it is particularly interesting that the mutation was a loss of a base pair leading to a frameshift causing significant changes in the gene, but apparently without carrying a deleterious effect to impede growth. Changes in many amino acid and protein synthesis pathways can also be important if the mutation affects the folding of the proteins. There were also many mutations - including 14 SNPs - that occurred on transposases, which can have a function in cell adaptability (Reznikoff, 2003).

Beyond genetic mutations that help with adaptation to new environments, mechanisms within the cells can allow cells to survive at low pH as well as increase the surrounding pH throughout growth. One such mechanism available within the *M. album* BG8 genome is arginine decarboxylase (Kits *et al.*, 2013), which is known to be present in other acid tolerance systems such as *E.coli* (Lin *et al.*, 1996; Lund *et al.*, 2014).

TEM images of unadapted and adapted cells at different pH show many differences based on carbon source, pH, or adaptation level (Figures 4.18 and 4.19). Noticeably, unadapted cells at pH 6.86 grown on either methane or methanol (Figures 4.18a and 4.19a) produce OMVs in large abundance; however after adaptation to low pH, these OMVs completely disappear from the media (Figures 4.18b and 4.19b). The most interesting feature here is that upon growth of adapted cells back into pH 6.86 (Figures 4.18c and 4.19c) the OMVs did not return, suggesting a loss of function in the adapted cell lines. This loss of OMV production is likely a function of the cells removing any unnecessary processes that might use up resources that do not contribute to cell growth

or survival at low pH. This is particularly interesting because of how adapted cells performed similarly to unadapted cells at pH 6.86 despite not producing the OMVs, which theoretically should allow these cells to have more resources to commit to growth. This suggests that although cell energy required to produce OMVs is not being used, this energy does not immediately get used towards growth. There is a possibility that energy is being utilized for mechanisms involved with acid tolerance, which can be taxing on the cells despite not being required in the current solution. This possibility is supported by the ability of cells to adapt quickly from passaging in low pH, to multiple passages at normal pH, and return to low pH without trouble (Figures 4.15 and 4.16). The loss of OMV production is also particularly interesting, as they have been determined to have a roll in stress response, including pH stress (McBroom & Kuehn, 2005).

In terms of morphology, cells grown at low pH (Figures 4.18b and 4.19b) appeared to be slightly smaller with a higher proportion were circular rather than oblong, and more translucent or completely dark cells, all signs of stress or poor health. This suggests that despite their ability to grow well at low pH, cells adapted cells were still affected by these harsh conditions. Other bacteria have been shown to demonstrate differences in cell morphology depending on pH, including unhealthy cells appearing more transparent (Rao *et al.*, 1984). Cells adapted in methanol (Figure 4.19) also appeared to be less healthy than those grown on methane (Figure 4.18), showing more transparency and less textural definition, possibly due to toxicity of methanol (Tays *et al.*, 2018). Some differences in cell shape and size can be due to stage of growth (van Teeseling *et al.*, 2017). This is likely also a cause for the differences seen here, as cells were taken from late log phase, when some cells were possibly already in stationary phase.

6 Conclusion and Future Works

6.1 Conclusion

The main goal of this study was to develop a version of *M. album* BG8 that is more amenable to industrialization in low pH conditions through adaptive evolution. This involved evaluating the growth of this organism at different pH, adapting cells to growth in the lowest possible pH using methane or methanol, characterizing the resulting adapted cell lines, and investigating the mechanisms of cellular adaptation.

Adaptation was performed in nitrate-based media as ammonium-based media were found to not be conducive to growth at lower pH. Adaptive evolution using sequentially transferred batch cultures led to cell lines that could grow using methane or methanol in media that are 3 pH units lower than the starting cultures (equivalent to 1000-fold increase in [H+]). The adaptations had little to no effect on the growth rate and maximum growth density of *M. album* BG8 cells, and this at both low pH and pH 6.86. In fact, the adapted cells performed as well as unadapted cells in terms of growth at pH 6.86. Adaptation was also found to remain stable after sequential passages at pH 6.86 followed by additional passages at low pH, suggesting that adaptations were not merely phenotypic.

The morphology of adapted cells did not change remarkably between growth at low pH and pH 6.86. Adapted cells growing at low pH showed slightly more discolouration and shrinking, both signs of cultures under stress, but the largest difference was the loss of OMV production after adaptation. The absence of OMVs was observed whether the adapted cells were grown at low pH or pH 6.86, suggesting this function loss could be of genetic origin.

Many genetic differences were discovered when comparing the genomes of adapted cells to a reference *M. album* BG8 genome. A significant number of mutations and SNPs were found to be similar between growth on methane or methanol –suggesting these were either present in the parental strain at the onset of adaptive evolution or resulted from the two adaptation trains investigated (one with methane and the other with methanol) – but there was also a number of differences between the mutations observed from the two adaptation trains. One limitation to this analysis is that adapted cells were compared to a reference genome of *M. album* BG8 which may have some differences with the strain used to perform the adaptation study. However, some of the similarity could in fact still be products of the mechanism of adaptation, as many genes affected are involved with membrane function, DNA/RNA function, or transposases, all related to cellular response to changing environments.

These findings provide important information on the adaptation of methanotrophs, which often grow poorly outside of laboratory conditions, to non-ideal conditions without consequential changes in growth performance. It also provides a starting point for the investigation of adaptive mechanisms of cells to new environments and process conditions. Finally, the study produced 2 variants of *M. album* BG8 that display traits favorable to implementation in industrial processes involving media at low pH, such as bioconversion in effluents from the pulp and paper industry and agriculture.

6.2 Future Works

The present study gives an early investigation and a starting point for further research into a number of possible areas involving methanotroph growth, adaptive evolution, and mechanisms of adaptation.

The first step for the improvement of methanotroph growth would be to adapt cells to other harsh conditions such as higher temperatures, higher concentrations of inhibitors, or in various concentrations of effluents from pulp and paper activities, which could, upon significant adaptation, provide a low to no cost medium for bioindustrial applications of *M. album* BG8.

Other methods of improving growth characteristics, such as genetically engineering pathways into or out of methanotrophs to improve bioproduction efficiency of growth, would also be beneficial. This method can also be used to engineer pathways to synthesize valuable products into the cells, such as putting the PHB cycle into Gammaproteobacteria like *M. album* BG8, which cannot themselves produce PHB but have faster and more abundant growth over most Alphaproteobacteria.

Finally, further investigation into the mechanisms of adaptation through methods such as RNA sequencing and analysis can provide valuable context into how these organisms are managing to survive and grow at such low pH.

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