MOLECULAR AND PHENOTYPIC CHARACTERIZATION OF ZINC-RESISTANT *Methylobacterium* SPECIES IN SOIL SYSTEMS

By

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A Dissertation submitted to the Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Environmental Sciences

Written under the direction of

Professor Robert L. Tate, III

and approved by

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New Brunswick, New Jersey

October, 2012

ABSTRACT OF THE DISSERTATION

Molecular and Phenotypic Characterization

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Methylobacterium species, pink-pigmented facultatively methylotrophic bacteria (PPFMs), have the potential facilitate agricultural and bioremediation processes through plant growth promotion, zinc cycling and biofilm production. Phenotypic and genetic studies of *Methylobacterium* strains were conducted to increase our understanding of the roles and evolution of PPFMs and to develop methods for characterizing environmental isolates to help realize their potential.

Chapter 1 describes the isolation of *Methylobacterium* isolates from a New Jersey agricultural soil. *mxaF*, the structural gene for the methanol dehydrogenase enzyme, was compared with the 16S rRNA gene during the characterization of 114 PPFM isolates from agricultural soil, forest soil and blueberry skin. PPFM isolate 16S rRNA sequences were too similar to distinguish them at the species level. 16S rRNA sequences clustered tightly phylogenetically whereas *mxaF* clustering paralleled phenotypic patterns. Soil type, pH and plant cover did not influence *Methylobacterium* geographic distribution or select for specific *mxaF* sequences, indicating a reason for the widespread nature and

versatility of PPFMs in the environment.

A phylogenetic survey of archived GenBank® sequences is presented in Chapter 2. *mxaF*, *mxaF*-like and *mxaF'* sequences were included with *xoxF* and other pyrroloquinoline-quinone-(PQQ)-dependent methanol/ethanol dehydrogenase sequences. *Methylobacterium mxaF* sequences clustered tightly, even with other bacterial genera similar methylotrophy genes present. *mxa* gene family operon construction revealed evidence of horizontal gene transfer (HGT). HGT events could not be confirmed by examining G+C content of *mxa* genes. It is unclear whether the PPFM *mxa* operon construction pattern is the ancestor or the descendant of those of other genera.

Biofilm production, a major mode of colonization for plant-associated bacteria, is addressed for *Methylobacterium* species in Chapter 3. The effects of various substrates and Zn concentrations on biofilm development were investigated. Low levels of Zn did not inhibit biofilm production but did affect time-dependently affect the amount of DNA per gram of biofilm material in a time-dependent manner.

Chapter 4 addresses Zn tolerance in PPFMs. Isolates exbitied precipitation of a white solid, clearing zone production, or growth with neither phenomenon. Isolates solubilized hopeite, a Zn phosphate compound. This ability can potentially raise the bioavailability of Zn in soils and was not described for this genus prior to this research.

Plant growth-promotion of red clover (*Trifolium pratense*) seeds and seedlings by PPFM isolates is described in Chapter 5. Not all *Methylobacterium* species promoted plant growth, indicating the importance of determining individual ability of PPFM isolates.

ACKNOWLEDGEMENTS

There are so many people I want to thank for their help and support during the completion of this research thesis. Above all, I want to thank my parents for their unending love and support. I am so thankful for the encouragement, guidance and advice they provided me with over the years. To my mom, for always supporting me and finding a way to make me laugh even through my most stressful and crazy times, I am eternally grateful. To my dad, your endless help with all things technical whenever I had need of it did not go unnoticed and was greatly appreciated. I love them both. What I owe them I feel can never be repaid.

I want to thank my best friend, Chris Reyes, for also being a source of support. Through good times and bad, he was always supportive of my drive to complete my thesis and often gave me much needed encouragement. He is a wonderful and irreplaceable friend and I thank him for it.

I want to thank H. Boyd Woodruff and his wife, Jeanette, for establishing the H. Boyd and Jeanette Woodruff Microbiology Fellowship. I was extremely honored to receive this fellowship early on in my graduate studies, as it is awarded by nomination only. The fellowship provided me with direct financial support initially and then stipulated that Rutgers would match this contribution in the years to come. Without this amazing opportunity for funding, I would have no doubt struggled more financially and may have had to hold a second job in order to afford to remain in graduate school. I am eternally grateful to the Woodruffs for their generosity as well as to Rutgers for the scholarships and scientific opportunities the college has afforded me over the years.

I also want to thank my professor, Dr. Robert L. Tate, III, for giving me the

opportunity to conduct research in his laboratory. His support has been invaluable and the time I have spent in his lab has been both enjoyable and enlightening. I also acknowledge all the former Tate Lab members who have helped me so much along the way. Special thanks to Cris San Miguel and Robyn Mikita for sharing so many interesting, informative, and helpful conversations with me in the lab and for becoming good friends. I want to thank also Ula Filipowicz, Melissa Dulinski and Scott Mittman for their help and support.

As nervous as I was to present my research every semester, the collective lab meetings of the Tate, Young, Yee and Reinfelder Labs have been instrumental for my development as a scientist. The opportunity to present my research has not only made me a stronger presenter, but has allowed me to discuss my work with many students, professors and other researchers who have now become friends. Over the years, there have been so many people who have helped me to reach my goals at these meetings, throughout Rutgers, and at ASM conferences each year. Thank you to Craig Phelps, Max Häggblom, Doug Eveleigh, Adam Mumford, Abbie Porter, Alexandra Walczak, Amita Oka, Erin Gallagher, Sinead Ní Chadhain, Madhavi Parikh, Elise Rodgers-Vieira, Siobhan Duffy, and John Kelly for their help.

I want to acknowledge Albrecht Koppenhöfer and Eugene M. Fuzy, not only for the opportunity to work in their entomology lab early on in my graduate career, but also for becoming good friends. Without my experience in their lab and the knowledge I gained there, Chapter 5 of this thesis probably would not exist and I would not have half the interest in soil and plants that I do today. I thank them for sparking my interest in environmental science and for helping to shape me as a scientist. Eugene has become a

close friend and I thank him for his advice and encouragement over the years.

I cannot thank the rest of my family enough for supporting me no matter what, not only in my decision to attempt higher degrees but also to follow my interests in science no matter where they might take me. My grandparents, aunts, uncles and cousins have given me the most loving encouragement over the years and I am eternally grateful to have such a large and wonderful family.

Last, but certainly not least, I feel I should acknowledge the largest contributors to this research, the genus *Methylobacterium*, or as I always called them, my "Pink People." I have grown very attached to all the striking shades of pink, the beautiful biofilms, the interesting colony textures and the general "personality" of each and every one of my PPFM isolates. As weird as it sounds, I will miss them dearly when I leave. Thank you, Pink People! I could not have done it without you.

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INTRODUCTION

Zinc as a Soil Contaminant

Heavy metals are prevalent contaminants of soil worldwide. They leech into groundwater, contaminate surface waters, and exert toxic effects on humans, animals, plants, and microorganisms. Zinc (Zn) is a common industrial byproduct, is one of many metals in sewage sludge soil amendments, and is also a contaminant found in mine tailings (Han et al., 2001). It is a micronutrient for humans, animals, plants, and microorganisms at low concentrations, but is toxic at high concentrations. Its chemical forms, bioavailability and mobility in soil and groundwater vary with soil texture, pH, temperature, and moisture (Han et al., 2001).

Remediation techniques like off-site disposal, caps or impermeable barriers to contain heavy metal contamination postpone the problem instead of decontaminating the soil. Biological methods like phytoremediation, specifically phytoextraction, take advantage of beneficial symbioses that occur between bacteria and plants in the rhizosphere. Soil can be decontaminated provided that environmental conditions are favorable and plants are subsequently harvested.

For heavy metals like Zn, however, phytoremediation is currently not a cost-effective method of remediating contaminated soil. Zinc is often one of many types of contaminants at a site and is found at nearly every superfund site in the United States in concentrations that are often several orders of magnitude higher than other heavy metals (DePue New Jersey Zinc/Mobil Chemical Site Fact Sheet #5, 1998). Limitations in extraction efficiency, toxicity to both plants and bacteria, and lack of available information on beneficial, exploitable bacteria that could improve the extraction rate are

just a few obstacles that need to be overcome for phytoextraction of Zn to be more extensively employed.

Remediation of Zn-Contaminated Soil

Remediation techniques that rely on purely physical or chemical removal of Zn from soil are difficult to employ because of the chemical properties of Zn. The most common form of Zn in soil is the Zn^{2+} cation, which complexes with other metals or organic molecules. Research indicating the utility of Zn phytoremediation is much less than that available for other heavy metals, like lead (Pb) (Huang at al., 1997; Römkens et al., 2002), arsenic (As) (Huang et al., 2004), cadmium (Cd) (Lombi et al., 2001; Römkens et al., 2002), copper (Cu) (Lombi et al., 2001; Römkens et al., 2002), nickel (Ni) (Lombi et al., 2001), or radioactive elements like uranium (U)(Huang et al., 1998). Papers that discuss the role of metal resistant, plant growth promoting bacteria in Zn-phytoextraction are emerging, but are vague as to the identities of the bacteria and the mechanisms involved (Whiting et al., 2001).

Monitored natural attenuation is often not a feasible option for heavily contaminated soils. High concentrations and mobility in the environment demand that Zn contamination be remediated quickly. Left unattended, Zn can leech into groundwater, become bound in sediments, and be transported from a site in storm water runoff (DePue New Jersey Zinc/Mobil Chemical Site IEPA Public Health Statement, 1998). At Superfund sites where high Zn contamination has gone untreated, clusters of multiple sclerosis cases in humans have correlated with proximity to the sites (DePue New Jersey Zinc/Mobil Chemical Site IEPA Public Health Statement, 1998). Therefore, a

remediation method that would biologically target the Zn but also be efficient in its removal is ideal.

Phytoextraction is a remediation technique in which contaminants (usually metals) are taken up from the environment into plants through roots and stored in shoots. Although all plants are capable of extracting metals from soil for nutrient purposes, some plants are hyperaccumulators. This means they can extract higher amounts of metals than regular plants before they experience any toxic effects (Whiting et al., 2001). A plant is defined as a hyperaccumulator of Zn when it can store greater than 10,000 ug/g of Zn in its stems and leaves (Baker and Brooks, 1989). *Thlaspi caerulescens*, one of the most studied Zn-hyperaccumulators, is capable of accumulating approximately 30,000 ug/g of Zn dw in its shoots without signs of toxicity (Brown et al., 1995).

The use of hyperaccumulator plants to biologically extract the Zn from the soil, is a thorough way of removing Zn if the plants are subsequently harvested, but it is an inefficient process. Limitations on plant ability to both extract and store Zn from soil are real obstacles faced when a Zn-contaminated site is considered for phytoremediation. If phytoremediation were to be employed regularly at Zn-contaminated sites, it would have to be more efficient than can be currently achieved.

Methods are being developed to improve the efficiency of phytoextraction for many metal contaminants in soil. The use of chemical chelators has been investigated by several research groups as a means to improve the phytoextraction of Cu, Cd, Zn, Pb, and Ni (Huang et al., 1997; Lombi et al., 2001; Römkens et al., 2002), but not without meaningful disadvantages and limitations. The non-specific binding of chemical chelators such as EDTA bring about the solubilization of multiple metals in a heterogeneous

environment like soil, causing the bioavailability of more than one metal to be increased (Lombi et al., 2001). This combined with the high efficiency of chelator compounds results in dangerous cocktails of metals being suddenly available to the plants in high concentrations (Römkens et al., 2002). Thus, even though solubility and bioavailability of the contaminants has been increased, plant toxicity occurs and phytoextraction is often hindered. Efficiency may also be hindered due to phytotoxicity and soil invertebrate mortality caused by the chelators themselves or the shift in pH their addition is likely to cause (Römkens et al., 2002). Human safety is also a concern, as metals in solution that are not quickly taken up by plants can leach into groundwater (Lombi et al., 2001). Those who have specifically studied Zn phytoextraction through the use of chelating agents noted that neither bioavailability nor extraction rate was increased enough to improve efficiency (Römkens et al., 2002).

Plant Growth Promoting Bacteria as a Means to Facilitate Zn Phytoextraction

It is possible to exploit the symbiosis between plants and plant growth-promoting bacteria to benefit processes like phytoextraction of metals and nutrient uptake of crop plants. Solubilization of metals by rhizobacteria associated with plant roots is long-term and gradual, preventing the phytotoxicity that results from a sudden spike in metal concentration frequently associated with chemical chelation. Associations between metal-resistant bacteria and hyperaccumulator plants can improve the efficiency of the phytoextraction process (Whiting et al., 2001). Increased metal solubility due to exudates from plant roots enables resistant bacteria to thrive in the rhizoplane. In Zn-contaminated soil, Zn-resistant bacteria are capable of living around the roots of hyperaccumulator

plants. Bacteria that are both Zn-resistant and known plant growth-promoters are beneficial to the phytoextraction process, as they improve not only extraction efficiency but also the storage capacity of the plant (Whiting et al., 2001). This is done either directly through the production of siderophores or indirectly through organic acids and other potential chelators produced during metabolic activity (Whiting et al., 2001).

Researchers have identified Zn-resistant bacteria associated with established *T. caerulescens* plants but have not linked them to a definitive increase in phytoextraction efficiency (Lodewyckx et al., 2002). Conversely, other research has shown that a relationship exists in which Zn-resistant bacteria mobilize zinc for easier uptake by the hyperaccumulator, but without identifying specific bacteria or a mechanism (Whiting et al., 2001). Similarly, there is research that presents real evidence of the potential for plant-associated bacteria to improve phytoextraction for other metals such as cadmium (Carlot et al., 2002). Bridging this knowledge gap and identifying key bacteria and their abilities will better allow researchers to understand, predict, and possibly stimulate these activities in the field.

Genus Methylobacterium*: Pink-Pigmented Facultative Methylotrophs*

The genus *Methylobacterium,* members of the α-2 subclass of Proteobacteria, is known to contain plant colonizers and growth-promoters. It consists of thirty-seven species of gram-negative, motile, rod-shaped bacteria (GenBank, 2012). They have the ability to use C_1 substrates (methanol, formate, formaldehyde, etc.) as sole C and energy sources (Omer *et al*., 2004; Van Aken *et al*., 2004; Chistoserdova, et al., 2003). They are

not limited to that, however, and can also metabolize C_2, C_3, and C_4 substrates (Van Aken *et al*., 2004; Omer *et al*., 2004). They are noted as being environmentally resilient in that they are dehydration, heat, cold, chlorine, ultraviolet, and ionizing radiation tolerant (Van Aken *et al.*, 2004).

Referred to as pink-pigmented, facultatively methylotrophic bacteria (PPFMs), these bacteria colonize a variety of plant species worldwide and are adapted to survival on plant surfaces. Their pink pigmentation is due to caroteinoids. Caroteinoid production may provide protection against ultraviolet (UV) radiation, encountered on the leaves and stems of plants. PPFMs are ubiquitous in soil and colonize the roots, leaves, stems, seeds and fruits of both aquatic and terrestrial plants (Van Aken *et al.*, 2004). PPFMs are found in natural and manmade environments, including sediments, fresh and saltwater systems, air, masonry, ventilation systems and hospitals (Van Aken *et al.*, 2004). They can be persistent contaminants in the laboratory. For certain strains, use of 70% ethanol or 10% bleach will not completely sterilize a surface against them and these methods must be used simultaneously (Holland and Polacco, 1992).

Methylobacterium *Species as Plant Growth Promoters*

Members of the genus *Methylobacterium* are known to colonize over 70 different species of plants (Holland and Polacco, 1992; Omer et al., 2004). By colonizing and forming a symbiotic relationship with the plant, PPFMs can both directly and indirectly improve plant health. Several research groups have noted that these associations improve the germination rate, growth, and yield of some plant species (Madhaiyan, *et al.*, 2004; Madhaiyan, *et al.*, 2005; Madhaiyan, *et al.*, 2006; Abanda-Nkpwatt *et al.*, 2006). PPFMs

can stimulate plant growth directly by producing phytohormones (e.g. IAA and zeatin) and vitamins (e.g. B_{12}) (Trotsenko et al., 2001; Lidstrom and Chistoserdova, 2002). The symbiotic relationship is fulfilled by the plants since the increased plant growth increases methanol production. Plants produce and release methanol as a byproduct of pectin metabolism during cell wall synthesis, during leaf expansion and development, and as a factor of stomatal conductance (Nemecek-Marshall et al., 1995; Galbally and Kirstine, 2002). This plant-derived methanol can then be metabolized by C_1 substrate specialists like PPFMs.

Indirectly, PPFMs stimulate the innate immune systems of plants by exposing it to proteins on the surface of the cells that trigger a defensive response. Even though PPFMs are not pathogens themselves, proteins on the cell surface or on the flagellum still stimulate a basal immune response in plants that prepares them for attacks by pathogens. This is a passive occurrence on the part of the bacteria and provides no benefit to them. The plant, however, is able to induce more complex pathogen-induced immune responses to both bacterial and fungal invaders, shortening response time and resulting in lower disease and mortality rates (Lidstrom and Chistoserdova, 2002; Van Aken *et al*., 2004).

Besides the bolstering of a plant's innate immunity to pathogens, PPFMs compete for phyllosphere niches that might otherwise be exploited by fungal and bacterial plant pathogens, causing a reduction in their growth (Romanovskaya et al., 2001). One method by which they successfully compete is through biofilm production, which can both physically and chemically exclude other microorganisms from plant surfaces (Morris et al., 2003). Understanding what affects biofilm production in the genus *Methylobacterium*

is key to ensuring that plant colonization by these bacteria will occur in a given soil system.

Zn Resistance in Methylobacterium *Species*

There is some debate as to the presence of Zn-resistance within the genus *Methylobacterium*. Some researchers have observed resistance while conducting viable plate count studies from environmental soil samples (Kunito, 1997), yet others have observed much lower resilience in the presence of Zn and would therefore classify *Methylobacterium* species as tolerant or even susceptible (Romanovskaya et al., 2001; Zarnowski *et al.*, 2002). *Methylobacterium radiotolerans* is radiation resistant, for example, but was found to be only moderately Zn tolerant with an ID_{50} of 135 ppm (Zarnowski *et al.*, 2002). My studies have suggested that, while tolerance and resistance levels vary between species, the genus *Methylobacterium* can survive in elevated $ZnSO_4$ concentrations. Lab strains were sustainable with no inhibitory growth effects at 5 mM $ZnSO_4$ or 1440 ppm. Enough doubt exists as to the Zn-resistance of *Methylobacterium* species to warrant further research.

Certain species of bacteria have been found to solubilize some metal compounds, placing them into forms that are more readily taken up and stored by the plant (Whiting et al., 2001). This effect is desirable when considering that only soluble forms of Zn are bioavailable for uptake by plant roots (Whiting et al., 2001). Other species precipitate Zn, which may be beneficial as far as lowering toxicity to both the rhizobacteria and the plant. These two behaviors, working in the same system, may help regulate the balance between the toxicity of the Zn and its uptake by the plant. In the laboratory, PPFM strains

either solubilized or precipitated Zn compounds on solid media (Kist and Tate, 2013). Understanding when and why PPFMs solubilize or precipitate Zn and being able to detect these potentially desirable phenotypes in contaminated systems is therefore important for improving phytoextraction efficiency. This knowledge would also be of importance for the agricultural industry when considering crop plants that may be deficient in the required amount of Zn for growth and favorable yields.

Methylotrophy in Methylobacterium *Species*

A methylotrophic lifestyle provides a competitive advantage for PPFMs when growing on plant surfaces in that they can take advantage of an ecological and metabolic niche in the phyllosphere not feasible for all other bacteria to utilize. *Methylobacterium* species catalyze the oxidation of methanol to formaldehyde, take that intermediate into their cells, and assimilate the carbon into their biomass (Chistoserdova et al., 1998; Chistoserdova, 2003). In doing this, they prevent a fraction of carbon dioxide (CO_2) from methanol resulting from the oxidation of methane in subsurface soils as well as plant-derived methanol from diffusing out of soil systems. In this manner, they reduce the potential for CO_2, a greenhouse gas, to enter the atmosphere.

The initial oxidation of methanol to formaldehyde is performed by the methanol dehydrogenase (MDH) enzyme. The structural gene for MDH is *mxaF*. This gene controls the resulting conformation of the enzyme and therefore its sequence can dictate not only enzyme shape but efficiency as well (McDonald and Murrell, 1997). *mxaF* is ubiquitous in *Methylobacterium* species (Kasprzak and Steenkamp, 1983) and contains several conserved regions across many genera that are considered to be essential for

proper MDH function (McDonald and Murrell, 1997). Mutations in these conserved regions of *mxaF* can effect enzyme configuration, resulting in decreased efficiency or a non-functional enzyme (McDonald and Murrell, 1997). Thus, *mxaF* sequence directly affects the methylotrophic ability of PPFMs. Characterizing soil isolates by *mxaF* sequence, therefore, distinguishes them based on methylotrophy, a functional trait, and targets bacteria that are methylotrophic via *mxa* gene family system. MxaF proteins in *Methylobacterium* species contain differences in some of the highly conserved regions of the *mxaF* gene, resulting in changes in amino acid residues (McDonald and Murrell, 1997). Those differences may be exploitable as a tool for isolate characterization in bacteria, however, recent research suggests that *mxaF* sequence is too well conserved to distinguish at a species or even genus level (McDonald and Murrell, 1997).

Importance of This Research

Developing methods for the phenotypic and genetic characterization of new environmental *Methylobacterium* isolates will help to realize the potential of this genus for use in agricultural and bioremediation applications. Understanding the limitations of biofilm production and nutrient cycling abilities of PPFM species will determine how the colonization and beneficial activities of these bacteria might be optimized to improve the health and yield of crop plants. Elucidating levels of Zn tolerance within this genus and investigating tolerance strategies that affect the bioavailability of Zn in soil will not only be useful for the agricultural industry but also for phytoremediation applications. This combined with employing a useful functional gene marker to characterize environmental strains and new species will help the scientific community move from exploring the

potential of this genus to actual application of beneficial traits to alleviate metal contamination and crop nutrient deficiency in the future.

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Chapter 1

Comparative *mxaF* Gene Sequence Phylogeny of *Methylobacterium* Isolates Obtained from Agricultural Soil, Forest Soil and Blueberry Skin

ABSTRACT

Pink-pigmented, facultatively methylotrophic bacteria (PPFMs) of the *Methylobacterium* genus utilize plant-derived methanol as a carbon and energy source when growing on plant surfaces both above and below the soil surface. To accomplish this, they employ the MDH enzyme, for which the *mxaF* gene is responsible for the structure of the final protein. *mxaF* encodes for the large or alpha subunit of MDH and changes in its sequence can result in decreased efficiency or elimination of a functional enzyme. This research examines the phylogeny of soil- and blueberry-derived PPFM isolates using both the 16S rRNA and *mxaF* genes in order to determine the feasibility of using *mxaF* as a genetic marker for this genus. *mxaF* is ubiquitous in the *Methylobacterium* genus and is highly conserved while still containing more variability as a functional gene than the 16S rRNA gene. Results indicate that PPFM 16S rRNA gene sequences are too highly similar to be useful for the species characterization of environmental isolates. PPFMs had distinct banding patterns on DGGE gels for *mxaF* that differed by species and *mxaF* sequences clustered tightly on phylogenetic trees. *mxaF* sequence and PPFM species distribution in soil was not selected for by environmental parameters such as pH, suggesting the robust and versatile nature of the *mxaF* gene in the *Methylobacterium* genus.

INTRODUCTION

Methylobacterium species are known as pink-pigmented, facultatively methylotrophic (PPFM) bacteria. They are ubiquitous in the environment and occupy several niches, such as plant leaves, stems, roots, and fruit, as well as fresh and salt water, air, dust, and soil (Omer et al., 2004; Van Aken et al, 2004; Madhaiyan et al., 2005). They have also been found in a number of manmade environments including masonry, ventilation systems, tabletops, showers, and hospitals (Omer et al., 2004; Van Aken et al, 2004; Madhaiyan et al., 2005). PPFMs specialize in the use of C_1 compounds, most notably methanol (Van Aken et al, 2004; Madhaiyan et al., 2005). Methanol is released as a byproduct of pectin metabolism during cell wall synthesis in actively growing areas of the plant (Nemecek-Marshall et al., 1995; Galbally and Kirstine, 2002). As methylotrophs, PPFMs take advantage of this metabolic ecological niche when colonizing plant surfaces. Methylotrophy confers a competitive advantage to PPFMs over other non-methylotrophic bacteria and fungi when growing in the rhizosphere and phyllosphere. Genes that control the production and conformation of the methanol dehydrogenase (MDH) enzyme are therefore essential to PPFMs living on plant surfaces.

mxaF is the structural gene for MDH, controlling the eventual conformation and thus the efficiency of the resulting enzyme (McDonald and Murrell, 1997). This gene is ubiquitous in the genus *Methylobacterium* (Kasprzak and Steenkamp, 1983). *mxaF* contains several regions that are conserved across all genera and are therefore considered to be essential for proper MDH function (McDonald and Murrell, 1997). Mutations in these regions can effect enzyme configuration, resulting in decreased efficiency or a non-

functional enzyme (McDonald and Murrell, 1997). Thus, *mxaF* sequence directly affects the ability of PPFMs to grow on methanol. Characterizing soil isolates by *mxaF* sequence, therefore, distinguishes them based on the functional trait of methylotrophy and specifically targets bacteria that are methylotrophic via the *mxa* gene family system. It has been noted in the literature that some PPFM MxaF proteins contained variation due to differences in some of the highly conserved regions of the *mxaF* gene, causing in changes in resulting amino acid residues (McDonald and Murrell, 1997). Those differences in *mxaF* sequence may be exploitable as a tool for isolate characterization, although some research suggests that *mxaF* is too highly conserve to distinguish at the species or even genus level (McDonald and Murrell, 1997).

Since the *mxaF* gene is ubiquitous in the *Methylobacterium* genus, we hypothesize that targeting the methylotrophy system that utilizes this gene is a useful genetic tool for the characterization of PPFM bacteria. We hypothesize that *mxaF* will prove to be a better tool for distinguishing species that are very closely related genetically, such as those in the genus *Methylobacterium*, than will the 16S rRNA gene. The reason for this is because *mxaF* is a conserved gene, but still a functional gene for which there will be selective pressure for the sequence to adapt to environmental conditions. The 16S rRNA gene is too well conserved to distinguish reliably enough at the species level for bacteria as similar as *Methylobacterium* species.

We investigated the validity of using *mxaF* sequence as a tool by which *Methylobacterium* species can be characterized and compare its usefulness to that of 16S rRNA gene sequence, a more traditional genetic tool for determining the relatedness of bacterial species. In addition, we investigated the genetic relatedness of

Methylobacterium isolates from agricultural soil, forest soil, and blueberry skin using their *mxaF* gene sequences. We observed any patterns that might exist between *mxaF* sequence and geographic location of the sample, sample source pH, soil type, and plant cover or source plant type to determine environmental selective factors. This helped us to understand what environmental parameters affect *mxaF* sequence and therefore potentially influence PPFM species distribution in different environments.

MATERIALS AND METHODS

Soil and Blueberry Skin Sample Information

Soil was collected from three locations in New Jersey (NJ), two in California (CA), and three in South Carolina (SC) for a total of five samples from NJ, five from CA, and nine from SC (see Tables 1, 2 and 3 for sample information and soil physical and chemical properties). Nutrient and pH data for each sample site were obtained with the help of the Rutgers Soil Testing Facility (Tables 2 and 3).

Blueberry skin biofilm *Methylobacterium* isolates were also obtained as a non-soil comparison source of PPFMs. A New Jersey-grown, store-bought blueberry visibly colonized by a pink biofilm was collected and the biofilm harvested. The biofilm was then smeared onto a series of 0.5 % (v/v) Ammonium Mineral Salts (AMS) Medium agar plates using a glass rod and bacterial colonies were allowed to grow for three weeks at 30°C in the dark. Colonies from the soil samples and from the blueberry skin biofilm were selected and grown for DNA extraction and PCR procedures. Both *mxaF* and 16S rRNA gene sequencing of each isolate verified those that were *Methylobacterium* species and all non-PPFM isolates were discarded.

Table 1. Soil Sample Characteristics (as of December, 2009). Soil samples ranged from active agricultural soils with current crops and fallow agricultural fields to forest soil.

Sample ID(s)	Sample Location	Latitude	Longitude	Soil Type & Texture	Land Use	Vegetation	Treatment	History
SC-1	Clemson University's Pee Dee Research & Education Center, Clemson, SC 29634	N34.28556	W79.74694	Noboco loamy sand (fine-loamy, siliceous, subactive, thermic Oxyaquic Paleudults)	Agricultural	Corn currently	Deep non-inversion tillage, no surface tillage	Long-term corn-ryegrass-soybean-wheat rotation
SC-2	Clemson University's Pee Dee Research & Education Center, Clemson, SC 29634	N34.28552	W79.74699	Noboco loamy sand (fine-loamy, siliceous, subactive, thermic Oxyaquic Paleudults)	Agricultural	Corn currently	Deep non-inversion tillage, no surface tillage	Long-term corn-ryegrass-soybean-wheat rotation
SC-3	Clemson University's Pee Dee Research & Education Center, Clemson, SC 29634	N34.28358	W79.74145	Lynchburg sandy loam (fine-loamy, siliceous, semiactive, thermic Aeric Paleudults)	Agricultural	Corn currently	Corn residue removed	Corn grown and residue removed for last 2 years
SC-4	Clemson University's Pee Dee Research & Education Center, Clemson, SC 29634	N34.28361	W79.74148	Lynchburg sandy loam (fine-loamy, siliceous, semiactive, thermic Aeric Paleudults)	Agricultural	Corn currently	Corn residue removed	Corn grown and residue removed for last 2 years
SC-5	Coastal Plains Soil, Water & Plant Research Center, Florence, SC 29501	N34.24572	W79.80910	Coxville sandy loam (Fine, kaolinitic, thermic Typic Paleaquults)	Agricultural	Peanut currently	None	Last 4 years: peanut, corn, peanut, peanut
SC-6	Coastal Plains Soil, Water & Plant Research Center, Florence, SC 29501	N34.24398	W79.80783	Norfolk sandy loam (Fine, kaolinitic, thermic Typic Kandiudults)	Agricultural	Corn currently	None	Last 4 years: Corn, peanut, corn, corn
SC-7	Coastal Plains Soil, Water & Plant Research Center, Florence, SC 29501	N34.24395	W79.80788	Norfolk sandy loam (Fine, kaolinitic, thermic Typic Kandiudults)	Agricultural	Rye currently	None	Last 3 years: perennial rye
SC-8	Coastal Plains Soil,	N34.24176	W79.81187	Norfolk sandy loam (Fine,	Agricultural	Fallow currently	None	Last 3 years: soybean,

	Water & Plant Research Center, Florence, SC 29501			kaolinitic, thermic Typic Kandiudults)				winter fallow
SC-9	Next to Coastal Plains Soil, Water & Plant Research Center, Florence, SC 29501	N34.24177	W79.81170	Norfolk sandy loam (Fine, kaolinitic, thermic Typic Kandiudults)	Agricultural	Woods	None	Wood lot next to CPSW&PRC
CA-1	University of California, Riverside Farm, Riverside, CA 92507	W33°58'18.48"	W117°20'28.02"	Arlington Fine Sandy Loam	Agricultural	Fallow, none	N/A	N/A
CA-2	University of California, Riverside Farm, Riverside, CA 92507	W33°57'49.59"	W117°20'12.80"	Buren Fine Sandy Loam	Agricultural	Fallow, none	N/A	N/A
CA-3	University of California, Riverside Farm, Riverside, CA 92507	W33°57'55.28"	W117°20'12.12"	Hanford Fine Sandy Loam	Agricultural	Fallow, none	N/A	N/A
CA-4	University of California, Riverside Farm, Riverside, CA 92507	W33°57'52.44"	W117°20'34.94"	Ramona Sandy Loam	Agricultural	Fallow, none	N/A	N/A
CA-5	Twisselman Rd. Kern County, CA	N35°44'03.51"	W119°43'13.87"	Twisselman Clay	Agricultural	Fallow, sparse weeds	N/A	N/A
NJ-1	Adelphia Farm in Freehold, NJ (Plot 306)	N/A	N/A	Holmdel sandy loam	Agricultural	Fallow	N/A	N/A
NJ-2	Hort. Farm II, Rutgers, New Brunswick, NJ (dirt pile)	N/A	N/A	Sandy Loam	Agricultural	Moonflower, grass weeds, wild daisy	Mixing and Additions made	N/A
NJ-3	Pine Barrens in Ocean County, NJ	N39°43' 28.4"	W74°22'21.2"	Downer loamy sand	Forest	Pitch pine trees, oak trees, huckleberries, lowbush blueberries, other small leafy shrubs, reddish brown moss	None	Burned Pitch Pine and Oak Forest (May 2007)
NJ-4	Pine Barrens in Ocean County, NJ	N39° 44' 32.8"	W74°20'43.1"	Downer loamy sand	Forest	Pitch pine trees, oak trees, huckleberries, lowbush blueberries, other small leafy shrubs, shelf fungus	None	Burned Pitch Pine and Oak Forest (May 2007)
NJ-5	Pine Barrens in Ocean County, NJ	N39° 44' 25.0"	W74°22'8.9"	Downer loamy sand	Forest	Pitch pine trees, oak trees, huckleberries, lowbush blueberries, other small leafy shrubs	None	Unburned Pitch Pine and Oak Forest

Table 2. Soil Sample Characteristics (as of December, 2009, *cont'd*.). Data in this table was taken from information about the soils in each location based upon latitude and longitude and/or state and town information. This data was acquired with the help of the USDA Web Soil Survey (WSS) resource (http://websoilsurvey.nrcs.usda.gov).

Sample ID(s)	Avg. GWC (%)	Depth to Water Table (in.)	Mean Annual Precip. (inches)	Mean Annual Temp. (degrees F)	Frost-Free Period (days)	$CaCO_3$ (%)	Salinity (mmhos/cm)	CFU of PPFM per Gram of Soil
SC-1	12.41	30-40	45	64	230	N/A	0.0	6.40E+01
SC-2	12.94	30-40	45	64	230	N/A	0.0	5.20E+01
SC-3	13.53	6	40-58	63-72	230-240	N/A	N/A	1.46E+02
SC-4	12.44	6	40-58	63-72	230-240	N/A	N/A	1.48E+02
SC-5	19.92	0	40-58	63-72	230-240	N/A	N/A	4.00E+01
SC-6	18.67	48	40-58	63-72	230-240	N/A	N/A	1.54E+03
SC-7	16.94	48	40-58	63-72	230-240	N/A	N/A	4.40E+01
SC-8	16.60	48	40-58	63-72	230-240	N/A	N/A	2.38E+02
SC-9	13.98	48	40-58	63-72	230-240	N/A	N/A	3.00E+01
CA-1	0.48	> 80	12	63	240-320	5	0.0-0.2	2.00E+00
CA-2	0.52	> 80	10-14	63	230-300	1	0.0-0.4	5.00E+01
CA-3	1.13	> 80	9-20	63-64	230-250	N/A	N/A	6.00E+00
CA-4	0.67	> 80	10-20	63	230-320	1	N/A	3.60E+01
CA-5	2.79	0	5	64	250-275	10	16.0	1.00E+02
NJ-1	6.60	6-36	40-48	48-55	160-190	N/A	N/A	2.30E+02
NJ-2	9.79	N/A	N/A	48-55	N/A	N/A	N/A	1.67E+02
NJ-3	1.11	> 80	40-48	50-57	180-210	N/A	N/A	1.8E+04
NJ-4	1.32	> 80	40-48	50-57	180-210	N/A	N/A	1.9E+04
NJ-5	1.24	> 80	40-48	50-57	180-210	N/A	N/A	3.0E+03

Table 3. Chemical data for the soil samples. Nutrient levels for each soil sample were obtained by the Rutgers Soil Testing Facility using Mehlich-3 extraction methods for nutrient values (Mehlich, 1984). Electronic pH electrodes were utilized in soil slurries to obtain pH values (McLean, 1982).

Sample ID(s)	P (lbs/A)	K(lbs/A)	Mg (lbs/A)	Ca (lbs/A)	Cu (ppm)	Mn (ppm)	Zn (ppm)	B (ppm)	Fe (ppm)	pH
SC-1	71	344	154	721	0.6	5.4	6.7	2.6	81.5	6.35
SC-2	103	172	232	957	0.5	4.8	3.4	2.5	60.9	7.25
SC-3	75	339	196	681	0.3	4.6	3.3	2.3	75.5	6.80
SC-4	106	279	206	669	0.2	3.3	3.1	2.4	94.3	6.80
SC-5	307	256	60	855	0.9	7.8	6.5	2.3	181.5	5.25
SC-6	258	165	46	494	0.2	7.6	6.2	2.7	112.8	5.40
SC-7	240	242	48	226	0.4	3.7	5.6	2.6	105.4	5.55
SC-8	171	102	103	362	1.0	11.3	12.1	2.5	98.5	5.10
SC-9	208	175	362	449	0.2	3.2	3.6	2.6	85.5	5.20
CA-1	592	601	332	4025	10.9	95.0	0.0	4.2	76.4	7.35
CA-2	125	479	185	1768	4.2	81.2	29.4	2.7	75.7	7.00
CA-3	252	397	622	4580	4.8	132	32	4.1	96.5	7.40
CA-4	109	289	248	2170	1.5	47.9	6.4	2.5	64.9	7.85
CA-5	245	1261	1214	10842	1.6	104.9	5.5	77.5	58.3	7.80
NJ-1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	6.30
NJ-2	204	327	224	1171	7.4	32.7	8.5	1.4	216.4	5.75
NJ-3	5-7	18-58	40-85	145-415	1.33-1.19	0.62-4.59	1.07-2.65	1.37-1.06	11.48-28.31	4.20-4.65
NJ-4	5-6	36-82	63-215	190-885	1.22-1.71	0.78-9.8	1.55-5.08	0.56-1.16	27.72-42.77	3.90-4.00
NJ-5	7	90-196	77-134	253-427	1.26-1.86	2.42-3.67	1.67-4.55	0.83-1.23	12.19-95.04	3.50-3.65

Bacterial Isolation and Cultivation

Bacterial isolates were obtained through dilution plating of the soil using Bis Tris Buffer solution onto Ammonium Mineral Salts (AMS) agar plates amended with 0.5 % (v/v) methanol as the sole and selective carbon source. AMS medium and agar for these experiments were prepared according to the protocol for ATCC Medium 784 (http://www.atcc.org/Attachments/4001.pdf; Table 4). ATCC Medium 784 is methanol-amended version of ATCC Medium 1683, which is a minimal medium containing basic nutrients and trace metals to which a desired carbon source can be added (http://www.atcc.org/Attachments/2880.pdf; Table 4). Except for the addition of methanol, the two protocols are identical. The methanol used (99.9 % methanol and 0.07 % H_2O, Fisher Scientific) was not sterilized prior to its addition to the agar or medium due to its antimicrobial properties. Methanol was added after autoclave sterilization of the medium or agar after it had sufficiently cooled to prevent the methanol from volatilizing.

***Methylobacterium* Isolation & Genetic Relatedness to Known Species**

Soil samples were diluted in Bis Tris Buffer and the supernatant was plated onto 0.5 % (v/v) methanol-amended AMS agar plates. Pink colonies were chosen and streaked onto new plates and left to grow for three weeks at 30°C in the dark. A total of 114 PPFM isolates were collectively obtained from the blueberry skin and NJ, SC and CA soil samples. *mxaF* gene fragments were sequenced from all 114 isolates, while it was only possible to obtain 16S rRNA gene sequences for only a subset of those isolates within the time constraints of this study.

Table 4. Ammonium Mineral Salts (AMS) Medium Information as per American Type Culture Collection (ATCC) Components and Protocols. These two media are identical but for the fact that ATCC 784 includes the addition of methanol after autoclaving, whereas ATCC 1683 is just the base medium to which a carbon source of choice can be added.

	ATCC Medium 784	ATCC Medium 1683
Components List (For One Liter)	K_2HPO_4 0.7 g* KH_2PO_4 0.54 g $MgSO_4 . 7H_2O$ 1.0 g $CaCl_2 . 2H_2O$ 0.2 g $FeSO_4 . 7H_2O$ 4.0 mg NH_4Cl 0.5 g $ZnSO_4 . 7H_2O$ 100.0 mcg $MnCl_2 . 4H_2O$ 30.0 mcg H_3BO_3 300.0 mcg $CoCl_2 . 6H_2O$ 200.0 mcg $CuCl_2 . 2H_2O$ 10.0 mcg $NiCl_2 . 6H_2O$ 20.0 mcg $Na_2MoO_4 . 2H_2O$ 60.0 mcg	K_2HPO_4 0.7 g* KH_2PO_4 0.54 g $MgSO_4 . 7H_2O$ 1.0 g $CaCl_2 . 2H_2O$ 0.2 g $FeSO_4 . 7H_2O$ 4.0 mg NH_4Cl 0.5 g $ZnSO_4 . 7H_2O$ 100.0 mcg $MnCl_2 . 4H_2O$ 30.0 mcg H_3BO_3 300.0 mcg $CoCl_2 . 6H_2O$ 200.0 mcg $CuCl_2 . 2H_2O$ 10.0 mcg $NiCl_2 . 6H_2O$ 20.0 mcg $Na_2MoO_4 . 2H_2O$ 60.0 mcg
Distilled Water	1 L	1 L
Agar (if needed)	15 g**	15 g**
Carbon Source	After autoclave sterilization, add sterile methanol to a concentration of 0.5 (% by volume).	None. (Researcher's choice, add as needed.)
Sterilization	Autoclave before addition of methanol carbon source to prevent volatilization.	Autoclave.
pH	Adjust pH to 6.8.	Adjust pH to 6.8.
Reference	http://www.atcc.org/Attachments/4001.pdf	http://www.atcc.org/Attachments/2880.pdf

* When amending with $ZnSO_4$, it is necessary to reduce the amount of K_2HPO_4 by a factor of 10 (to 0.07 g/L) to prevent excess precipitation. This lowers the overall buffering capacity and pH of the medium, but does not affect the ability of the PPFM isolates to grow in the medium (at $ZnSO_4$ concentrations of 1 to 25 mM).

The first nine *Methylobacterium* isolates were obtained from plot 306 at of Adelphia Farm in Freehold, NJ. Initial enrichment culturing was done to select for zinc-(Zn)-resistant bacteria, and therefore these nine isolates were picked along with non-PPFMs for a total of 42 isolates. This set was later narrowed down to PPFM isolates only. These and all subsequent isolates obtained from other soil and plant samples were named as in the following example for isolate NJ1104:

NJ	**1**	**1**	**04**
a	*b*	*c*	*d*

a = General location of soil sample from which the organism was obtained. A state label ("NJ" for New Jersey in this example).

b = Indicates the sample number from which the isolate was obtained.

c = Indicates the isolation trial number from that sample that yielded the isolate.

d = Specifies the isolate with a number in the order in which the colony was picked from dilution plates of the sample.

Blueberry skin PPFM isolates were simply labeled, for example, as BB01, with BB indicating that the isolate came from a blueberry skin biofilm, followed by the double-digit isolate number. There was only one sample and only one isolation attempt for blueberry skin isolates and therefore no other designations were needed.

DGGE of *Methylobacterium* 16S rRNA and *mxaF* Gene Sequences

A subset of nine isolates from plot 306 of the Adelphia Farm in Freehold, New

Jersey were selected for the comparison of *mxaF* to the 16S rRNA gene for its ability to be used as a characterization tool. Single-colony (SC)-PCR was performed on colonies from each isolate using primers for a 560 bp region of the *mxaF* gene. *mxaF* PCR primers (f1003 and r1561) were taken from the *mxaF* gene conserved region of *mxa* gene family (McDonald *et al*., 1997). The sequences were obtained from the sequenced genome of *Methylobacterium organophilum* XX (Madhaiyan, *et al*., 2005; McDonald *et al*., 1997). PCR product of approximately 520 bp for 16S rRNA and 560 bp for *mxaF* was used to generate acrylamide DGGE gels and phylogenetic trees to assess isolate relatedness. Both whole culture *mxaF* PCR product and excised dominant DGGE band DNA were sequenced and compared for each isolate to confirm that dominant bands did in fact represent the dominant sequence in the original PCR product. In addition, ten phenotypic variants with disparate colony shapes, colors and textures on AMS agar plates were sequenced using single colony PCR product to verify that *mxaF* was conserved enough to distinguish at the species level but not the strain level.

Phylogeny of Soil *Methylobacterium mxaF* Sequences

To apply *mxaF* sequence as a characterization tool and to further investigate the impact of environmental factors on *mxaF* sequence, *mxaF* gene sequences of all 114 isolates were used for phylogenetic analysis. Initial sequence cleanup and trimming was conducted using LaserGene 7.1 software (DNAStar, 2006). Alignment of the sequences, using both computed and manual aligning methods, and all phylogenetic analyses were conducted using MEGA4.1 Beta software (Benson et al. 2008; Tamura et al., 2007). The evolutionary history of all organisms presented in each phylogenetic tree was inferred

using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches (Felsenstein, 1985), and only bootstrap values greater than 50 % are shown. All trees were drawn to scale, with the scale bar and label representing the number of nucleotide mismatches per scale bar length. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and were in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). Phylogenetic trees included lab isolates from soil from Adelphia Farm in Freehold, NJ, five ATTC acquisitions, and reference sequences from as many *Methylobacterium* species as were available from GenBank. Physical and chemical patterns of each source were investigated.

RESULTS AND DISCUSSION

Comparison of *Methylobacterium* 16S rRNA and *mxaF* Gene Sequences: DGGE Analysis

16S rRNA gene PCR product approximately 520 bp in length and *mxaF* PCR product approximately 560 bp in length were sequenced for each of nine New Jersey *Methylobacterium* isolates to determine genetic relatedness to known species. This was done prior to DGGE analysis in order to establish putative species groups that would then be expected to have disparate banding patterns on a DGGE gel. *mxaF* and 16S rRNA gene sequencing of the isolates verified the *Methylobacterium* genus, but 16S rRNA gene sequences yielded ambiguous results at the species level and produced several top results

during a BLAST search with the same identity (Tables 5.1 and 5.3) (NCBI BLAST, 2009). Isolate *mxaF* sequences varied more than those of 16S rRNA, species relatedness could be better resolved, and in all cases only one top result was obtained from the BLAST search (Tables 5.2 and 5.4) All isolate *mxaF* sequences were then compared further using the construction of a phylogenetic tree, with a subset of nine bacteria used for 16S rRNA and *mxaF* DGGE gel comparison and in-depth phenotypic study.

PCR product (approximately 520 bp) of the 16S rRNA gene gave identical banding patterns for nine NJ isolates of varying phenotype on a DGGE gel (Fig. 1). For *mxaF*, an approximately 560 bp PCR product yielded multiple bands, suggesting sequence variation within each isolate. A dominant band, denoted by the darkest band in each lane, was observed for each isolate and was unique for each putative species within the group (Fig. 2). Dominant bands were reproducible on subsequent DGGE gels, while other minor bands varied with each run of the DGGE analysis.

Whereas the banding patterns are nearly identical across all isolates for the 16S rRNA gene, the common band for 16S and "dominant" bands for *mxaF* that were sequenced (red boxes) for the *mxaF* gene corresponded with the putative species groups observed from sequencing data and are described in Table 4. For example, NJ1106 and NJ1107 are thought to be the same species, NJ1101 and NJ1104 are each unique, and NJ1103, NJ1105, NJ1108, NJ1126, and NJ1128 may all be the same, as reflected by the positions of the dominant bands reflect this. Minor bands that were sequenced (red arrows) were more variable in sequence and those sequences were not as clean. Sequences of excised dominant *mxaF* DGGE bands were identical to the sequences generated by the original PCR product used to make the DGGE gel for each

Table 5.1 – Initial 16S rRNA Gene Sequence Relatedness of 9 NJ isolates as of March 5, 2009. Genetic relatedness of 9 NJ isolates to known species in GenBank database using the Basic Local Alignment Search Tool (BLAST) Search Tool for 16S rRNA gene fragments. Only the matches with the highest Max Score and that have a complete genus and species name are shown.

	16S rRNA Short Sequences (27f, 529r)						16S rRNA Long Sequences (27f, 1525r)					
Isolate Name	*Frag-ment Length (bp)*	*Closest Related Sequence (Top BLAST Search Result)*	*Match Accn #*	*Sequence Identity (% Query Matched % of Reference Sequence)*	*Max Score*	*E Value (Search Error)*	*Frag-ment Length (bp)*	*Closest Related Sequence (Top BLAST Search Result)*	*Match Accn #*	*Sequence Identity (% Query Matched % of Reference Sequence)*	*Max Score*	*E Value (Search Error)*
NJ1101	476	*Methylobacterium radiotolerans* JCM 2831, complete genome *Methylobacterium radiotolerans* JCM 2831 plasmid pMRAD01, complete sequence	CP001001.1 CP001002.1	99 to 99	852	0.0	1,382	*Methylobacterium radiotolerans* JCM 2831, complete genome *Methylobacterium radiotolerans* JCM 2831 plasmid pMRAD01, complete sequence	CP001001.1 CP001002.1	99 to 99	2534	0.0
NJ1103	477	*Methylobacterium fujisawaense* strain MP1 16S ribosomal RNA gene, complete sequence*	EF015477.1	99 to 99	852	0.0	1,383	*Methylobacterium oryzae* strain CBMB110 16S ribosomal RNA gene, partial sequence *Methylobacterium oryzae* CBMB20 strain CBMB20 16S ribosomal RNA, partial sequence	AY683046.1 NR_043104.1	99 to 97	2329	0.0
NJ1104	479	*Methylobacterium podarium* strain FM1 16S	AY468363.1	99 to 99	857	0.0	1,377	*Methylobacterium populi* BJ001, complete genome	CP001029.1	100 to 99	2531	0.0

		ribosomal RNA gene, complete sequence											
NJ1105	476	*Methylobacterium fujisawaense* strain MP1 16S ribosomal RNA gene, complete sequence*	EF015477.1	99 to 99	852	0.0	1,384	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial sequence *Methylobacterium fujisawaense* gene for 16S rRNA, partial sequence *Methylobacterium* sp. F48 partial 16S rRNA gene, strain F48	HQ647259.1 AB558142.1 AM910540.1	99 to 100	2547	0.0
NJ1106	479	*Methylobacterium fujisawaense** strain MP1 16S ribosomal RNA gene, complete sequence*	EF015477.1	98 to 98	846	0.0	1,372	*Methylobacterium radiotolerans* JCM 2831, complete genome *Methylobacterium radiotolerans* JCM 2831 plasmid pMRAD01, complete sequence	CP001001.1 CP001002.1	99 to 100	2460	0.0
NJ1107	476	*Methylobacterium fujisawaense** strain MP1 16S ribosomal RNA gene, complete sequence	EF015477.1	99 to 98	839	0.0	1,306	*Methylobacterium radiotolerans* JCM 2831, complete genome	CP001001.1	99 to 100	2364	0.0
NJ1108	475	*Methylobacterium fujisawaense** strain MP1 16S ribosomal RNA gene, complete sequence	EF015477.1	99 to 99	852	0.0	1.372	*Methylobacterium radiotolerans* JCM 2831, complete genome *Methylobacterium radiotolerans* JCM 2831 plasmid pMRAD01, complete sequence	CP001001.1 CP001002.1	99 to 100	2484	0.0
NJ1126	478	*Methylobacterium fujisawaense** strain MP1 16S	EF015477.1	99 to 99	852	0.0	1.375	*Methylobacterium radiotolerans* JCM 2831, complete	CP001001.1	99 to 100	2490	0.0

		ribosomal RNA gene, complete sequence						genome *Methylobacterium radiotolerans* JCM 2831 plasmid pMRAD01, complete sequence	CP001002.1			
NJ1128	477	*Methylobacterium fujisawaense** strain MP1 16S ribosomal RNA gene, complete sequence	EF015477.1	99 to 99	852	0.0	1.386	*Methylobacterium radiotolerans* JCM 2831, complete genome *Methylobacterium radiotolerans* JCM 2831 plasmid pMRAD01, complete sequence	CP001001.1 CP001002.1	99 to 99	2497	0.0

***Note:** EF015477.1, previously listed in GenBank as *Methylobacterium fujisawaense* MP1 is now listed as *Methylobacterium oryzae* MP1.

Table 5.2 – Initial *mxaF* Gene Sequence Relatedness of 9 NJ isolates as of March 5, 2009. Genetic relatedness of 9 NJ isolates to known species in GenBank database using the Basic Local Alignment Search Tool (BLAST) Search Tool for *mxaF* gene fragments. Only the matches with the highest Max Score and that have a complete genus and species name are shown.

	***mxaF* Short Sequences (1003f, 1561r)**						***mxaF* Long Sequences (769f, 1561r)**					
Isolate Name	*Frag-ment Length (bp)*	*Closest Related Sequence (Top BLAST Search Result)*	*Match Accn #*	*Sequence Identity (% Query Matched % of Reference Sequence)*	*Max Score*	*E Value (Search Error)*	*Frag-ment Length (bp)*	*Closest Related Sequence (Top BLAST Search Result)*	*Match Accn #*	*Sequence Identity (% Query Matched % of Reference Sequence)*	*Max Score*	*E Value (Search Error)*
NJ1101	562	*Methylobacterium radiotolerans* JCM 2831, complete genome	CP001001.1	99 to 99	1024	0.0			N/A			
NJ1103	562	*Methylobacterium fujisawaense** strain MP1 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds	EF030547.1	99 to 99	1024	0.0			N/A			
NJ1104	559	*Methylobacterium populi* BJ001, complete genome	CP001029.1	99 to 100	1002	0.0	648	*Methylobacterium populi* BJ001, complete genome	CP001029.1	99 to 96	1057	
NJ1105	549	*Methylobacterium phyllosphaerae* strain CBMB27 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds	EF562496.1	99 to 100	1009	0.0			N/A			
NJ1106	556	*Methylobacterium phyllosphaerae* strain CBMB27 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds	EF562496.1	94 to 100	856	0.0			N/A			
NJ1107	565	*Methylobacterium phyllosphaerae* strain CBMB27 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds	EF562496.1	94 to 97	859	0.0			N/A			
NJ1108	509	Methylobacterium oryzae	EF562478.1	99 to 98	922	0.0			N/A			

		strain CBMB20 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds					
NJ1126	559	Methylobacterium fujisawaense* strain MP1 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds	EF030547.1	99 to 98	1013	0.0	N/A
NJ1128	515	Methylobacterium fujisawaense* strain MP1 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds	EF030547.1	99 to 99	939	0.0	N/A

*__Note:__ EF015477.1, previously listed in GenBank as *Methylobacterium fujisawaense* MP1 is now listed as *Methylobacterium oryzae* MP1.

Table 5.3 – Updated 16S rRNA Gene Sequence Relatedness of 9 NJ isolates as of October 27, 2011. Genetic relatedness of 9 NJ isolates to known species in GenBank database using the Basic Local Alignment Search Tool (BLAST) Search Tool for 16S rRNA gene fragments. Only the matches with the highest Max Score and that have a complete genus and species name are shown.

	16S rRNA Short Sequences (27f, 529r)						16S rRNA Long Sequences (27f, 1525r)					
Isolate Name	*Frag-ment Length (bp)*	*Closest Related Sequence (Top BLAST Search Result)*	*Match Accn #*	*Sequence Identity (% Query Matched % of Reference Sequence)*	*Max Score*	*E Value (Search Error)*	*Frag-ment Length (bp)*	*Closest Related Sequence (Top BLAST Search Result)*	*Match Accn #*	*Sequence Identity (% Query Matched % of Reference Sequence)*	*Max Score*	*E Value (Search Error)*
NJ1101	476	*Methylobacterium radiotolerans* strain 7212 16S ribosomal RNA gene, partial sequence	GU294333.1	99 of 99	857	0.0	1,382	*Methylobacterium radiotolerans* strain 7210 16S ribosomal RNA gene, partial sequence	GU294325.1	99 to 99	2540	0.0
		Methylobacterium radiotolerans strain TD15 16S ribosomal RNA gene, partial sequence	GU294330.1					*Methylobacterium radiotolerans* strain LM2 16S ribosomal RNA gene, partial sequence	GU294321.1			
		Methylobacterium radiotolerans strain 7210 16S ribosomal RNA gene, partial sequence	GU294325.1					*Methylobacterium radiotolerans* partial 16S rRNA gene, strain F2	AM910531.1			
		Methylobacterium radiotolerans strain 6514 16S ribosomal RNA	GU294323.1					*Methylobacterium radiotolerans* gene for 16S ribosomal	AB175637.1			

		gene, partial sequence *Methylobacterium radiotolerans* strain LM2 16S ribosomal RNA gene, partial sequence *Methylobacterium radiotolerans* strain IMBG187 16S ribosomal RNA gene, partial sequence	GU294321.1 EF583689.2					RNA, partial sequence, strain:DSM 760				
NJ1103	477	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial sequence *Methylobacterium fujisawaense* strain SNA5 16S ribosomal RNA gene, partial sequence *Methylobacterium fujisawaense* gene for 16S rRNA, partial sequence *Methylobacterium oryzae* strain MP1 16S ribosomal RNA gene, complete sequence	HQ647259.1 HQ220063.1 AB558142.1 EF015477.1	99 of 99	852	0.0	1,383	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial sequence *Methylobacterium fujisawaense* gene for 16S rRNA, partial sequence	HQ647259.1 AB558142.1	99 to 97	2333	0.0
NJ1104	479	*Methylobacterium podarium* strain FM1 16S ribosomal RNA gene, complete sequence	AY468363.1	99 of 99	857	0.0	1,377	*Methylobacterium populi* BJ001, complete genome	CP001029.1	100 to 99	2531	0.0
NJ1105	476	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA	HQ647259.1	99 of 99	854	0.0	1,384	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial	HQ647259.1	100 to 99	2547	0.0

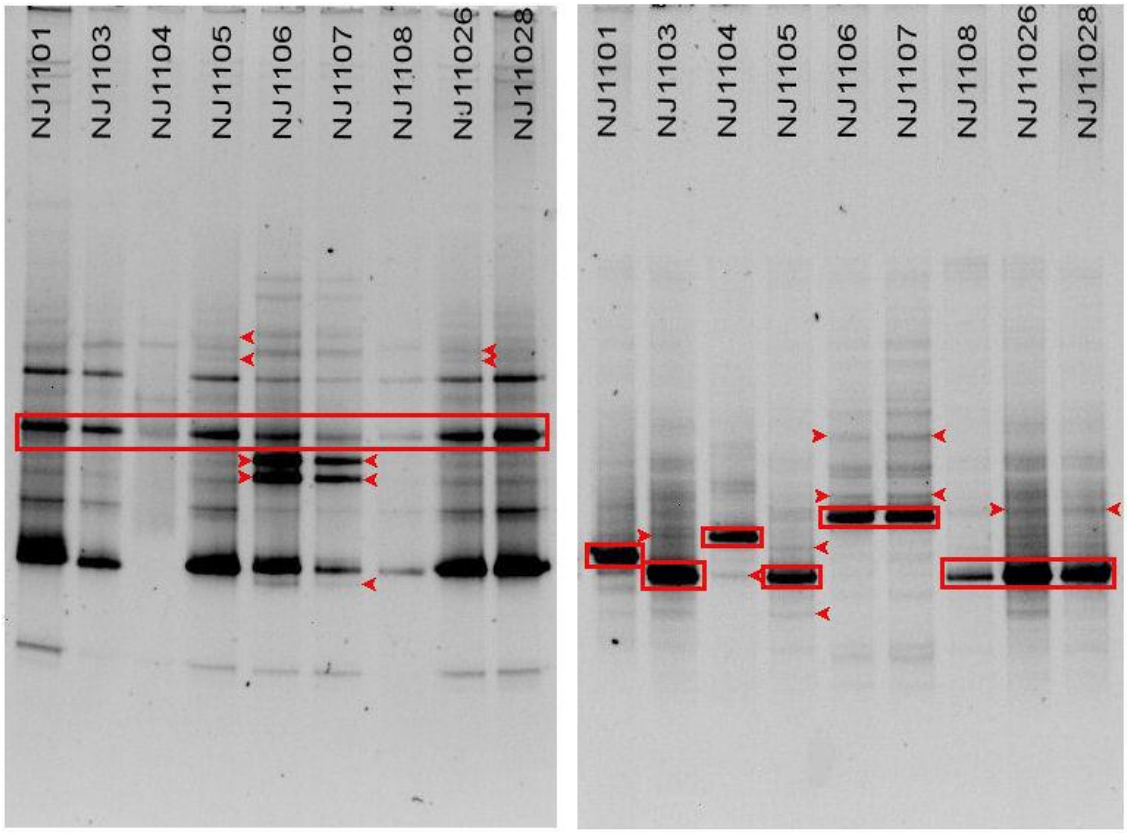
		gene, partial sequence						sequence AB558142.1 *Methylobacterium* fujisawaense strain STY1 16S ribosomal RNA gene, partial sequence AM910540.1 *Methylobacterium* sp. F48				
NJ1106	479	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial sequence	HQ647259.1	99 of 98	850	0.0	1,372	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial sequence AB558142.1 *Methylobacterium* fujisawaense strain STY1 16S ribosomal RNA gene, partial sequence AM910540.1 *Methylobacterium* sp. F48	HQ647259.1	99 to 100	2499	0.0
NJ1107	476	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial sequence	HQ647259.1	99 of 98	843	0.0	1,306	*Methylobacterium oryzae* strain 1021b 16S ribosomal RNA gene, partial sequence	GU294332.1	99 to 100	2394	0.0
NJ1108	475	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial sequence	HQ647259.1	100 of 99	854	0.0	1.372	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial sequence AB558142.1 *Methylobacterium* fujisawaense strain STY1 16S ribosomal RNA gene, partial sequence AM910540.1 *Methylobacterium* sp. F48	HQ647259.1	100 to 100	2534	0.0
NJ1126	478	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial sequence	HQ647259.1	99 of 99	856	0.0	1.375	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial sequence AB558142.1 Methylobacterium	HQ647259.1	100 to 100	2540	0.0

								fujisawaense strain STY1 16S ribosomal RNA gene, partial sequence *Methylobacterium* sp. F48	AM910540.1				
NJ1128	477	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial sequence	HQ647259.1	99 of 99	854	0.0	1.386	*Methylobacterium fujisawaense* strain S13 16S ribosomal RNA gene, partial sequence *Methylobacterium* fujisawaense strain STY1 16S ribosomal RNA gene, partial sequence *Methylobacterium* sp. F48	HQ647259.1 AB558142.1 AM910540.1	100 to 99	2547	0.0

Table 5.4 – Updated *mxaF* Gene Sequence Relatedness of 9 NJ isolates as of October 27, 2011. Genetic relatedness of 9 NJ isolates to known species in GenBank database using the Basic Local Alignment Search Tool (BLAST) Search Tool for *mxaF* gene fragments. Only the matches with the highest Max Score and that have a complete genus and species name are shown.

	mxaF Short Sequences (1003f, 1561r)						*mxaF* Long Sequences (769f, 1561r)					
Isolate Name	*Frag-ment Length (bp)*	*Closest Related Sequence (Top BLAST Search Result)*	*Match Accn #*	*Sequence Identity (% Query Matched % of Reference Sequence)*	*Max Score*	*E Value (Search Error)*	*Frag-ment Length (bp)*	*Closest Related Sequence (Top BLAST Search Result)*	*Match Accn #*	*Sequence Identity (% Query Matched % of Reference Sequence)*	*Max Score*	*E Value (Search Error)*
NJ1101	562	*Methylobacterium radiotolerans* JCM 2831, complete genome	CP001001.1	99 of 99	1022	0.0	N/A					
NJ1103	562	*Methylobacterium oryzae* strain MP1 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds	EF030547.1	99 of 99	1024	0.0	N/A					
NJ1104	559	*Methylobacterium populi* BJ001, complete genome	CP001029.1	99 of 99	1002	0.0	648	*Methylobacterium populi* BJ001, complete genome	CP001029.1	99 of 96	1057	0.0
NJ1105	549	*Methylobacterium phyllosphaerae* strain CBMB27 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds	EF562496.1	99 of 99	1009	0.0	N/A					
NJ1106	556	*Methylobacterium phyllosphaerae* strain CBMB27 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds	EF562496.1	100 of 94	856	0.0	N/A					
NJ1107	565	*Methylobacterium oryzae* strain MP1 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds	EF030547.1	99 of 94	863	0.0	N/A					
NJ1108	509	*Methylobacterium oryzae* strain CBMB20 methanol dehydrogenase alpha subunit (*mxaF*) gene,	EF562478.1	98 of 99	922	0.0	N/A					

		partial cds					
NJ1126	559	*Methylobacterium oryzae* strain MP1 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds	EF030547.1	98 of 99	1013	0.0	N/A
NJ1128	515	*Methylobacterium oryzae* strain CBMB20 methanol dehydrogenase alpha subunit (*mxaF*) gene, partial cds	EF562478.1	99 of 99	941	0.0	N/A



Figures 1 (16S rRNA gene DGGE gel, left) and 2 (*mxaF* gene DGGE gel, right), DGGE gels of 9 NJ PPFM Isolates. DGGE acrylamide gels were run at 55V for 16.5 hours with a 55-90% gradient for 16S and 50-90% gradient for *mxaF*. For the 16S rRNA gene gel (left), the red box indicates bands common to all isolates that were excised and red arrows point to excised unique minor bands. For the *mxaF* gene gel (right), boxes indicate "dominant" genes for a given isolate banding pattern and arrows indicate minor unique bands that were excised. All excised bands were further analyzed for sequencing and determination of genetic relatedness to other bacterial sequences.

isolate. This suggests that the high intensity of the band indicates a real dominance of that sequence within the PCR product and is not merely an artifact of the DGGE process. The sequences of minor bands excised from *mxaF* DGGE gels varied slightly from that of the dominant band of PCR product sequences. These data suggest that *mxaF* is a better indicator of species than the 16S rRNA gene when comparing species from the genus *Methylobacterium* since the 16S rRNA gene DGGE banding patterns were nearly identical.

Comparison of *Methylobacterium* 16S rRNA and *mxaF* Gene Sequences: Sequencing Analyses

Isolate sequences were reproducible across different sequencing methods (whole culture PCR and SC-PCR), multiple sequencing trials from different cultures of the same isolate, and sequencing of various phenotypes of the same isolate. A total of 21 NJ1104 colonies were compared for *mxaF* sequence using the SC-PCR method. They possessed varied phenotypic characteristics indicating differential growth rate or behavior while metabolizing the methanol carbon source in amended AMS agar plates, indicated by variations in colony size, color and texture. All NJ1104 sequences clustered together on an *mxaF* phylogenetic tree (Fig. 3) and were extremely similar to themselves, indicating conservation of *mxaF* sequencing within an isolate regardless of phenotypic variation. NJ1104 *mxaF* sequences were highly similar to *Methylobacterium thiocyanatum* DSM 11490, indicating the species to which this isolate is most closely related.

This correlates with the findings of the DGGE analysis in that *mxaF* sequence is a consistent characteristic of an isolate strain and is not influenced by culturing protocols,

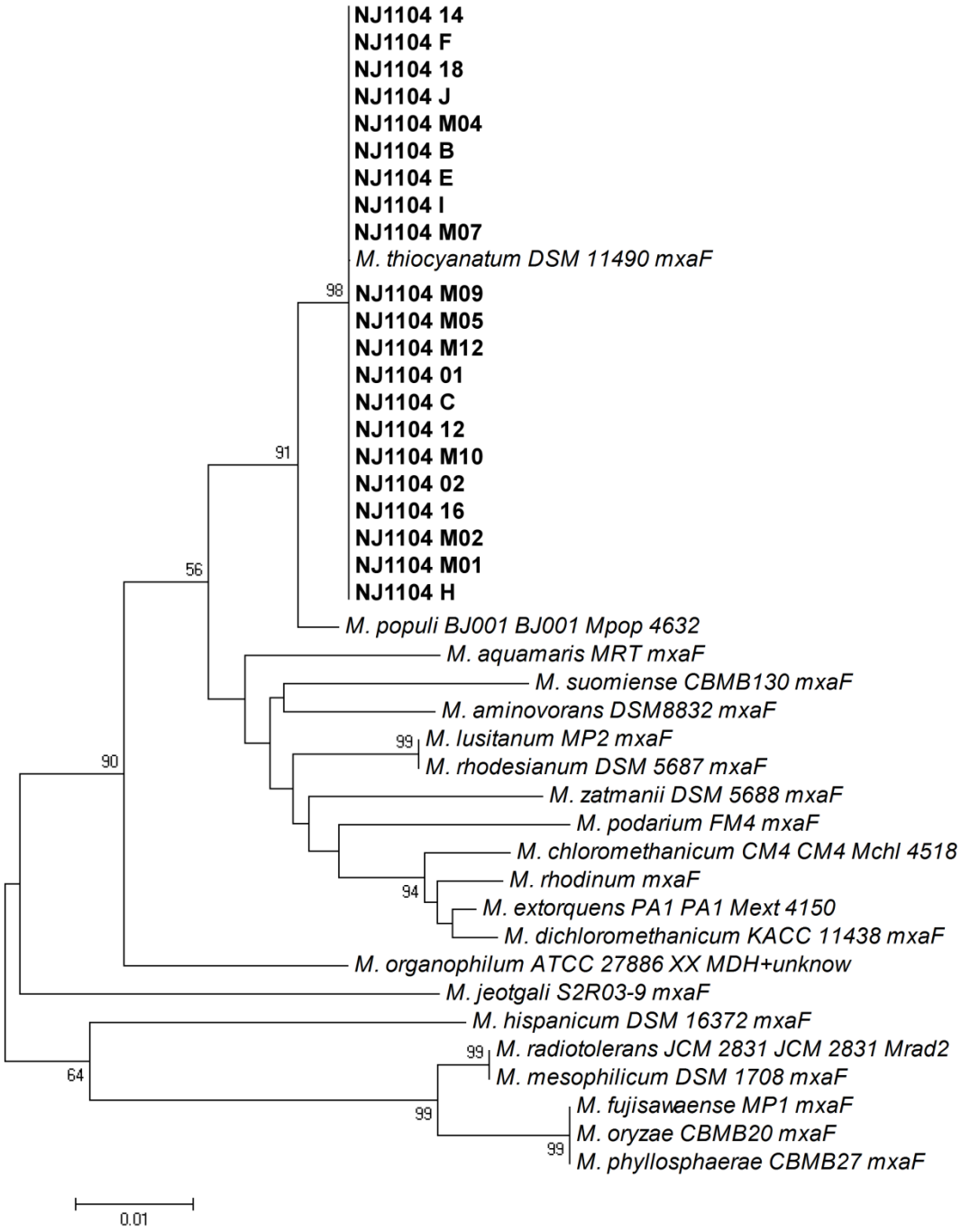


Figure 3. *mxaF* Phylogenetic Tree of NJ1104 Phenotypic Variants.

This is an *mxaF* Neighbor-Joining phylogenetic tree depicting the evolutionary relationships of 42 taxa. There were a total of 517 positions in the final dataset. Bootstrap values above 50 % are shown and were calculated based on the construction of 500

replicate trees. The sequences of phenotypic variants of NJ isolate NJ1104 that differed in colony color, texture, and shape are shown boldfaced.

current isolate phenotype, or different PCR methods. Because of this, *mxaF* sequence is a useful tool for the genetic characterization of PPFM species from environmental soil samples.

Comparison of *Methylobacterium* 16S rRNA and *mxaF* Gene Sequences: Phylogenetic Analysis

Isolate 16S rRNA gene sequences were all very similar to each other and to GenBank reference sequences (98-100% homology). Because of this, eight out of nine NJ isolates clustered together on a phylogenetic tree (Fig. 4), suggesting close relatedness if not that they are the same species. The addition of GenBank PPFM and non-PPFM reference sequences to the phylogenetic analysis and the acquisition of a longer gene fragment did result in slightly better resolution of species sequences within the *Methylobacterium* genus (Fig. 5). However, while NJ1101 in Fig. 5 did separate from the larger cluster of isolates seen in Fig. 4, all other isolates besides NJ1101 and NJ1104 still grouped tightly together, even though there are three putative species groups within that cluster.

Isolate *mxaF* sequences were separated throughout the genus in groups that corresponded to putative species groups assumed from phenotypic and sequencing results (Fig. 6). All ATCC sequences clustered near the species that they were identified as by ATCC (Fig. 6). In addition, non-*Methylobacterium* sequences used as side groups to root the tree clustered according to their genus (Fig. 6). The *Methylobacterium* genus clustered separately from other genera and sequences resolved as expected according to their GenBank species annotations in most cases. An updated tree containing additional

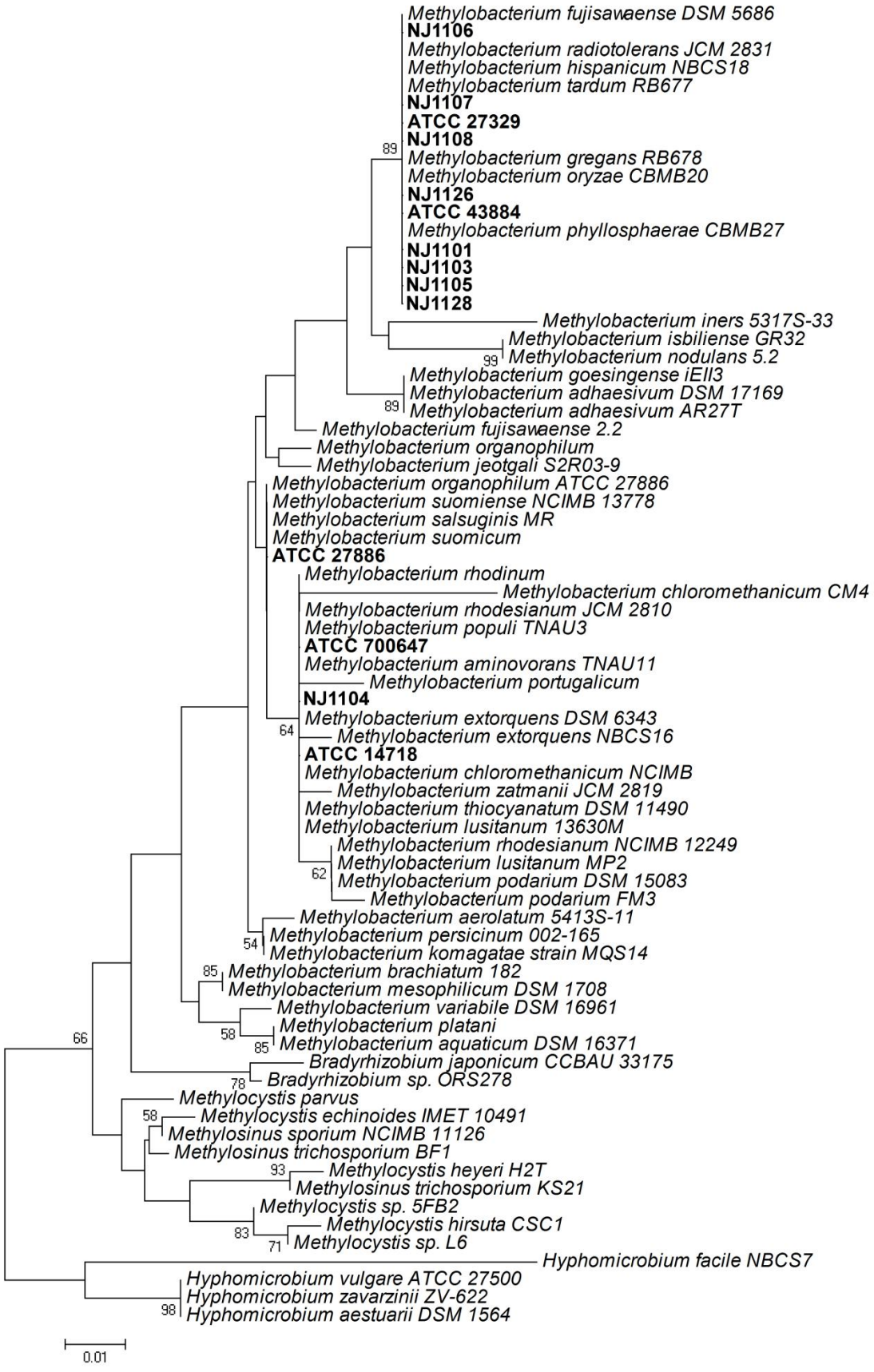

Methylobacterium fujisawaense DSM 5686
NJ1106
Methylobacterium radiotolerans JCM 2831
Methylobacterium hispanicum NBCS18
Methylobacterium tardum RB677
NJ1107
ATCC 27329
NJ1108
89
Methylobacterium gregans RB678
Methylobacterium oryzae CBMB20
NJ1126
ATCC 43884
Methylobacterium phyllosphaerae CBMB27
NJ1101
NJ1103
NJ1105
NJ1128
Methylobacterium iners 5317S-33
Methylobacterium isbiliense GR32
99
Methylobacterium nodulans 5.2
Methylobacterium goesingense iEII3
89
Methylobacterium adhaesivum DSM 17169
Methylobacterium adhaesivum AR27T
Methylobacterium fujisawaense 2.2
Methylobacterium organophilum
Methylobacterium jeotgali S2R03-9
Methylobacterium organophilum ATCC 27886
Methylobacterium suomiense NCIMB 13778
Methylobacterium salsuginis MR
Methylobacterium suomicum
ATCC 27886
Methylobacterium rhodinum
Methylobacterium chloromethanicum CM4
Methylobacterium rhodesianum JCM 2810
Methylobacterium populi TNAU3
ATCC 700647
Methylobacterium aminovorans TNAU11
Methylobacterium portugalicum
NJ1104
Methylobacterium extorquens DSM 6343
64
Methylobacterium extorquens NBCS16
ATCC 14718
Methylobacterium chloromethanicum NCIMB
Methylobacterium zatmanii JCM 2819
Methylobacterium thiocyanatum DSM 11490
Methylobacterium lusitanum 13630M
Methylobacterium rhodesianum NCIMB 12249
Methylobacterium lusitanum MP2
62
Methylobacterium podarium DSM 15083
Methylobacterium podarium FM3
Methylobacterium aerolatum 5413S-11
54
Methylobacterium persicinum 002-165
Methylobacterium komagatae strain MQS14
85
Methylobacterium brachiatum 182
Methylobacterium mesophilicum DSM 1708
Methylobacterium variabile DSM 16961
58
Methylobacterium platani
66
85
Methylobacterium aquaticum DSM 16371
Bradyrhizobium japonicum CCBAU 33175
78
Bradyrhizobium sp. ORS278
Methylocystis parvus
58
Methylocystis echinoides IMET 10491
Methylosinus sporium NCIMB 11126
Methylosinus trichosporium BF1
93
Methylocystis heyeri H2T
Methylosinus trichosporium KS21
Methylocystis sp. 5FB2
83
Methylocystis hirsuta CSC1
71
Methylocystis sp. L6
Hyphomicrobium facile NBCS7
Hyphomicrobium vulgare ATCC 27500
Hyphomicrobium zavarzinii ZV-622
98
Hyphomicrobium aestuarii DSM 1564
0.01

Figure 4. 16S rRNA Neighbor-Joining Phylogenetic Tree of 9 NJ Isolates, ATCC acquisitions, and Minimal Reference Sequences. This is an early 16S rRNA gene tree of the evolutionary relationships of 73 taxa using the 16S rRNA gene fragments. There were a total of 489 positions in the final dataset. Bootstrap values above 50 % are shown and were calculated based on the construction of 500 replicate trees. Sequences of nine New Jersey *Methylobacterium* isolates from agricultural soil as well as those of ATCC acquisitions cultured in the laboratory are boldfaced.

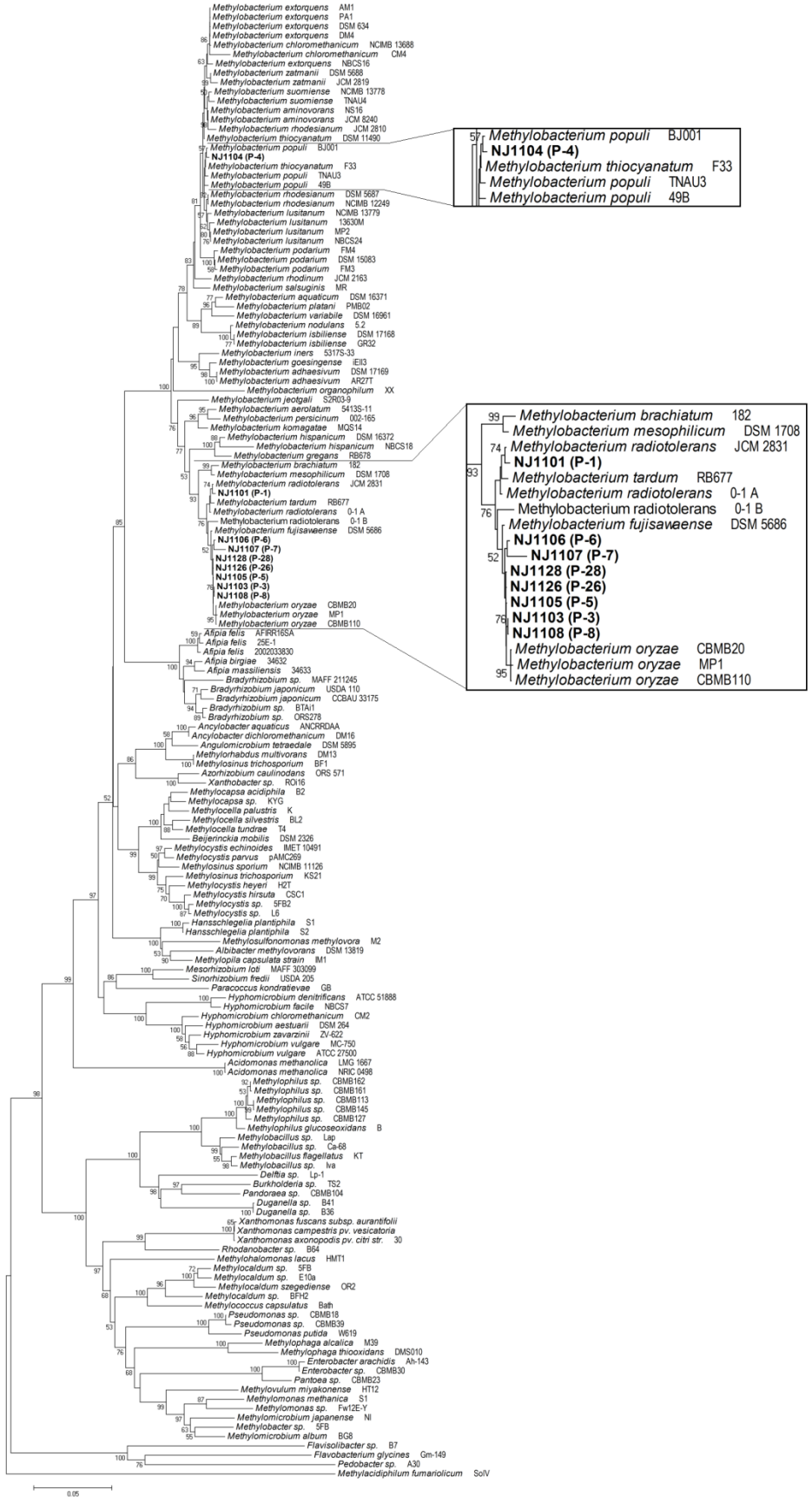

Methylobacterium extorquens AM1
Methylobacterium extorquens PA1
Methylobacterium extorquens DSM 634
Methylobacterium extorquens DM4
Methylobacterium chloromethanicum NCIMB 13688
Methylobacterium chloromethanicum CM4
Methylobacterium extorquens NBCS16
Methylobacterium zatmanii DSM 5688
Methylobacterium zatmanii JCM 2819
Methylobacterium suomiense NCIMB 13778
Methylobacterium suomiense TNAU4
Methylobacterium aminovorans NS16
Methylobacterium aminovorans JCM 8240
Methylobacterium rhodesianum JCM 2810
Methylobacterium thiocyanatum DSM 11490
Methylobacterium populi BJ001
NJ1104 (P-4)
Methylobacterium thiocyanatum F33
Methylobacterium populi TNAU3
Methylobacterium populi 49B
Methylobacterium rhodesianum DSM 5687
Methylobacterium rhodesianum NCIMB 12249
Methylobacterium lusitanum NCIMB 13779
Methylobacterium lusitanum 13630M
Methylobacterium lusitanum MP2
Methylobacterium lusitanum NBCS24
Methylobacterium podarium FM4
Methylobacterium podarium DSM 15083
Methylobacterium podarium FM3
Methylobacterium rhodinum JCM 2163
Methylobacterium salsuginis MR
Methylobacterium aquaticum DSM 16371
Methylobacterium platani PMB02
Methylobacterium variabile DSM 16961
Methylobacterium nodulans 5.2
Methylobacterium isbiliense DSM 17168
Methylobacterium isbiliense GR32
Methylobacterium iners 53175-33
Methylobacterium goesingense IEII3
Methylobacterium adhaesivum DSM 17169
Methylobacterium adhaesivum AR27T
Methylobacterium organophilum XX
Methylobacterium jeotgali SJR03-9
Methylobacterium aerolatum 5413S-11
Methylobacterium persicinum 002-165
Methylobacterium komagatae MQS14
Methylobacterium hispanicum DSM 16372
Methylobacterium hispanicum NBCS18
Methylobacterium gregans RB678
Methylobacterium brachiatum 182
Methylobacterium mesophilicum DSM 1708
Methylobacterium radiotolerans JCM 2831
NJ1101 (P-1)
Methylobacterium tardum RB677
Methylobacterium radiotolerans 0-1 A
Methylobacterium radiotolerans 0-1 B
Methylobacterium fujisawaense DSM 5686
NJ1106 (P-6)
NJ1107 (P-7)
NJ1128 (P-28)
NJ1126 (P-26)
NJ1105 (P-5)
NJ1103 (P-3)
NJ1108 (P-8)
Methylobacterium oryzae CBMB20
Methylobacterium oryzae MP1
Methylobacterium oryzae CBMB110
Afipia felis AFRR18SA
Afipia felis 25E-1
Afipia felis 2002033830
Afipia birgiae 34632
Afipia massiliensis 34633
Bradyrhizobium sp. MAFF 211245
Bradyrhizobium japonicum USDA 110
Bradyrhizobium japonicum CCBAU 33175
Bradyrhizobium sp. BTAi1
Bradyrhizobium sp. ORS278
Ancylobacter aquaticus ANCRRDAA
Ancylobacter dichloromethanicum DM16
Angulomicrobium tetraedale DSM 5895
Methylorhabdus multivorans DM13
Methylosinus trichosporium BF1
Azorhizobium caulinodans ORS 571
Xanthobacter sp. ROI16
Methylocapsa acidiphila B2
Methylocapsa sp. KYG
Methylocella palustris K
Methylocella silvestris BL2
Methylocella tundrae T4
Beijerinckia mobilis DSM 2326
Methylocystis echinoides IMET 10491
Methylocystis parvus pAMC269
Methylosinus sporium NCIMB 11126
Methylosinus trichosporium KS21
Methylocystis heyeri H2T
Methylocystis hirsuta CSC1
Methylocystis sp. 5FB2
Methylocystis sp. L6
Hansschlegelia plantiphila S1
Hansschlegelia plantiphila S2
Methylosulfonomonas methylovora M2
Albibacter methylovorans DSM 13819
Methylopila capsulata strain IM1
Mesorhizobium loti MAFF 303099
Sinorhizobium fredii USDA 205
Paracoccus kondratievae GB
Hyphomicrobium denitrificans ATCC 51888
Hyphomicrobium facile NBCS7
Hyphomicrobium chloromethanicum CM2
Hyphomicrobium aestuarii DSM 264
Hyphomicrobium zavarzinii ZV-622
Hyphomicrobium vulgare MC-750
Hyphomicrobium vulgare ATCC 27500
Acidomonas methanolica LMG 1667
Acidomonas methanolica NRIC 0498
Methylophilus sp. CBMB162
Methylophilus sp. CBMB161
Methylophilus sp. CBMB113
Methylophilus sp. CBMB145
Methylophilus sp. CBMB127
Methylophilus glucoseoxidans B
Methylobacillus sp. Lap
Methylobacillus sp. Ca-68
Methylobacillus flagellatus KT
Methylobacillus sp. Iva
Delftia sp. Lp-1
Burkholderia sp. TS2
Pandoraea sp. CBMB104
Duganella sp. B41
Duganella sp. B36
Xanthomonas fuscans subsp. aurantifolii
Xanthomonas campestris pv. vesicatoria
Xanthomonas axonopodis pv. citri str. 30
Rhodanobacter sp. B64
Methylohalomonas lacus HMT1
Methylocaldum sp. 5FB
Methylocaldum sp. E10a
Methylocaldum szegediense OR2
Methylocaldum sp. BFH2
Methylococcus capsulatus Bath
Pseudomonas sp. CBMB18
Pseudomonas sp. CBMB39
Pseudomonas putida W619
Methylophaga alcalica M39
Methylophaga thiooxidans DMS010
Enterobacter arachidis Ah-143
Enterobacter sp. CBMB30
Pantoea sp. CBMB23
Methylovulum miyakonense HT12
Methylomonas methanica S1
Methylomonas sp. Fw12E-Y
Methylomicrobium japanense NI
Methylobacter sp. 5FB
Methylomicrobium album BG8
Flavisolibacter sp. B7
Flavobacterium glycines Gm-149
Pedobacter sp. A30
Methylacidiphilum fumariolicum SolV
0.05

Figure 5 (previous page). Updated 16S rRNA Neighbor-Joining Phylogenetic Tree of 9 NJ Isolates, and All GenBank Reference Sequences. This is an updated 16S rRNA gene phylogenetic tree depicting the evolutionary relationships of 158 taxa. There were a total of 1495 positions in the final dataset. Bootstrap values above 50 % are shown and were calculated based on the construction of 2,000 replicate trees. Sequences of nine New Jersey (NJ) *Methylobacterium* isolates from agricultural soil are boldfaced.

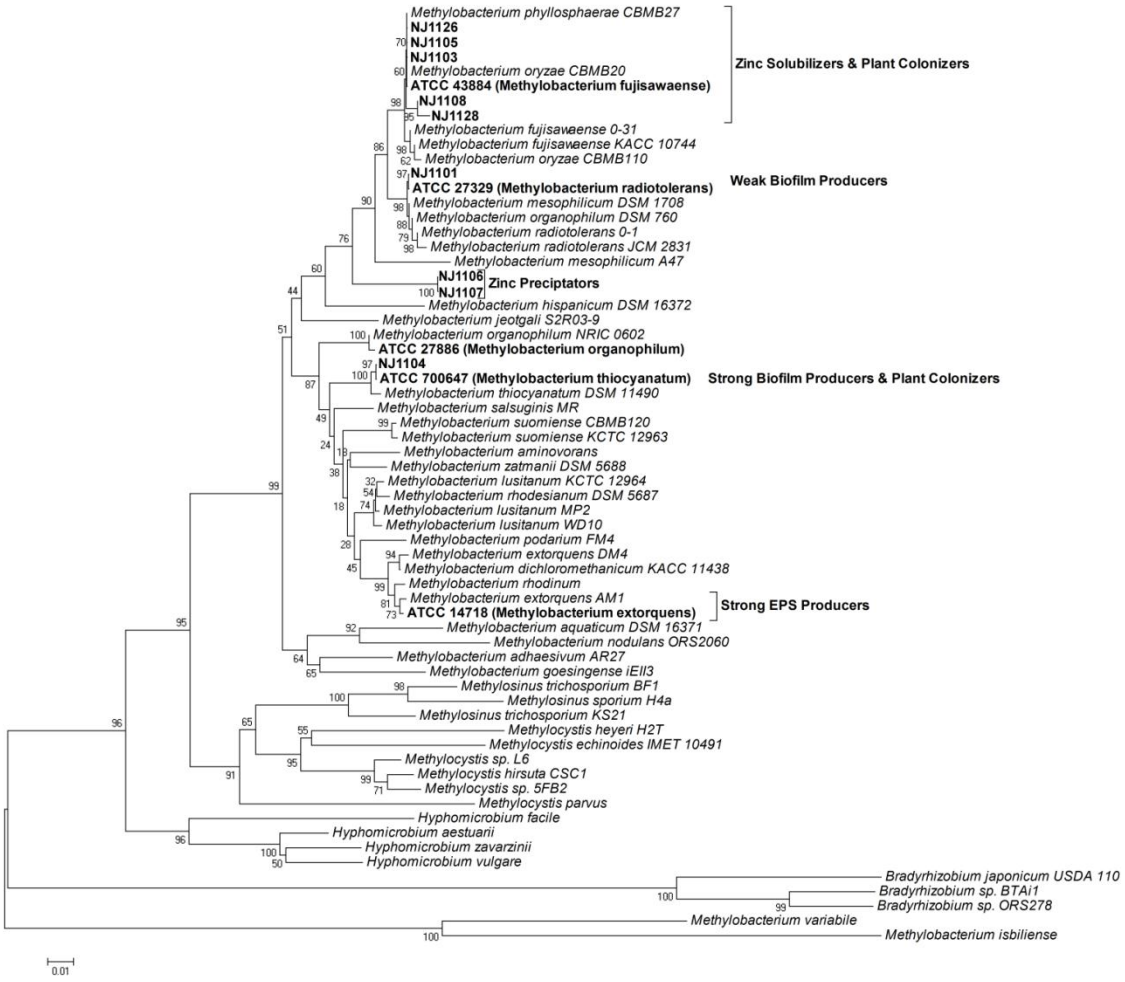


Figure 6. *mxaF* Neighbor-Joining Phylogenetic Tree of 9 NJ Isolates, ATCC acquisitions, and Minimal GenBank Reference Sequences. This is an early *mxaF* tree of the evolutionary relationships of the evolutionary relationships of 64 taxa. There were a total of 567 positions in the final dataset. Bootstrap values above 50 % are shown and were calculated based on the construction of 500 replicate trees. Sequences of nine New Jersey *Methylobacterium* isolates from agricultural soil as well as those of ATCC acquisitions are boldfaced.

GenBank references sequences maintained the same species grouping results for isolates (Fig. 7) Based on the separation of isolate sequences and their varying phenotypic ability (data not shown), putative functional groups are labeled and required further study to confirm.

mxaF gene sequences are highly conserved while still maintaining more variability than those of the 16S rRNA gene sequences. Because of this, the *mxaF* phylogenetic tree organized species into more genetically variable species clades for both isolates and GenBank archived sequences (compare Fig. 4, 16S and Fig. 6, *mxaF*). Based upon phenotypic analyses, isolate behaviors and abilities varied in 0.5 % methanol-amended AMS liquid medium, particularly as regards biofilm production (see Chapter 3) and Zn tolerance mechanisms (see Chapter 4). The separation of these isolates on an *mxaF* phylogenetic tree suggests that species with specific *mxaF* sequences conferring differential activity of the MDH enzyme may group together phylogenetically. However, this was not observed, as the pattern does not appear to be maintained when further GenBank reference sequences are added to the phylogenetic analysis for better resolution (Fig. 7).

The existence of identical *mxaF* sequences for phenotypic variants of a single isolate culture but disparate and reproducible sequences between species suggests that *mxaF* sequence as a genetic tool is capable of characterizing PPFMs at the genus and species level, but that it does not distinguish between transient phenotypic variants. The addition of further GenBank PPFM and non-PPFM sequences to the original subset of nine NJ bacteria only served to further separate the putative species groupings for the isolate sequences. In Fig. 7, the close genetic relatedness of *Methylobacterium* species

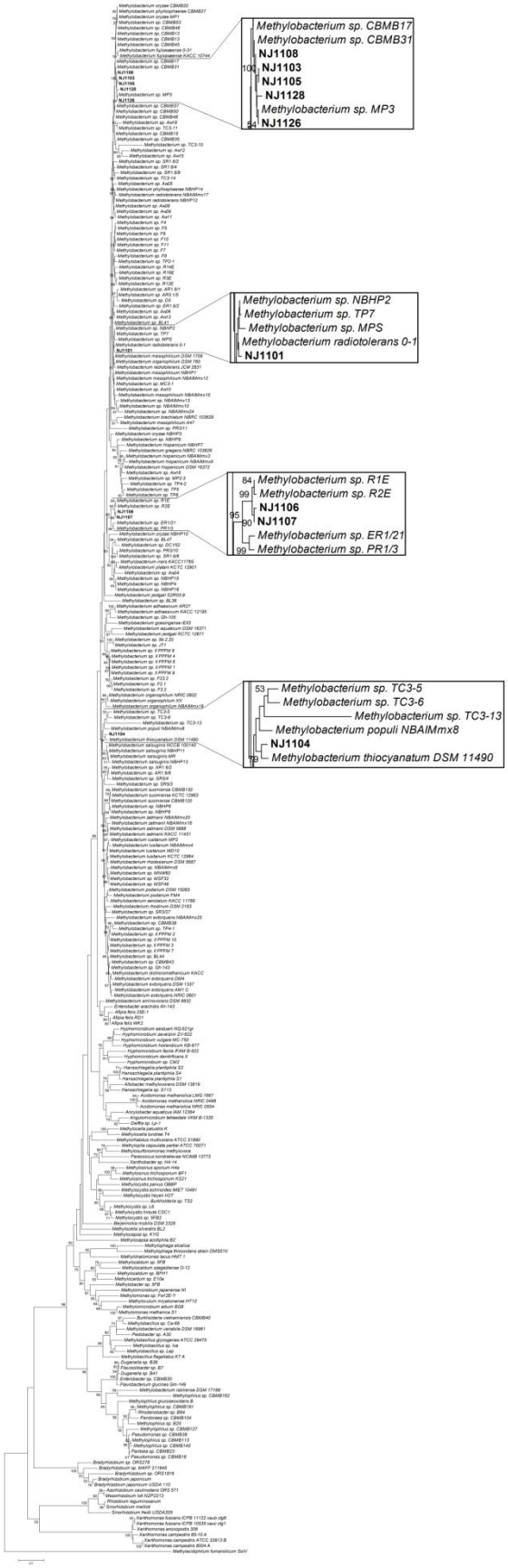

Methylobacterium sp. CBMB17
Methylobacterium sp. CBMB31
NJ1108
NJ1103
NJ1105
NJ1128
Methylobacterium sp. MP3
NJ1126
Methylobacterium sp. NBHP2
Methylobacterium sp. TP7
Methylobacterium sp. MPS
Methylobacterium radiotolerans 0-1
NJ1101
Methylobacterium sp. R1E
Methylobacterium sp. R2E
NJ1106
NJ1107
Methylobacterium sp. ER1/21
Methylobacterium sp. PR1/3
Methylobacterium sp. TC3-5
Methylobacterium sp. TC3-6
Methylobacterium sp. TC3-13
Methylobacterium populi NBAIMmx8
NJ1104
Methylobacterium thiocyanatum DSM 11490

Figure 7 (previous page). **Updated *mxaF* Neighbor-Joining Phylogenetic Tree of 9 NJ Isolates, and All GenBank Reference Sequences.** (Note: Tree is halved for ease of reading, starting from the top left to the bottom, then upper right to bottom again.) This is an updated *mxaF* phylogenetic tree depicting the evolutionary relationships of 279 taxa. There were a total of 697 positions in the final dataset. Bootstrap values above 50 % are shown, calculated based on the construction of 2,000 replicate trees. Sequences of nine New Jersey (NJ) PPFM isolates from agricultural soil are boldfaced.

mxaF sequences becomes starkly apparent. This strengthens the argument for the utility of *mxaF* as a characterization tool for *Methylobacterium* species.

mxaF Phylogenetic Analysis of Soil and Blueberry Skin Isolates

There was no relationship observed on the *mxaF* phylogenetic tree between PPFM species and soil sample geography, plant cover or source plant species, soil type, or sample pH (Fig. 8, showing only geographic location and pH coding). *Methylobacterium mxaF* sequences spanned the genus regardless of sample source, geography, or pH (indicated in Fig 8) and regardless of plant cover or source plant, soil type, or annual rainfall (not shown), suggesting that none of these parameters are selective factors for *mxaF* sequence in the environment. All isolate sequences and GenBank *Methylobacterium* reference sequences were highly genetically related based upon their *mxaF* genes, indicated by the close branching of *Methylobacterium* sequences and distant branching of the *Xanthomonas axonopodis* root reference sequence. The only exceptions were *M. variabile* and *M. isbiliense*, which had annotated *mxaF* sequences that were noticeably divergent.

These findings were surprising, especially when considering pH, as MDH is a periplasmic enzyme with only a semi-permeable membrane between it and the extracellular environment (Kasprzak and Steenkamp, 1983; Nunn and Lidstrom, 1986). It would have seemed intuitive that certain species would be selected that possessed *mxaF* genes with sequences that give rise to MDH enzymes capable of performing optimally at those pH values. This was not found to be true for PPFMs, however. *Escherichia coli* induces the production of various periplasmic enzymes and varies their levels depending

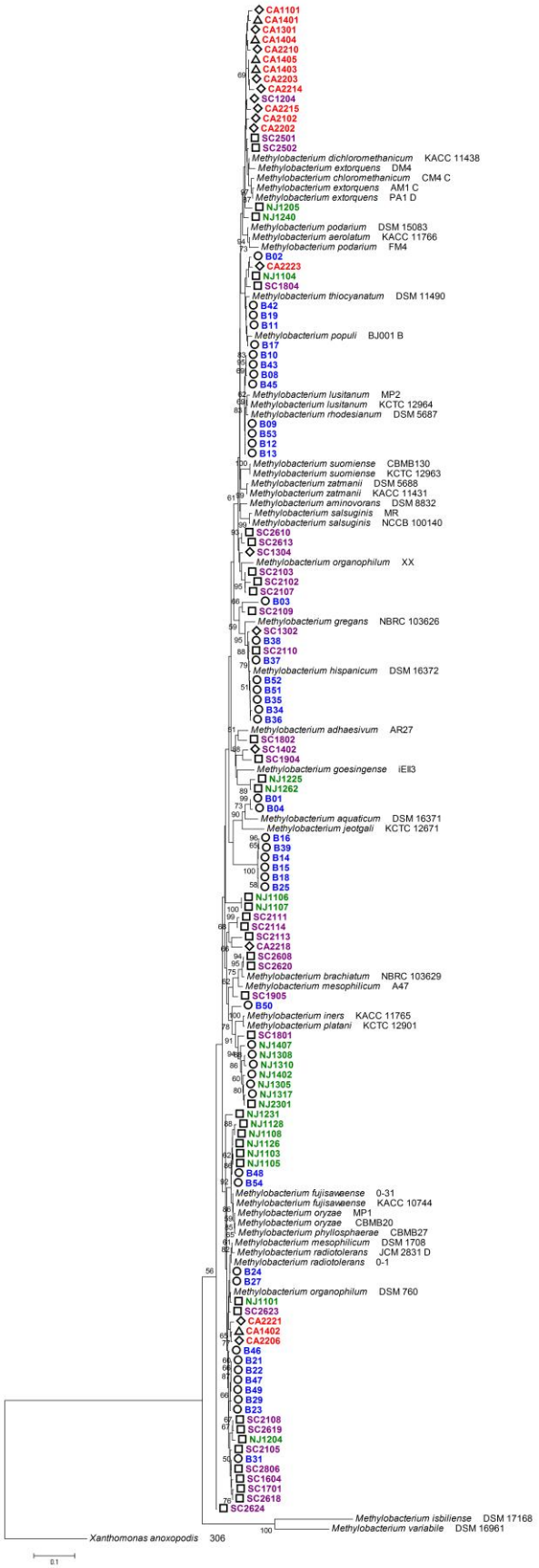

CA1101
CA1401
CA1301
CA1404
CA2210
CA1405
CA1403
CA2203
CA2214
SC1204
CA2215
CA2102
CA2202
SC2501
SC2502
Methylobacterium dichloromethanicum KACC 11438
Methylobacterium extorquens DM4
Methylobacterium chloromethanicum CM4 C
Methylobacterium extorquens AM1 C
Methylobacterium extorquens PA1 D
NJ1205
NJ1240
Methylobacterium podarium DSM 15083
Methylobacterium aerolatum KACC 11766
Methylobacterium podarium FM4
B02
CA2223
NJ1104
SC1804
Methylobacterium thiocyanatum DSM 11490
B42
B19
B11
Methylobacterium populi BJ001 B
B17
B10
B43
B08
B45
Methylobacterium lusitanum MP2
Methylobacterium lusitanum KCTC 12964
Methylobacterium rhodesianum DSM 5687
B09
B53
B12
B13
Methylobacterium suomiense CBMB130
Methylobacterium suomiense KCTC 12963
Methylobacterium zatmanii DSM 5688
Methylobacterium zatmanii KACC 11431
Methylobacterium aminovorans DSM 8832
Methylobacterium salsuginis MR
Methylobacterium salsuginis NCCB 100140
SC2610
SC2613
SC1304
Methylobacterium organophilum XX
SC2103
SC2102
SC2107
B03
SC2109
Methylobacterium gregans NBRC 103626
SC1302
B38
SC2110
B37
Methylobacterium hispanicum DSM 16372
B52
B51
B35
B34
B36
Methylobacterium adhaesivum AR27
SC1802
SC1402
SC1904
Methylobacterium goesingense iEII3
NJ1225
NJ1262
B01
B04
Methylobacterium aquaticum DSM 16371
Methylobacterium jeotgali KCTC 12671
B16
B39
B14
B15
B18
B25
NJ1106
NJ1107
SC2111
SC2114
SC2113
CA2218
SC2608
SC2620
Methylobacterium brachiatum NBRC 103629
Methylobacterium mesophilicum A47
SC1905
B50
Methylobacterium iners KACC 11765
Methylobacterium platani KCTC 12901
SC1801
NJ1407
NJ1308
NJ1310
NJ1402
NJ1305
NJ1317
NJ2301
NJ1231
NJ1128
NJ1108
NJ1126
NJ1103
NJ1105
B48
B54
Methylobacterium fujisawaense 0-31
Methylobacterium fujisawaense KACC 10744
Methylobacterium oryzae MP1
Methylobacterium oryzae CBMB20
Methylobacterium phyllosphaerae CBMB27
Methylobacterium mesophilicum DSM 1708
Methylobacterium radiotolerans JCM 2831 D
Methylobacterium radiotolerans 0-1
B24
B27
Methylobacterium organophilum DSM 760
NJ1101
SC2623
CA2221
CA1402
CA2206
B46
B21
B22
B47
B49
B29
B23
SC2108
SC2619
NJ1204
SC2105
B31
SC2806
SC1604
SC1701
SC2618
SC2624
Methylobacterium isbiliense DSM 17168
Methylobacterium variabile DSM 16961
Xanthomonas anoxopodis 306
0.1

Figure 8 (previous page). Neighbor-Joining Phylogenetic Tree of All 114 Isolate *mxaF* Gene Fragments and Limited GenBank References Sequences (*Methylobacterium* and Minimal Side Reference Sequences).

This *mxaF* phylogenetic tree depicts the evolutionary relationships of 156 taxa, including all PPFM isolates sequenced to date and GenBank reference sequences. There were a total of 652 positions in the final dataset. Bootstrap values above 50 % are shown and were calculated based on the construction of 2,000 replicate trees. The sequences of *Methylobacterium* isolates from soil samples and the blueberry skin biofilm are shown boldfaced. Isolate names and colors denote the sample source and location. CA (red) = California soil, SC (purple) = South Carolina soil, NJ (green) = New Jersey soil and BB (blue) = blueberry skin biofilm. Node markers indicate the pH of the soil or sample source. Categories of soil pH acidity and alkalinity used were established from Bradley and Weil, 2002. The pH of blueberry skin was estimated from that of blueberry skin processing waste materials (Lee and Wrolstad, 2004). *Circle* = 2.5 to 4.7 (extremely acidic), *square* = 4.8 to 6.5 (moderately acidic), *diamond* = 6.6 to 7.4 (neutral), and *triangle* = 7.5 to 7.9 (slightly alkaline).

upon in what pH the cells are being made to function (Stancik et al., 2002). While PPFM *mxaF* sequence does not seem to be affected by varying pH, perhaps other aspects of *mxaF* transcription and function are affected by pH or other environmental parameters and further research would be necessary to determine that.

It was also surprising that plant cover or source plant species did not seem to select for specific *Methylobacterium* species based upon their *mxaF* sequences, as this is contrary to the research of other groups that found that *Methylobacterium* species distribution both in soil and above-ground was plant species specific (Knief et al., 2010).

These data indicate that *mxaF* sequence is a robust genetic tool that can reliably characterize *Methylobacterium* species from environmental samples, particularly from soil. Species distribution did not correlate with geographical source or pH (Fig. 7), nor with land use or annual rainfall (data not shown). This suggests that PPFMs occupy a metabolic niche in the environment (utilization of plant-derived methanol) for which they are extremely well adapted. It also suggests a reason for the ubiquity of the *Methylobacterium* genus.

CONCLUSIONS

The DGGE, sequencing and phylogenetic analyses presented here confirm that *mxaF* sequence is a useful characterization tool for *Methylobacterium* species. *mxaF* sequences were identical within lab isolate species and highly similar between archived species. Species groupings on Neighbor-Joining phylogenetic trees were tighter and more reliable with *mxaF* than when using the 16S rRNA gene. *Methylobacterium mxaF* sequences have a “dominant band” representing the dominant sequence in the PCR

product that is species-specific, while 16S rRNA banding patterns are similar and indistinguishable even for phenotypically dissimilar species. *mxaF* gene sequence contains enough variability to distinguish between *Methylobacterium* species but is conserved enough for species to have a unique and reproducible *mxaF* sequence.

Methylobacterium species are environmental specialists that engage in both methanol utilization and plant colonization in soil environments. Data presented here shows evidence of PPFM *mxaF* sequence being varied throughout the genus. However, there does not appear to be a subset of organisms possessing certain sequences for the environmental and sample parameters investigated here that are selected for under those conditions. This suggests not only variability in PPFM *mxaF* sequence but versatility as well. The fact that no trend was seen linking the chemical or soil property data (e.g. pH) to PPFM species distribution might suggest a reason for the widespread nature of both the genus and the *mxaF* gene. The ability of *mxaF* to function in various environmental conditions, such as soils with differing pH, suggests versatility of function as a reason for the gene's ubiquity in PPFMs and soil.

When considering bioremediation processes, where environmental conditions can be harsh, and agricultural crop stimulation, for which certain species are preferred, the versatility of PPFM *mxaF* sequence is promising. The application of bioremediation methods, particularly phytoremediation methods that involve plants, may be made easier through the exploitation of PPFM metabolism and versatility. It may mean that in a given environment, PPFMs with beneficial qualities may be present and need only be stimulated, rather than that bacteria have to be added to the soil and acclimated before bioremediation can begin.

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Chapter 2

Phylogenetic Characterization of Bacterial *mxaF*, *xoxF* and PQQ-Dependent Dehydrogenase Genes Reveals Robustness in *Methylobacterium mxaF* Gene Sequences and *mxa* Operon Construction

ABSTRACT

Methylobacterium species, or pink-pigmented facultatively methylotrophic bacteria (PPFMs) are plant colonizing bacteria that utilize plant-derived methanol as a carbon and energy source during phyllosphere and rhizosphere growth. The *mxaF* gene is the structural gene for the methanol dehydrogenase (MDH enzyme) and is responsible for the conformation of the resulting protein. It encodes for the large or alpha subunit of the MDH enzyme. It is one of a few genes within the *mxa* family for which a working gene is required for the production of a functional MDH enzyme. Here, *mxaF* sequence is explored as a potential genetic tool for the characterization of PPFM species, due to its ubiquity and widespread activity in the genus. Phylogenetic comparison of 311 archived GenBank bacterial sequences of *mxaF*, *xoxF*, and generically annotated PQQ-dependent dehydrogenase genes in the methanol/ethanol family was conducted, including 270 *mxaF*, *mxaF'* and *mxaF*-like sequences. These sequences spanned multiple genera including *Methylobacterium*. *mxaF* gene sequences were compared with those of the *xoxF* homolog, which encodes for only a single-unit MDH enzyme, and those of other methanol/ethanol dehydrogenases to investigate the robustness of *mxaF* sequence as a genetic marker for this genus. In addition, construction of operons within the *mxa* gene

family were compared using complete genome sequences from GenBank to investigate the evolution of *mxaF* and the *mxa* system is methylotrophs. *mxaF* sequence was found to be a robust genetic marker for the genus *Methylobacterium*, showing tight clustering for that genus when compared to others. *Mxa* gene family operon construction appears to be more highly conserved for the *Methylobacterium* genus than for other genera, however, the presence of anomalous species (both PPFM and non-PPFM) and the limited number of completed genomes containing *mxa* family genes suggests that more data and further investigation is needed to understand how the *mxa* system has evolved in methylotrophs. Evidence of horizontal gene transfer of *mxaF* and other *mxa* genes can be inferred from discrepancies in the 16S rRNA and *mxaF* phylogenetic trees as well as the comparison of *mxa* operons between genera. This was not validated with GC content or presence/absence of mobile genetic elements, however, indicating that HGT events occurred very early in the history of these bacteria, that GC content was too similar between the species to show significant differences, or that some species may be incorrectly identified or annotated in the GenBank database.

INTRODUCTION

The genus *Methylobacterium* consists of pink-pigmented, facultatively methylotrophic (PPFM) bacteria. These bacteria specialize in the use of C_1 compounds as their sole carbon and energy sources (Omer *et al*., 2004; Van Aken *et al*., 2004; Chistoserdova, et al., 2003). Although these bacteria are not limited to one-carbon substrates, their ability to utilize methanol confers an advantage when colonizing the phyllosphere (Omer *et al*., 2004; Van Aken *et al*., 2004). PPFMs are known plant

colonizers and plant growth promoters. They can directly improve the health of the plant by occupying niches that could otherwise be occupied by pathogens and by producing vitamins and growth hormones that improve the plant's metabolism (Lidstrom and Chistoserdova, 2002; Hornschuh et al., 2006). They can also promote plant growth indirectly by boosting the plant's innate immune system against pathogens simply by being present on plant surfaces (Madhaiyan, et al., 2004; Madhaiyan, et al., 2005; Madhaiyan, et al., 2006; Abanda-Nkpwatt et al., 2006). There are both epiphytic and soil strains of PPFMs, with the epiphytic bacteria being found on leaves, stems, roots and leaf buds (Holland and Polacco, 1992; Romanovskaya et al., 2001).

The genus was first described by Patt et al (1976). It now includes all known pink-pigmented bacteria that are facultatively methylotrophic, following the renaming of several species formerly classified as Pseudomonas species (Green & Bousfield, 1983) and one genus formerly classed as a *Protomonas* (Bousfield & Green, 1985). It consists of 38 published species and one awaiting publication for a total of 39 species as of September 13, 2011 (Table 1). That number changes often not only with the publishing of new species but also with ongoing species reclassification. Some reclassification has resulted in separately described *Methylobacterium* species being merged under a single species name (Kato et al., 2005). It was noted that such reclassification need not be adopted by every researcher (Euzeby, 2006). This resulted in ambiguity as to whether the previously distinct species names are still in existence. Confusion arises especially when browsing the GenBank online database for *Methylobacterium* species sequences, as outdated or reclassified names for species are still being used in combination with new classifications. Although confusing, this constant reclassification of *Methylobacterium*

species and uncertainty surrounding distinctions between species attests to the high degree of genetic similarity and yet multifaceted phenotypic variation observed with these species.

One type of metabolism in *Methylobacterium* species with the potential to result in multiple phenotypes and behaviors is methylotrophy. PPFMs are capable of oxidizing naturally occurring methanol in soil. *Methylobacterium* species are found in soil associate with plant roots, which release methanol as a result of the growth of plant cells. Because of this, methylotrophy plays a large role in PPFM metabolism in the environment, whether they are colonizing plants above ground or are part of the rhizosphere community. Methanol is produced by plants in areas of active growth as new cells are created, elongated, and split into smaller cells. Pectin, a component of plant cell walls, is synthesized and broken down many times during the growth process as new cells are created (Nemecek-Marshall et al., 1995; Galbally and Kirstine, 2002). When pectin is degraded, it is demethylated, resulting in a release of methanol Plants produce and release methanol as a byproduct of pectin metabolism during cell wall synthesis, during leaf expansion and development, and as a factor of stomatal conductance (Nemecek-Marshall et al., 1995; Galbally and Kirstine, 2002). Therefore, methanol is present in both growing, living plant tissue and in debris that falls to the ground. Phyllosphere PPFMs can utilize plant-produced methanol directly. Soil PPFMs can utilize that which is released from decaying plant matter near the soil surface. Methanol is also produced in subsurface soils by methanotrophs as methane is oxidized into methanol. When this methanol volatilizes and makes its way upwards into aerobic soils, PPFMs can utilize that source as well.

Methylotrophy in PPFMs has been studied using traditional culturing methods, but the genetics of methylotrophy are also being investigated. As more is discovered about the *Methylobacterium* genus and their ability to utilize methanol competitively in plant phyllosphere and rhizosphere environments, it is important to understand the genes involved and how they relate to methylotrophy genes of other genera.

The environmental impact of methylotrophy in the *Methylobacterium* genus lies in its potential to reduce the amount of carbon dioxide (CO_2) that is released into the atmosphere as a result of the oxidation of methane in soils. A simplified chemical pathway for the complete oxidation of methane to CO_2 and water as it can occur in PPFMs is detailed below (Chistoserdova et al., 1998; Chistoserdova, 2003):

CH_4	→	CH_3OH	→	HCOH	→	$CHOO^-$	→	CO_2	+	H_2O
Methane		Methanol		Formaldehyde		Formate		Carbon Dioxide		Water

Because *Methylobacterium* species are widespread in soil, their methylotrophy system, the *mxa* gene family, is also widespread. As PPFMs utilize methanol produced by plants or by subsurface anaerobes, they oxidize it to methanol using methanol dehydrogenase (MDH), forming formaldehyde. A few species can oxidize methane first and then methanol, but the majority can only oxidize methanol. The formaldehyde is then taken into the cells and enters into biomass assimilation pathways. Formate is not formed and therefore neither are CO_2 and water. This results in lower potential for CO_2 to be released into the atmosphere as a result of methane oxidation. In this way, PPFMs play a role in the cycling of both methane and CO_2, two important greenhouse gases.

MDH is a heterotetrameric enzyme, consisting of two large alpha subunits and two small beta subunits. The enzyme also has Ca^{2+} and pyrrolo quinolone-quinone (PQQ) associations. The oxidation of methanol is coupled with the reduction of PQQ to $PQQH_2$. PQQ is reduced, re-oxidized, and then reduced once more (Anthony et al., 1994; Anthony and Ghosh, 1998; Duine, 1999). It is this exchange of electrons that catalyzes the oxidation of the methanol. Calcium ions stabilize the attack site of PQQ so that it can be reduced and the reaction can proceed (White et al., 1993; Duine, 1999).

Genes of the *mxa* family control the production and arrangement of the components of the MDH enzyme, its calcium and PQQ associations, and its eventual shape. The conformation of the MDH enzyme is encoded for by the *mxaF* gene, one of seventeen genes in the *mxa* family (Madhaiyan, *et al.*, 2005; McDonald and Murell, 1997). *mxaF* is the structural gene for the MDH enzyme, encoding for the large, alpha subunit. It is essential for the formation of a properly functioning MDH enzyme. It is a highly conserved gene, but it is still a functional gene and is therefore not as conserved as the housekeeping gene, 16S rRNA. It is ubiquitous in the *Methylobacterium* genus, but not so in other genera. Within the gene are specific conserved regions wherein, if mutations occur, the conformation and thus the resulting efficiency of the MDH enzyme may be compromised (McDonald and Murell, 1997).

It is known that other genes in the *mxa* family are also involved in the production of the MDH enzyme. The beta subunit is encoded for by the *mxaI* gene. The transcription of the *mxaF* and *mxaI* genes is controlled by other response-regulator genes in the *mxa* family, such as *mxaB* (Springer et al., 1998). Also, genes that are involved in the acquisition and/or the incorporation of either Ca^{2+} ions or PQQ are essential for a fully

functioning MDH enzyme. It still remains unclear what functions and gene products many genes in the *mxa* family have. The *mxaAKL* genes appear to be involved with Ca2+ acquisition (Morris et al., 1995; Springer et al., 1998), while the functions of the *mxaCDJ* genes are unknown. Knockouts of *mxaJ* prevent a functional MDH enzyme (Amaratunga et al., 1997), however, and *mxaD* is thought to be involved with cytochrome C_L transport inside the periplasm (Anderson and Lidstrom, 1988).

Another gene, *xoxF*, is a homolog of *mxaF* and encodes for a single-unit MDH enzyme reminiscent of the large alpha subunit that *mxaF* encodes for. It is unclear what the purpose of this gene is beyond creating a different type of MDH enzyme, but that enzyme is known to have advantages over the MDH that results from the *mxaF* and *mxaI* subunit system of genes, especially in the phyllosphere. It is highly expressed in the phyllosphere and is therefore a gene to be considered alongside *mxaF* for the importance of methylotrophy in PPFM species. If *mxaF* is to be considered a true biomarker for *Methylobacterium* species, its sequence must be distinctive not only for that genus but also from other similar methylotrophy genes. For this reason, *xoxF* sequences in addition to those of other generically labeled (pyrrolo-quinoline quinone) PQQ-dependent dehydrogenase genes in the methanol/ethanol family will be considered in this chapter.

The *mxa* family consists of 17 genes with the following terminology: *mxaA, mxaB, mxaC, mxaD, mxaE, mxaF, mxaG, mxaH, mxaI, mxaJ, mxaK, mxaL, mxaR, mxaS, mxaW, mxaX, mxaY* and *mxaZ* (Lidstrom, et al., 1994). Annotated and published gene names have changes over the years, due to further information being revealed about the location, function, and gene product, but attempts have been made to standardize the nomenclature and clear up confusion (Lidstrom, et al., 1994).

PPFMs have the potential to increase seed germination and crop yields (Madhaiyan, *et al.*, 2005; Madhaiyan, *et al.*, 2006). We believe as well that they can participate in bioremediation processes, such as the phytoextraction of Zn. But not all species promote plant growth or do so in the same manner, and they vary in their tolerance strategies regarding Zn. Before we can begin to exploit the potential of this genus for various agricultural and environmental applications, we must first find a way of rapidly and reliably characterizing them in the environment. Chapter 1 delved into the *mxaF* gene and its usefulness as a genetic tool to characterize *Methylobacterium* species compared to that of the 16S rRNA gene. In this chapter, we will further investigate *mxaF* sequence as a genetic tool and potential biomarker for the *Methylobacterium* genus compared to other genera.

We hypothesize that studying archived sequences in the GenBank database can provide insight into the evolution of the *mxa* methylotrophy system in *Methylobacterium* and other genera, with a focus on the *mxaF* gene. If *mxaF* sequence can affect the performance of the resulting MDH enzyme, then it is not unreasonable to propose that genetic characterization using *mxaF* sequences will distinguish a group of bacteria based upon the single trait of methylotrophy. In addition, comparison of *mxaF* and related gene sequences to their GenBank annotations may reveal inconsistencies in those annotations that can be cleared up and better explained once erroneously annotated sequences are placed together with others on the phylogenetic tree. We also hypothesize that a comparison between archived GenBank *mxaF* sequences and *mxa* gene family operon structure from PPFMs and non-PPFMs will further illustrate whether or not *mxaF*

sequence can be considered a biomarker for the *Methylobacterium* genus. Studying *mxa* operon construction will shed light on the evolution of the *mxa* system in methylotrophs.

The specific objectives of this research were as follows:

1) to examine the feasibility of using *mxaF* as a biomarker (a genetic marker or tool) for characterizing species in the genus *Methylobacterium* using sequences from the GenBank database;

2) to further assess the robustness of *mxaF* sequence as a characterization tool for this genus by compounding the *mxaF* sequence data with that of *xoxF* and other PQQ-dependent dehydrogenase gene sequences;

3) to examine *mxa* gene family operon construction, G+C content and potential mobile genetic elements of several completed bacterial genomes in order to see what such a comparison might tell us about the evolution of the *mxa* methylotrophy system in bacteria.

MATERIALS AND METHODS

General Methods for Obtaining Phylogenetic Trees

Clustering patterns on Neighbor-Joining phylogenetic trees were observed using MEGA 4 software (Tamura, 2007). Bootstrapping was implemented as a means of adding statistical relevance to any resulting clustering patterns. For each tree, 2,000 replicate trees were produced and consensus trees were developed. Bootstrap values are not shown due to the circular nature of the trees and the fact that they only depict topology and not evolutionary distance. The bootstrapping and obtaining of a consensus tree, however, still

served as a method by which some of the variability and uncertainty could be removed from the dataset.

Phylogenetic Analysis of GenBank *Methylobacterium* and Non-PPFM Sequences with *mxaF, mxaF'* and *mxaF*-like Annotations

Initially, only *mxaF, mxaF'* (the apostrophe is pronounced "prime," and is another way of denoting "*mxaF*-like" genes) and *mxaF*-like sequences from *Methylobacterium* and non-PPFM GenBank species were considered for phylogenetic analysis. The purpose of this analysis was to theoretically assess the feasibility of using *mxaF* sequence as a genetic characterization tool for *Methylobacterium* species. A reference list of current species comprising the genus *Methylobacterium* and where each was first isolated can be found in Table 1. Utilizing GenBank sequences allowed for a larger and more varied subset of bacteria to be examined beyond the isolates that were obtained in our laboratory. Non-PPFM sequences were considered along with PPFM sequences to determine whether PPFM *mxaF* sequences were unique and conserved enough to act as genetic markers for the genus. A total of 270 bacterial sequences were chosen, from GenBank as of October 30, 2011. Detailed information regarding the definitions of each gene annotation can be found in Table 2.

Phylogenetic Analysis of GenBank *Methylobacterium* and Non-PPFM Sequences with *mxaF, mxaF', mxaF*-like, *xoxF*, and PQQ-Dependent Methanol/Ethanol Dehydrogenase Annotations

Table 1. List of Described Species in the Genus *Methylobacterium*.

Species	Isolation Source	Reference
M. adhaesivum	Drinking water	Gallego et al., 2006
M. aerolatum	An air sample in Korea	Weon et al., 2008
M. aminovorans		Urakami et al., 1993
M. aquamaris (renamed: see *M. salsuginus)*	N/A	N/A
M. aquaticum	Drinking water	Gallego et al., 2005a
M. brachiatum	Freshwater	Kato et al., 2008
M. chloromethanicum	A petrochemical factory in Tartarstan, Russia	McDonald et al., 2001
M. dankookense	Drinking water	Lee et al., 2009
M. dichloromethanicum		Doronina et al., 2000
M. extorquens		Urakami & Komagata, 1984; Bousfield & Green, 1985 (reclassified)
M. fujisawaense	Fujisawa, Japan	Green et al., 1988
M. gregans	Isolated from freshwater	Kato et al., 2008
M. hispanicum	Drinking water	Gallego et al., 2005a
M. iners	An air sample in Korea	Weon et al., 2008
M. isbiliense	Drinking water system in Sevilla, Spain	Gallego et al., 2005c
M. jeotgali	Korean traditional fermented seafood called joetgal	Aslam et al., 2007
M. komagatae	Freshwater	Kato et al., 2008
M. lusitanum	Sewage station in Portugal	Doronina et al., 2002
M. marchantiae	Liverwort thallus	Schauer et al., 2011
M. mesophilicum		Austin & Goodfellow, 1979; Green & Bousfield, 1983 (reclassified)
M. nodulans	Root nodule samples from legume *Crotalaria* spp.	Jourand et al., 2004
M. organophilum		Patt et al., 1976
M. oryzae	Rice	Madhaiyan et al., 2007
M. persicinum	Freshwater	Kato et al., 2008
M. phyllosphaerae	Rice phyllosphere	Madhaiyan et al., 2009
M. platani	Leaf from the tree *Platanus orientalis*	Kang et al., 2007
M. podarium	Human foot	Anesti et al., 2004
M. populi	Poplar tree (*Populus deltoides*) tissues	Van Aken et al., 2004
M. radiotolerans		Ito & Iizuka, 1971; Green & Bousfield, 1983 (reclassified)
M. rhodesianum	A fermentor operating with formaldehyde as the sole carbon source.	Green et al. 1988
M. rhodinum		Heumann, 1962; Green & Bousfield, 1983 (reclassified)
M. salsuginis	Seawater	Wang et al. 2007

M. soli	Forest soil	Cao et al. (in press, 2011)
M. specialis		Unpublished source, 2011.
M. suomiense	Soil samples in Finland	Doronina et al. 2002
M. tardum	Freshwater	Kato et al. 2008
M. thiocyanatum	Rhizosphere soil from A. aflatunense	Wood et al. 1998
M. variabile	Drinking water	Gallego et al. 2005b
M. zatmanii	A fermentor operating with formaldehyde as the sole carbon source.	Green et al. 1988

Table 2. GenBank Gene Annotations and their Meanings.

Gene Annotation	Description	References
mxaF	Denotes a sequence encoding for the alpha (large) subunit of the methanol dehydrogenase (MDH) enzyme.	McDonald and Murrell, 1997 Machlin and Hanson, 1988 Anderson and Lidstrom, 1988
mxaF' or *mxaF*-like	Denotes a sequence with high homology to that of the *mxaF* gene sequence, but that the submitting author has deemed not homologous enough to identify it definitively as *mxaF*.	*(Described as mxaF but the prime or –like designation is made at the author's discretion.)*
xoxF	Denotes a sequence for a homolog of *mxaF* that encodes for a single-unit MDH enzyme.	Schmidt et al., 2010 Skovran, et al., 2011
PQQ-dependent dehydrogenase, methanol/ethanol family	Denotes a sequence encoding for a PQQ-dependent dehydrogenase in the methanol/ethanol family that has not been identified more specifically to indicate a specific gene or enzyme product.	Anthony et al., 1994; Anthony and Ghosh, 1998.; Duine, 1999

In order to further examine the robustness of *mxaF* sequence as a characterization tool, genes with similar sequences, protein products, and product functions were considered alongside *mxaF*-related genes. Five categories of sequences were used for the phylogenetic analysis, including *mxaF*, *mxaF'*, *mxaF*-like, *xoxF*, and several PQQ-dependent dehydrogenase genes in the methanol/ethanol family (Table 2). All gene sequences were categorized based upon their annotations in the GenBank online database (Dennis et al., 2005).

Capital letters "A," "B," "C" or "D" following the strain designation for a species label on the tree denote the order in which that sequence is found in the genome. For example, *Methylobacterium* sp. 4-46 possesses four genes that are all annotated as "PQQ-dependent dehydrogenase in the methanol/ethanol family," but to tell them apart, letters were added according to the order in which they occur in the genome sequence. "*Methylobacterium* sp. 4-46 A," therefore, is the first PQQ-dependent dehydrogenase gene found in that organism's completed genome sequence, "B" is the second, and so on. These labels were added after sequences were compiled from GenBank for clarification and are not part of the original sequence annotation.

A total of 311 bacterial sequences were chosen for this analysis, from GenBank as of October 30, 2011. No lab isolate sequences were included in this particular analysis. While only some generic PQQ-dependent dehydrogenase gene sequences were included in the analysis, all *mxaF* and *xoxF* sequences were used, excluding limited exceptions. Those exceptions fell into the categories detailed in Table 3.

Table 3. Specifications for Sequences Omitted from the GenBank *mxaF*, *xoxF*, and PQQ-Dependent Dehydrogenase Phylogenetic Study.

Reason for Omission	Detailed Explanation
Low sequence quality	Sequences contain indeterminate data (designated by "N," meaning the base could be any letter) greater than 2.0 % of the sequence that was not limited to ends .
Lack of reference paper	Sequences do not cluster as expected on a phylogenetic tree and there are no published methods or other information regarding the source or obtaining of that sequence.
Publication reveals potential errors in gene sequencing or bacterial species classification	Sequences do not cluster as expected on a phylogenetic tree and there is reason to suspect based on information contained in the reference paper that the source organism or sequence identification are not what they are annotated as in GenBank.
Short sequence length	Once an alignment has been constructed with all sequences intended for the analysis, a sequence has only 60.0 % or less of the number of bases of the rest after all ends have been suitably trimmed.
Duplicate submissions for the same bacterium	Two or more submissions for the same gene exist for the same bacterial strain and are homologous enough to fall on the same branch of a phylogenetic tree, duplicates are omitted to reduce the complexity of the dataset and the size of the final tree.
Highly divergent solitary sequences	Bacteria with solitary sequences deposited in GenBank were reviewed for their importance if the sequences were very divergent. Omitted sequences were so divergent as to lie on long branches that made the tree unwieldy but whose presence or absence did not drastically change the placement of other sequences on the tree.
Highly similar submissions of research isolates	Research isolates with only genus and strain designations were submitted by the same researcher or research group and clustered very closely. Of ten isolates, two or three would be kept to reduce the dataset.
Sequences were submitted after May 12, 2011	Sequences submitted between May 12, and September 12, 2011 were repetitive submissions of isolate strains and were not predicted to meaningfully affect the outcome of the overall analysis, and were not included.

All definitively annotated *mxaF* genes (excluding those annotated as *mxaF'*, *mxaF*-like, *xoxF*, or generic PQQ-dependent dehydrogenase) from completed bacterial genomes were collected from the GenBank online database. These *mxaF* gene sequences were used to pinpoint the location of the *mxa* operons on the chromosome map of each bacterial strain with a complete genome sequence. From the *mxaF* genes, the chromosome was searched both left and right to discern the genes adjacent to *mxaF*. In addition to this, *mxa* gene sequences were searched to confirm that they were not simply found elsewhere in the genome and not near *mxaF*. Once all available *mxa* genes had been located for each completed genome, an operon construction map was built that included the operons for the major clusters of *mxa* genes. Lined up according to their *mxaF* genes, bacterial strains were then compared for the sizes, transcriptional directions, chromosome locations, and gene products of their *mxa* genes. Detailed information on the *mxa* genes considered in this analysis, including *mxaF*, are listed in Table 4.

Investigation into Horizontal Gene Transfer of *mxa* Genes

The G+C contents of completed genomes and *mxa* genes existing in *Bradyrhizobium* and *Xanthomonas* spp. were calculated based on available sequences in GenBank, calculated as percentages of the total number of nucleotides present. Genomes were also screened for annotated mobile genetic elements or insertion sequences in the vicinities of *mxa* genes. The purpose of this analysis was to provide a preliminary approach to investigating the evolution of the *mxa* methylotrophy system in these bacteria. Because *Bradyrhizobium* and *Xanthomonas* species only possess *mxaF* and one other *mxa* gene (either *mxaG* or *mxaJ*, respectively), they were identified from the many

Table 4. Information Regarding the *mxa* Genes Considered in the Operon Construction Comparison Analysis.

Gene Name	Gene Sequence Length (Approx.)	Gene Product	Product Function	Relation to *mxaF*	References
mxaA		MxaA protein	Ca2+ insertion; stabilization of PQQ attack site during reduction to $PQQH_2$		Amaratunga et al., 1997 Anthony & Williams, 2003 Dewanti & Duine, 2000 Morris et al., 1995
mxaB		Response regulator protein	Regulation of transcription some *mxa* genes	Essential for *mxaF* transcription	Springer et al., 1998
mxaC		MxaC	Ca^{2+} insertion	Unknown	Amaratunga et al., 1997 Morris et al., 1995
mxaD		MxaD, a putative periplasmic protein	Electron transfer during methanol oxidation		Amaratunga et al., 1997 Morris et al., 1995 Toyama et al., 2003
mxaF	1.8 kB	MDH α-subunit	MDH structural gene; encodes for MDH alpha subunits	*(Same gene)*	Amaratunga et al., 1997 Anderson and Lidstrom, 1988 Machlin and Hanson, 1988 McDonald and Murrell, 1997
mxaG		Cytochrome c_L or c_{551i}	Electron acceptor for MDH; participates in electron transfer during oxidation of $PQQH_2$ to PQQ		Van Spanning, 1991 Amaratunga et al., 1997 Anderson and Lidstrom, 1988 Anthony & Williams, 2003
mxaH					
mxaI	290 bp	MDH β-subunit	Two β-subunits of heterotetrameric MDH enzyme	Encodes for the MDH beta subunit that binds to the alpha subunit	Amaratunga et al., 1997 McDonald and Murrell, 1997 Nunn et al., 1989
mxaJ		MxaJ protein	Putative fifth subunit of MDH (only in *Acetobacter methanolicus*)	Product potentially associated with alpha subunit in MDH configuration (*Acetobacter methanolicus* only)	Amaratunga et al., 1997 Matsushita et al., 1993
mxaK		MxaK protein	Ca2+ insertion; stabilization of PQQ		Amaratunga et al., 1997

		attack site during reduction to $PQQH_2$		Anthony & Williams, 2003 Dewanti & Duine, 2000 Morris et al., 1995
mxaL	MxaL protein	Ca2+ insertion; stabilization of PQQ attack site during reduction to $PQQH_2$		Amaratunga et al., 1997 Anthony & Williams, 2003 Dewanti & Duine, 2000 Morris et al., 1995
mxaR	MxaR protein	Putative ATP-binding protein	Necessary for active MDH	Amaratunga et al., 1997; Van Spanning et al., 1991
mxaS	MxaS protein	Unknown	Unknown	Amaratunga et al., 1997
mxaW	MxaW protein	Unknown	Unknown	Springer et al., 1998
mxaX	MxaX protein	Regulation	Unknown	Harms, et al., 1993
mxaY	MxaY protein	Methanol utilization control sensor	Unknown	Harms, et al., 1993
mxaZ	MxaZ protein	Regulation	Unknown	Harms, et al., 1993

genera considered in the operon construction analysis as potential candidates for horizontal gene transfer events involving PPFMs.

RESULTS AND DISCUSSION

Phylogenetic Analysis of GenBank *Methylobacterium* and Non-PPFM Sequences with *mxaF, mxaF'* and *mxaF*-like Annotations

The phylogenetic analysis of GenBank *mxaF, mxaF'* and *mxaF*-like nucleotide sequences showed clustering of sequences from several genera, *Methylobacterium*, *Hyphomicrobium*, *Hansschlegelia*, *Acidomonas*, *Xanthomonas*, *Bradyrhizobium* and *Methylosinus* (Fig. 1). Other *mxaF* and *mxaF*-similar sequences from species in genera such as *Methylocystis*, *Methylomonas*, *Methylocaldum*, and *Methylophilus* did not cluster closely together (Fig. 1). This suggests that *mxaF* sequence, despite being a conserved functional gene, is more highly conserved in some genera than in others. Many genera were represented only by a single sequence from one species, and therefore no comment can be made on whether *mxaF* sequence is conserved within that genus. However, it does indicate that more research and archived sequences are necessary to draw further conclusions on the conservation of *mxaF* sequence in bacterial genera.

Of 176 *Methylobacterium mxaF* and *mxaF*-similar sequences, 174 (98.9 %) of them fell within one large cluster on a phylogenetic tree (Figure 1, #1). *M. variabile* and *M. isbiliense* (Fig. 1, #1 and #2, respectively) did not cluster with the rest of the *Methylobacterium mxaF* gene sequences. These anomalies represented of 2 out of 176 (1.1 %) *Methylobacterium* sequences. Due to the existence of error both in obtaining gene sequences and in depositing them into GenBank and considering the potential for

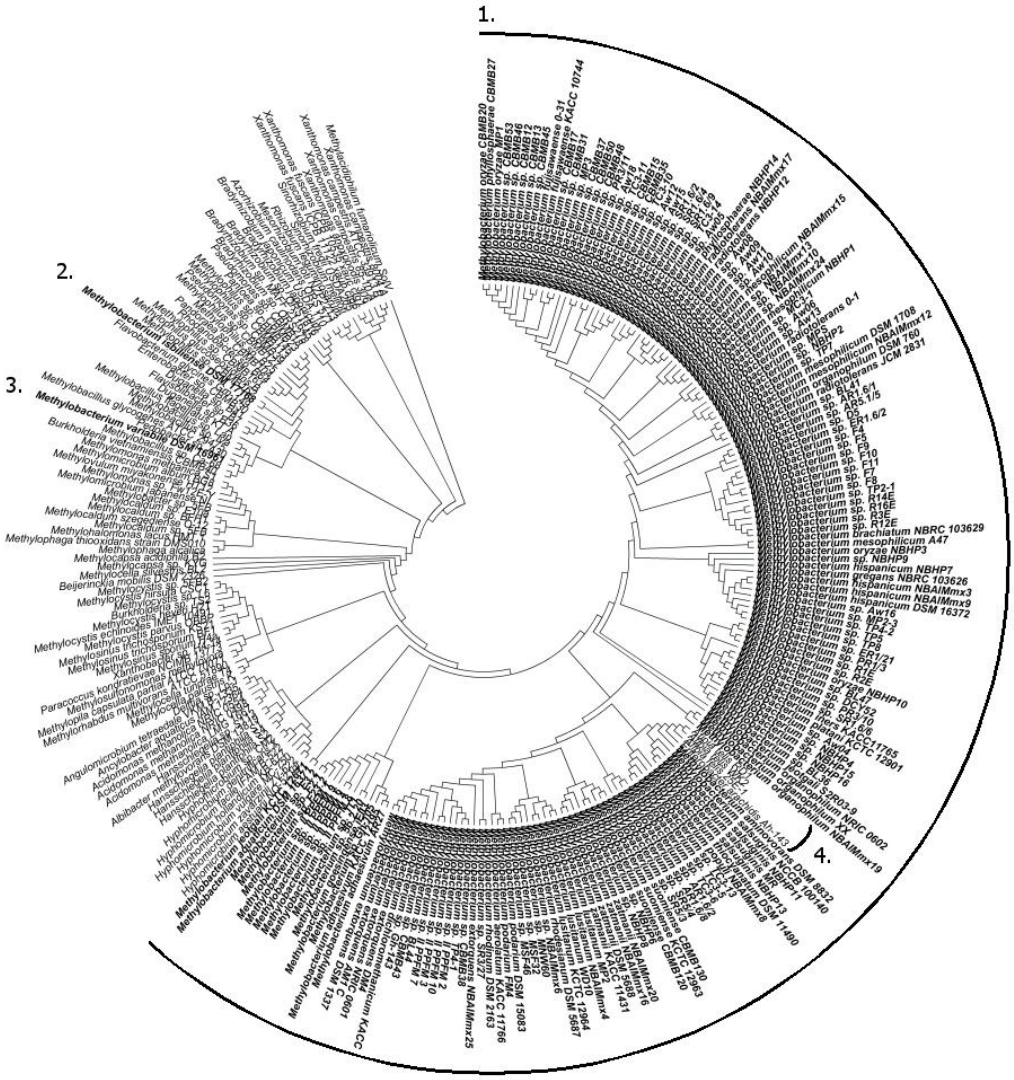

Figure 1. Neighbor-Joining, phylogenetic tree of GenBank sequences annotated as *mxaF*, *mxaF'*, and *mxaF*-like genes (MEGA 4 software). This tree includes *Methylobacterium* species and non-PPFMs alike (topology only, no evolutionary distance, branches not to scale). 697 base pair positions and 270 GenBank sequences were utilized in this analysis. *Methylobacterium* sequences are boldfaced. 174 of 176 (98.9 %) of PPFM *mxaF* sequences fall within one clade, designated by the #1. Two PPFM sequences (#2 and #3; 1.1 %) fell outside this main clade. Four non-PPFM

sequences (#4) fell within the major PPFM clade, indicating conservation of *mxaF* sequence within the genus *Methylobacterium*.

incorrect annotation in the database, this is an acceptable level of anomaly. It is unclear why these two species have *mxaF* gene sequences that are so divergent from the rest of the genera. These results either indicate an error in obtaining the *mxaF* sequences or in the annotation of these genes in Genbank, or could possibly represent genes that have evolved differently than the majority of *Methylobacterium mxaF* genes.

Four non-PPFM sequences fell inside the major *Methylobacterium* clade (Fig. 1, #4). Three of these anomalies were *Afipia felis* strains RD1, WK2, and 25E-1. It has been noted by O'Conner et al., (1991) that some *Afipia* spp. have 16S rRNA gene sequences that are highly similar to those of other species from the alpha-2 subclass of *Proteobacteria,* although it is unclear at this time why *A. felis*, best known as the "cat scratch disease bacillus (Brenner et al., 1991)," might possesses methylotrophy genes.

The fourth anomaly that fell inside the main *Methylobacterium* cluster was *Enterobacter arachidis* strain Ah-143 (Fig. 1, #4). The *mxaF* gene sequence for *E. arachidis* Ah-143 did not cluster with the only other member of its genus present on the tree, *Enterobacter* sp. CBMB30, and was the only gammaproteobacterium that fell within the *Methylobacterium* cluster. The *Enterobacter* sp. CBMB30 did not have a species designation, therefore it is difficult to consider its relationship to *E. arachidis* Ah-143. Because they are both *Enterobacter* species, however, one might expect the two sequences to cluster together on a phylogenetic tree. The methods contained in the reference paper for the *E. arachidis* Ah-143 *mxaF* sequence may indicate the opportunity for horizontal gene transfer (HGT) with *Methylobacterium* species in the rhizosphere from which it was isolated (Madhaiyan et al., 2010). If an HGT event did occur, that may

explain the similarity of the *E. arachidis* Ah-143 *mxaF* sequences to that of *Methylobacterium mxaF* sequences.

Even with the anomalies mentioned, this analysis shows that *mxaF* sequence is highly conserved among *Methylobacterium* spp. This conservation was seen in some other genera but not in all, and not with as many species sequences as are available for the *Methylobacterium* genus. This indicates a high degree of *mxaF* sequence conservation not only within that one genus but also compared to other genera. Because of this, the analysis suggests that *mxaF* sequence is a useful genetic characterization tool for *Methylobacterium* species. Therefore, in completion of our first objective, we have established that it is feasible to use *mxaF* sequences as genetic marker for the *Methylobacterium* genus.

Phylogenetic Analysis of GenBank *Methylobacterium* and Non-PPFM Sequences with *mxaF, mxaF', mxaF*-like, *xoxF,* and PQQ-Dependent Methanol/Ethanol Dehydrogenase Annotations

Phylogenetic analysis of *mxaF, mxaF', mxaF*-like, *xoxF*, and select PQQ-dependent dehydrogenase gene sequences from GenBank resulted in some unexpected clustering. As before, 98.3% (176/179) of *Methylobacterium mxaF*, *mxaF'* or *mxaF*-like sequences still formed a single cluster, even when sequences of genes other than *mxaF* were considered (Fig. 2, #1), This analysis demonstrated the same high degree of conservation of *mxaF* in the genus, as seen with the previous analysis involving only *mxaF*-related genes. This further indicates the robustness of *Methylobacterium mxaF* gene sequence and its usefulness as a genetic characterization tool for this genus.

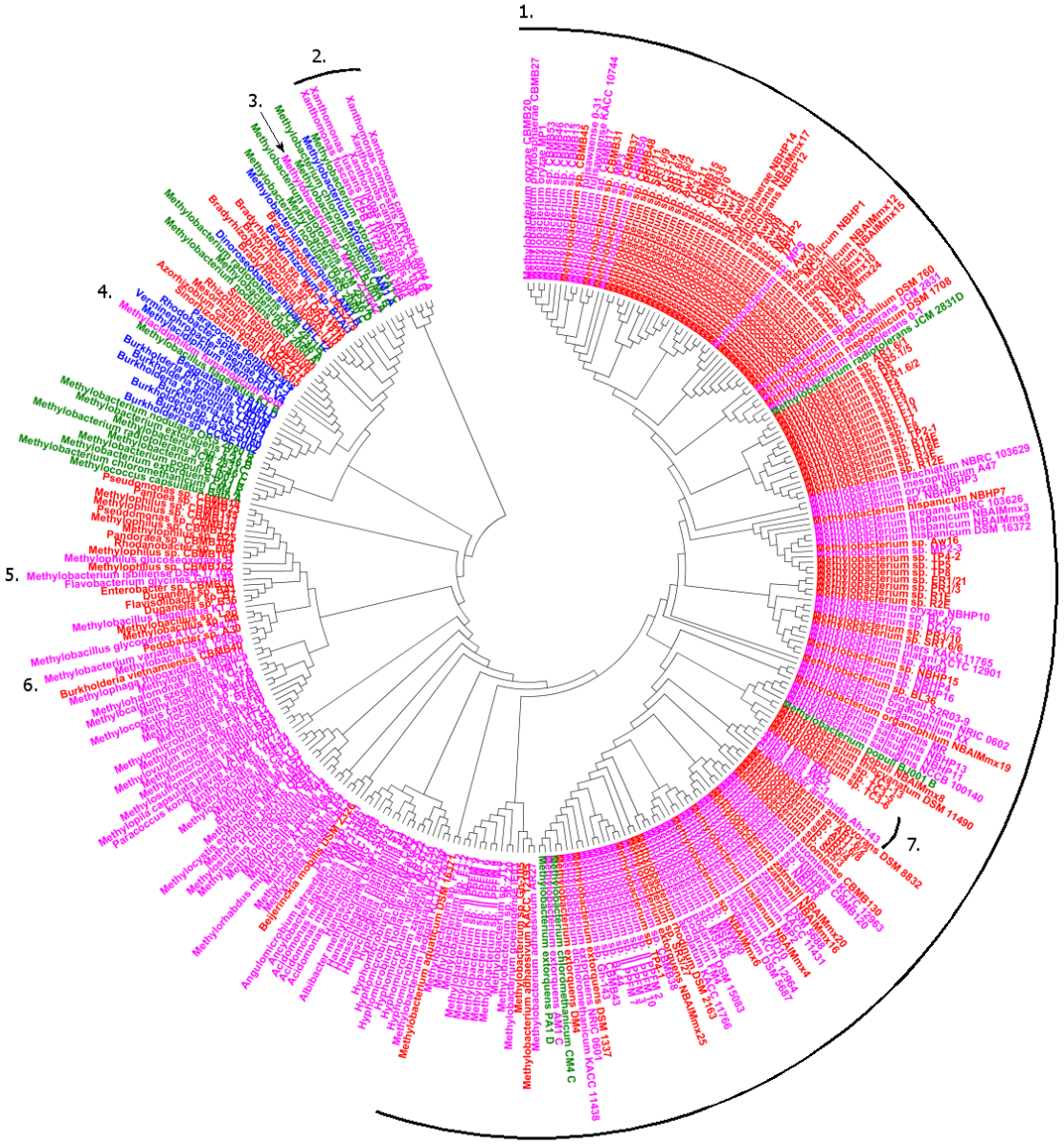

Figure 2. Neighbor-Joining, phylogenetic tree of GenBank (Dennis et al., 2005) sequences annotated as *mxaF*, *xoxF* and PQQ-dependent methanol/ethanol dehydrogenase gene sequences, constructed using MEGA 4 software. This tree includes *Methylobacterium* species and non-PPFMs alike and shows topology only (no evolutionary distance, branches not drawn to scale). 715 base pair positions and 311 GenBank sequences were utilized in this analysis. *mxaF* sequences are shown in pink,

mxaF' or *mxaF*-like in red, *xoxF* in blue, and PQQ-dependent dehydrogenase genes in the methanol/ethanol family are in green. In this tree, 176 of 179 (98.3 %) of PPFM *mxaF* sequences on this tree fall within one clade, designated by the large bracket, #1. The #2 indicastes *mxaF* sequences that were extremely divergent and differently sized than all other *mxaF* sequences on the tree, and are therefore suspected of as being mis-annotated. Only three PPFM sequences (#3, #5 and #6; 1.7 %) fell outside this main clade. The #4 indicates an *mxaF* sequence inside a *xoxF* clade, suspected as well of being mis-annotated. Only four non-PPFM sequences (#7) fell within the major PPFM clade. This indicates that PPFM *mxaF*, *mxaF*-like and *mxaF'* sequences are still conserved when the phylogenetic analysis is expanded to include other close-related methylotrophy gene sequences.

Other observations were made that suggest annotation inaccuracy in the GenBank database as well as possible misclassification of gene sequences by submitting authors. *mxaF'* and *mxaF*-like annotations did not cluster separately regardless of genus, (Fig. 2, red). This indicates uncertainty as to whether a sequence is sufficiently homologous to be annotated as a true *mxaF* gene or dissimilar enough to receive a *mxaF'* or *mxaF*-like annotation.

Sequences *M. radiotolerans* JCM 2831 D, *M. populi* BJ001 B, *M. chloromethanicum* CM4 C and *M. extorquens* PA1 D were annotated PQQ-dependent methanol/ethanol dehydrogenase genes (Fig. 2, shown in green), but they were most closely related to *mxaF* and *mxaF*-related genes. They fell inside the main PPFM *mxaF* and *mxaF*-related gene clade. These sequences all came from genomes with multiple generically annotated PQQ-dependent dehydrogenases. Only one gene from each of these genomes fell inside the main PPFM clade. These sequences may represent *mxaF* genes that have not been fully investigated or definitively annotated as such. This exposes the potential for an analysis like this involving hundreds of previous archived sequences to help researchers make that distinction between *mxaF*, *mxaF*-like, and generic PQQ-dependent methanol/ethanol dehydrogenase annotations when submitting their sequences.

Xanthomonas spp. (Fig. 2, #2), *Methylobacterium* sp. MAFF 211642 (Fig. 2, #3), *Methyloacidiphilum fumariolicum* SolV (Fig. 2, #4), *Methylobacterium variabile* (Fig. 2, #5) and *Methylobacterium isbiliense* (Fig. 2, #6) all possessed gene sequences annotated as true *mxaF* genes. These genes did not cluster as expected with other *mxaF* or even *mxaF'* and *mxaF*-like sequences. Several *Xanthomonas* genome sequences contained more than one annotated *mxaF* sequence, however, the lengths of some of those gene

were surprisingly small. *mxaF* is known to be approximately 1,800 bp long. Some *Xanthomonas mxaF* sequences were only 300 to 400 bp long. This combined with the fact that these *mxaF* genes fell so far outside the section of the phylogenetic tree that contained most *mxaF*, *mxaF'* and *mxaF*-like genes suggests incorrect *mxaF* annotation for these bacterial species.

In completion of our second objective, this analysis showed that even in the presence of genes with similar nucleotide sequences, protein products, and product function, *mxaF* sequences remains a robust characterization tool. *mxaF* and *mxaF*-related sequences still cluster tightly for the *Methylobacterium* genus in the presence of similar gene sequences, indicating PPFM *mxaF* sequence robustness and near exclusivity. This further supports our earlier conclusion that *mxaF* is a useful genetic marker for the characterization of *Methylobacterium* species.

mxa Gene Family Operon Construction Analysis

Comparison of the configuration of GenBank completed genome operons for the *mxa* gene family revealed conservation of gene arrangement among seven *Methylobacterium* species compared to those of other genera (Fig. 3). Many non-PPFM species lack some or nearly all *mxa* family genes besides *mxaF*, most starkly illustrated with *Bradyrhizobium* and *Xanthomonas* species (Fig. 3), raising questions as to the functionality of *mxaF* and the MDH enzyme in those species. The lack of the gene essential for the transcription of *mxaF* (*mxaB*) or the gene that encodes for the small subunit (*mxaI*) hint at the lack of a functional MDH enzyme in the *Bradyrhizobium* and *Xanthomonas* genera. Based upon this observation, then the presence of an *mxaF* gene in

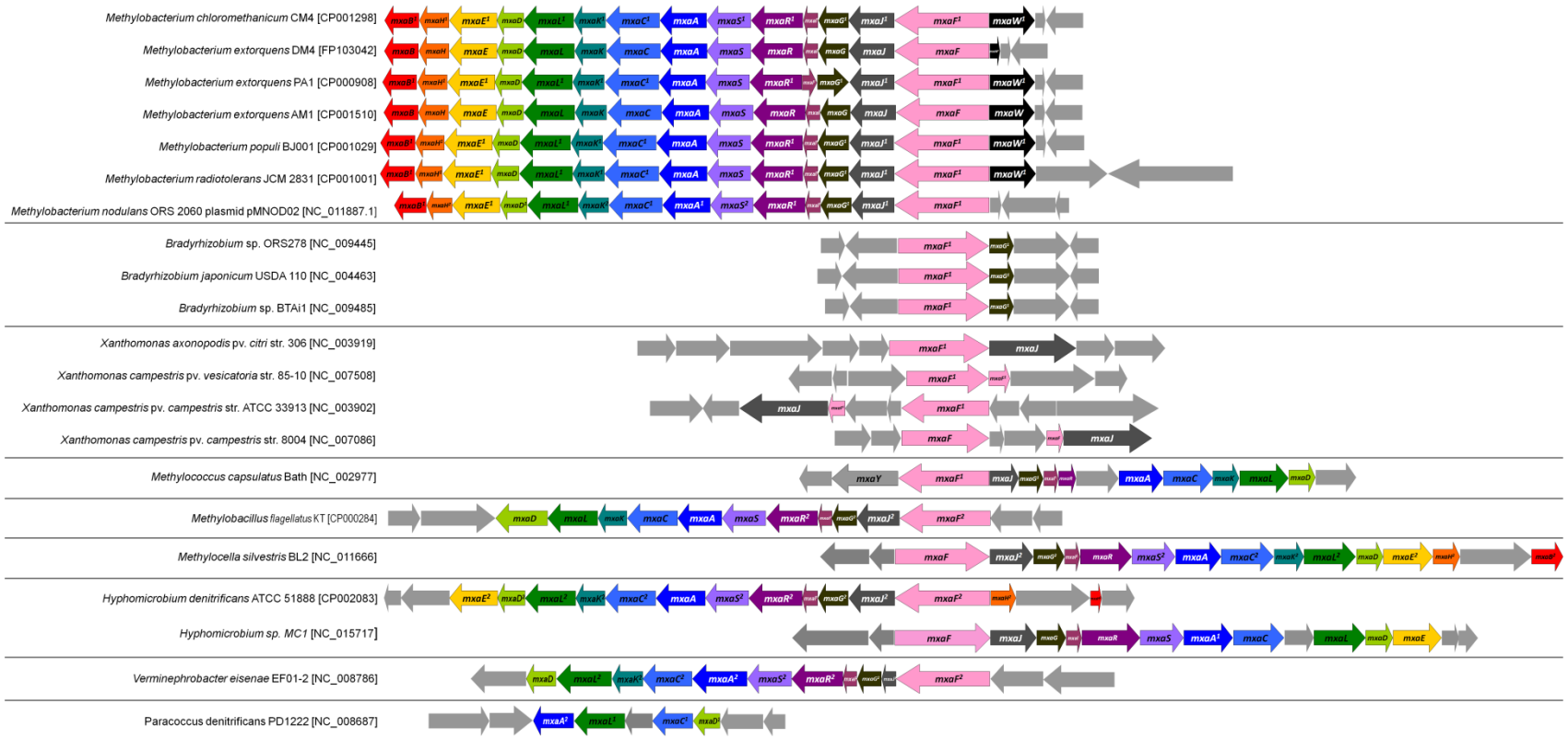

Figure 3. Diagram of *mxa* Gene Family Configuration of Multiple Genera. *mxa* gene family (colored arrows) shown for available completed GenBank (Dennis, et al., 2005) genomes as of October 30, 2011. Arrows indicate direction of transcription for each gene from positive strand DNA. Superscripts 1 and 2 indicate gene designations inferred from gene product and BLAST database

(http://blast.ncbi.nlm.nih.gov/Blast.cgi) nucleotide or protein searches, respectively. The *Methylobacterium* pattern of *mxa* operon construction is conserved compared with those of other bacterial genera. Evidence of horizontal gene transfer is present, as *mxa* genes are contained on PPFM plasmids (*M. nodulans* ORS 2060 plasmid pMNOD02 [NC_011887.1]). Also, some genera (*Xanthomonas* and *Bradyrhizobium*) are missing many of the genes necessary for a functional MDH enzyme and could have received them from other genera.

these species with one or two other *mxa* genes surrounding it could indicate that HGT events have taken place. This would make sense in the case of the *Bradyrhizobium* genus, known rhizosphere dwellers, and *Xanthomonas* species, known plant pathogens. The potential for these genera to encounter PPFMs in a plant phyllosphere or rhizosphere setting and engage in gene transfer is there.

In reference to our third objective involving evolution of the *mxa* methylotrophy system in bacteria, this analysis raises several questions. Is the conserved configuration of PPFMs containing nearly all the *mxa* genes in the family the ancestor of the system or the result of a new system evolving later on in a genus of closely related bacteria like *Methylobacterium*? The latter concept, that the PPFM system is the ancestor, would suggest that bacteria of other genera have picked up genes from PPFMs over time. This seems plausible when considering that many genes are missing from other genera, that species in other genera do not show conserved configurations, and that genes essential for a fully formed and functional enzyme are missing in other genera. The presence of certain anomalies not able to be shown in the operon construction map, however, may suggest that PPFMs are acquiring *mxa* genes through plasmids. These anomalies are discussed below.

There are a few PPFM anomalies worthy of note that either had *mxaF* genes that fell outside the main *Methylobacterium* clade on the phylogenetic trees described in Chapter 1 or that do not have completed genome sequences. Although the PPFM genomes appear to have conserved *mxa* operon construction, it is important to note that other patterns may exist, but more completed genome sequences for these bacteria are not currently available in the GenBank database. *Methylobacterium* sp. MAFF 211642

[AB550662](Fig. 2, #3), *M. variabile* [EU194913](Fig. 2, #5), and *M. isbiliense* [EU194912](Fig. 2, #6) all had *mxaF* genes that fell outside the main phylogenetic PPFM clade, but completed genomes for these organisms are not available. At this time, little can be concluded regarding the presence of other *mxa* genes in these genomes and what the arrangement of genes within the *mxa* operons might be.

Methylobacterium sp. 4-46 had four genes annotated as PQQ-dependent dehydrogenases in the methanol/ethanol family, but does not appear to have an *mxaF* gene. Because no completed genome or publication exists for this organism, we can only speculate on why this anomaly exists. It is possible that the bacterium was misidentified as a PPFM, or it may be that this represents a true anomaly in the *Methylobacterium* genus and does not possess an *mxaF* gene. Lastly, due to the often ambiguous and convoluted nature of GenBank annotation and sequence deposition, it may be that further scrutiny of the genome may reveal a previously hypothetical gene to be *mxaF*. Without further information, it may not be possible to answer these questions at this time.

The final anomaly was of great interest when considering the objectives of this research. Our third objective was to investigate what information *mxa* operon construction in bacteria could tell us about the evolution of this gene system. From the operon construction analysis, we were able to discern that PPFM *mxa* gene family operon configuration appears to be conserved compared to that of other genera when considering the genomes available in GenBank. One PPFM arrangement of genes on the operon construction map (Fig. 3) was not from a chromosomal sequence, however, but from a plasmid. *M. nodulans* ORS 2060 has a single *mxaF* sequence [AF220764] that was not found in its completed genome sequence [NC_011894] by either searching the genome

manually or using BLAST to compare the two sequences. *mxaF* and other *mxa* genes were found in a sequence for a plasmid belonging to *M. nodulans* ORS 2060, pMNOD02 [NC_011887]. This bacterium may represent an organism in genetic transition. *M. nodulans* ORS 2060 may have acquired the *mxa* methylotrophy system via a plasmid. This is plausible when considering that *M. nodulans* ORS 2060 both nodulates roots and fixes nitrogen, practices that are very rare in PPFMs. *M. nodulans* could be misidentified as a PPFM and is in fact another species that has acquired the *mxa* system from a plasmid of another species. The 16S rRNA gene sequence for *M. nodulans* ORS 2060 [AF220763], however, does show a close genetic relationship to *Methylobacterium* species. Another explanation could be that *M. nodulans* ORS 2060 engages in the dissemination of *mxa* genes to other bacteria. We can only speculate at this time, and as more completed bacterial genomes become available in GenBank, more information regarding the evolution of the *mxa* methylotrophy system in bacteria may be found.

Investigation into Horizontal Gene Transfer of *mxa* Genes

Investigation into G+C content of *Bradyrhizobium* and *Xanthomonas* species *mxaF*, *mxaG* and *mxaJ* genes, however, remains inconclusive as all G+C percentages were comparable (Table 5). This indicates that either no HGT event has occurred or it occurred so long ago that the *mxa* genes have had sufficient time to be assimilated into the host genome. A third explanation, which is highly probable, is that HGT events have occurred that cannot be detected by this type of analysis. The reason for that might be that *Bradyrhizobium*, *Xanthomonas* and *Methylobacterium* species are found above ground in the phyllosphere or in surface soils, so they will experience sunlight on a regular basis.

Table 5. G+C Contents and Presence of Annotated Mobile Genetic Elements from GenBank *Bradyrhizobium* and *Xanthomonas* mxa Genes and Completed Genome Sequences. G+C content and the presence of mobile genetic elements were investigated as evidence of horizontal gene transfer (HGT) events. Searchable genome maps in GenBank (Dennis et al., 2005) were used to look for mobile genetic elements around genes of interest and genome sequences were used to calculate the % G+C content of both full genomes and *mxa* genes. G+C contents of genomes and genes were similar across all species and therefore not indicative of HGT events. No pattern was found concerning mobile genetic elements. It may be that HGT has not occurred, that it occurred so long ago that acquired gene sequences have acclimated to their parent genomes, or that G+C contents between these genera and PPFMs are too similar.

Bacterial Genome Sequence	Accession #	Genome G+C (%)	*mxaF* G+C (%)	*mxaG* G+C (%)	*mxaJ* G+C (%)	Mobile Genetic Elements Present?
Bradyrhizobium sp. BTAi1	[NC_009485]	64.9	62.5	59.8	N/A	No
Bradyrhizobium sp. ORS278	[NC_009445]	65.5	63.0	61.9	N/A	No
Bradyrhizobium japonicum str. USDA 110	[NC_004463]	64.1	63.2	61.4	N/A	No
Xanthomonas axonopodis pv. *citrumelo* str. F1	[NC_016010]	64.9	64.0	N/A	N/A	No
Xanthomonas axonopodis pv. citri str. 306	[NC_003919]	64.8	64.2	N/A	68.2	No
Xanthomonas campestris pv. *campestris* str. ATCC 33913*	[NC_003902]	65.1	65.6, 62.6	N/A	67.1	No
Xanthomonas campestris pv. *campestris* str. B100*	[NC_010688]	65.0	64.3, 64.0	N/A	N/A	No
Xanthomonas campestris pv. *vesicatoria* str. 85-10*	[NC_007508]	64.5	63.3, 66.2	N/A	N/A	No
Xanthomonas campestris pv. *campestris* str. 8004*	[NC_007086]	65.0	63.2, 67.6	N/A	67.4	Yes

Having high G+C content protects their genomes from ultraviolet (UV) damage, and so it is not surprising that these bacteria would all have a comparable high G+C content in their genomes.

An annotated mobile genetic element was found for *Xanthomonas campestris* pv. *campestris* str. 8004 [NC_007086], indicating that a genetic transfer event has occurred in that strain, but it is unclear whether that event involved genes acquired from other bacteria or simply an event isolated within its own genome. The presence of a mobile genetic element does indicate the exchanging of *mxa* genes, whether inside genomes or between them. It can be concluded from this study and the operon construction analysis that there is transfer of *mxa* genes occurring, but that more completed genomes are necessary to produce a more comprehension data set and comparison between these organisms.

CONCLUSIONS

A high degree of robustness in *Methylobacterium mxaF* sequence was seen both in the exclusively *mxaF*-related phylogenetic tree and the tree that included *xoxF* and PQQ-dependent dehydrogenase genes as well. *mxaF'* and *mxaF*-like annotations did not cluster separately regardless of genus, suggesting annotation inaccuracy in GenBank database (Fig. 2, red). Some species, e.g. *Xanthomonas* spp. (Fig. 2, #2), *Methylobacterium* sp. MAFF 211642 (Fig. 2, #3), *Methyloacidiphilum fumariolicum* SolV (Fig. 2, #4) may have other methanol/ethanol dehydrogenase genes misidentified as *mxaF*. Furthermore, some generically annotated PQQ-dehydrogenase genes fell into the PPFM *mxaF* clade, suggesting that phylogenetic analyses prior to sequence submission

could help researchers to definitively and more accurate decide upon an annotation for their sequence. Despite these potential inaccuracies in annotation in the database, *Methylobacterium mxaF* and *mxaF*-related sequences clustered tightly, indicating a robustness and high degree of conservation in *mxaF* gene sequence for this genus.

Just as *mxaF* sequence was very highly conserved among *Methylobacterium* species, the *Methylobacterium mxa* system also had a high level of conservation in its construction. The implications of the conservation of *mxa* gene family operon construction in *Methylobacterium* species are uncertain. It is possible that *mxa* system seen in the *Methylobacterium* genus is an ancestor of genes that were then transferred to other genera during HGT events. This theory is plausible when one considers the predominance of *Methylobacterium* species in the plat phyllosphere and rhizosphere and the interactions they might have with bacteria from many other plant-colonizing genera. 16S rRNA phylogenetic trees (not shown), however, suggest that *Methylobacterium* is a genus that evolved later than other *mxaF*-containing genera. Also, the existence of other bacteria with the same or comparable number of *mxa* genes, albeit arrange in a different configuration, discounts *Methylobacterium* as the only possible ancestor of this methylotrophy system.

Conversely, it could also be possible that the *Methylobacterium mxa* system is a newly-evolved system derived from more ancient bacterial lineages that previously only contained *mxaF* and a limited number of other *mxa* genes. This would agree with 16S rRNA phylogenetic analyses that suggest that *Methylobacterium* species evolved later. Operon construction and G+C content analyses neither confirmed nor ruled out the possibility of HGT events between plant-associated bacteria.

Analysis of G+C content and the presence of annotated mobile genetic elements in *Bradyrhizobium* and *Xanthomonas* spp. was inconclusive, largely because G+C percentages were comparable across all species and whether considering single gene or complete genome sequences. While no further conclusion can be drawn as to how these genes may have been transferred from PPFMs or other bacteria, it can be said that % G+C and the presence of mobile genetic elements alone is not a sufficient method to determine HGT events between soil and plant-associated bacteria. The comparable high G+C content encountered in the genomes of surface soil and plant phyllosphere bacteria makes discerning which genes might have come from what species difficult using this method.

There were several bacterial *mxaF* sequences that did not correspond with 16S rRNA phylogeny, either because *Methylobacterium* species identified by their 16S rRNA genes had *mxaF* sequences outside the *Methylobacterium mxaF* clade or because bacteria identified as non-PPFMs by 16S rRNA phylogeny had *mxaF* sequences inside the PPFM clade. This, when considered with the G+C content analysis shows that there are clear discrepancies between 16S rRNA and *mxaF* phylogeny than cannot be validated by G+C content analysis or the presence of mobile genetic elements. *mxa* genes are found on some plasmids of the *Methylobacterium* genus (*M. nodulans* ORS 2060), hinting that HGT events are possible. Horizontal gene transfer appears to play a role in the evolution of the *mxaF* genes and perhaps the *mxa* system as a whole, but it may also be that some species have been misidentified and therefore their genes misclassified.

Without more data on the functionality of all the *mxaF* genes, information about resultant MDH enzymes in non-PPFM species, and more completed genomes for

bacterial genera, more research is needed to make further conclusions on the evolution of the *mxa* gene system in bacteria. It can be said, however, that *mxaF* sequence is a useful genetic characterization tool for *Methylobacterium* species due to the highly conserved nature of those sequences compared to those of other genera. Preliminary analysis suggests that *mxa* gene family operon construction may also be highly conserved. These conclusions open the door for definitive characterization of *Methylobacterium* species from environmental samples and further investigation into how the *mxa* methylotrophy system evolved in bacteria. The *mxa* gene system is the largest known methylotrophy system in bacteria. The role of these genes in soil and phyllosphere processes that lower the potential for greenhouse gas CO_2 to enter the atmosphere warrants further study, as does the story behind how these genes may have been transferred between bacterial genera.

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Chapter 3

Effects of Substrate Variability and Elevated Zinc on Biofilm Production in Soil-Derived *Methylobacterium* Species

ABSTRACT

In order to realize the full potential of plant growth promoting bacteria for agricultural and bioremediation applications, it is important to understand how they are able to survive on plant surfaces. The ability of beneficial bacteria to retain competence in biofilm production under stressful conditions and therefore to colonize plant surfaces is of value to agricultural and bioremediation sciences for improving plant heath and participating in the removal of toxic metals from soil. Biofilm production and parameters affecting its occurrence have not been studied in detail for *Methylobacterium* species. This research studies biofilms in *Methylobacterium* soil isolates from New Jersey using both traditional culture methods as well as molecular methods to determine the effects of substrate variation, substrate and metal (zinc) toxicity, light, and sheer stress on biofilm production. Variation of methanol and ethanol substrate concentrations as well as sheer stress affected the color, weight, and structure of the biofilms. Darker color was associated with methanol and ethanol toxicity, and biofilms grown with ethanol as the sole carbon source tended to be heavier than those grown on methanol, possibly indicating an increase in EPS for protective purposes. Low levels of $ZnSO_4$ (0.1 and 1 mM) did not inhibit biofilm production, but did affect the amount of DNA per gram of biofilm material depending upon when the amendment was made. A trend of more

DNA/g was seen if a 1 mM $ZNSO_4$ amendment was made at 24 hrs or later, indicating a time-dependent effect on the ratio of DNA to EPS within the biofilms. Biofilm production varied by species, with some isolates producing thick, strongly adhered biofilms and others producing thin biofilms that were easily dissociated and detached by perturbation. Combining isolates of disparate ability within a single culture resulted in color and texture variations of colonies on plates, suggesting cooperative interspecies communication. This research has expanded out knowledge of the biofilm production capabilities of the genus *Methylobacterium* and has laid the groundwork for future research regarding this fascinating activity by these soil microbes.

INTRODUCTION

While it is known that quorum-sensing is widespread in the *Methylobacterium* genus (Poonguzhali et al., 2007), researchers have not yet linked this activity to biofilm production. Quorum sensing behavior has been linked to the ability to form biofilms in other species (Parsek and Greenberg, 2005; Sakuragi and Kolter, 2007), but not in PPFMs. *Methylobacterium* spp. are sometimes listed among the participants of multi-species biofilms in drinking water systems (Simões et al., 2007), on dental apparatuses (Barbeau et al., 1996), in printing machines (Väisänen et al., 1998), and on shower curtains (Kelley et al., 2004), but investigations into PPFM biofilm production by individual species have not been performed. Biofilm production is an important topic of discussion for PPFMs, as the ability to form biofilms can be a competitive mode of life for bacteria when colonizing plant surfaces both above and below the soil surface (Morris and Monier, 2003). We hypothesize that understanding the nature of PPFM biofilm

production and the factors that affect biofilm development will elucidate PPFM resilience and versatility, shedding light on how they remain a competitive force in the plant phyllosphere and rhizosphere.

Methanol is a major growth substrate for PPFMs on plant surfaces. It has been noted that lab isolates can be sustained at 1.6% methanol without inhibitory effects on growth (Bormann *et al*., 1997), but the most common concentration used in PPFM culture is 0.5% methanol as a sole carbon and energy source. A strain of *M. radiotolerans* was found by one research group to be capable of growing in 30 g/L methanol, or 3.8 %, although it was noted that toxicity to methanol was dependent upon the strain used and the experimental conditions (Bormann *et al*., 1997). The elucidation of methanol-resistant strains of PPFMs is important for the agricultural industry as it has been suggested that foliar-applied methanol is a way to stimulate PPFM growth on plant surfaces (Madhaiyan et al., 2006).

It is important to understand the effects of substances like methanol and ethanol on PPFM growth. Although they are both carbon sources, they are also toxic above threshold concentrations. Both methanol and ethanol are substrates for the MDH enzyme. MDH has a 25-fold higher affinity for ethanol than methanol (Bruice, 2001). The result is a faster rate of ethanol utilization compared to methanol. Therefore, given two cultures growing in medium with the same concentrations of ethanol and methanol, the ethanol-fed culture will experience toxicity before the methanol-fed culture due to increased uptake of ethanol compared to methanol as a result of this higher affinity. Ethanol also contains twice the amount of carbon compared to methanol. Thus, a culture

will receive more carbon per unit of ethanol compared to methanol, provided the culture is able to survive the associated toxic effects.

When considering the environmental relevance and potential application of PPFMs, the ability to withstand and degrade multiple contaminants in soil is a necessity. Methanol can become a soil contaminant as a result of industrial processes or when excess amounts of it are foliar applied to crops to stimulate PPFM growth and plant association (Madhaiyan et al., 2006). Ethanol becomes a soil contaminant as well during ethanol-amended gasoline spills (Corseuil and Moreno, 2001). Phytoremediation has been investigated for the removal of ethanol from contaminated soils (Corseuil and Moreno, 2001), but this method had not been applied using PPFMs for either methanol- or ethanol-contaminated soils. How PPFMs utilize and tolerate both methanol and ethanol is important to understanding the phytoremediation of Zn-contaminated soil and to improving upon crop-stimulation methods that encourage PPFM associations. Ethanol has also been suggested as a biostimulant for the bioremediation of U-contaminated soils (Gu et al., 2005) as well as As-contaminated munitions sites (Köhler *et al*., 2001). Through application of carbon sources like ethanol, other mineralization and transformation processes for metals and other contaminants may be stimulated as a byproduct of ethanol metabolism. If ethanol is to be investigated as a biostimulant for the phytoremediation of metal contaminated soils, information is needed about how ethanol application will affect plant-growth promoting bacteria such as *Methylobacterium* species.

In addition to methanol and ethanol, PPFMs are also being considered for the removal of other contaminants such as formaldehyde. In the pathway of methanol

assimilation in PPFMs, formaldehyde is created when methanol is oxidized and it is this intermediate that is internalized by cells for further mineralization (Chongcharoen *et al.*, 2005). Some PPFM strains are able to not only utilize formaldehyde as a carbon source (Mitsui et al., 2005), but to thrive in high concentrations (Chongcharoen *et al.*, 2005). It is important to understand the limits of formaldehyde utilization in PPFMs, as it becomes toxic at high concentrations. In contaminated soil, concentrations would be much higher than that which is created by the activities of the MDH alone.

As was the situation with ethanol, some substrates can be used as biostimulants. Sodium acetate, a common root exudate, is a readily bioavailable carbon source that many rhizobacteria can utilize. It has been investigated as a biostimulant for the remediation of metal-contaminated soil (Köhler *et al*., 2001). Sodium acetate is likely to be an available substrate that *Methylobacterium* species will encounter while colonizing plant roots. Studying how PPFMs respond to sodium acetate as a growth substrate will determine whether it can be used as a biostimulant for Zn-contaminated soil as well. Understanding how biofilm production is affected by the presence of elevated Zn as well as varying substrates like methanol and ethanol will begin to elucidate the potential for PPFMs to participate in nutrient cycling and plant colonization in the environment.

In order to document the physical characteristics of PPFM biofilms and to understand what factors affect biofilm production, the objectives of this study were as follows:

1) to identify the nature and extent of PPFM biofilm production, qualitatively determining the growth stages and physical structures involved;

2) to investigate how substrate variation (methanol, ethanol, etc.) affects the

ability of PPFM isolates to produce biofilms;

3) to observe how a concentration of Zn comparable to that which might be produced by microbial solubilization of Zn salts in the rhizosphere affects biofilm onset and development.

MATERIALS AND METHODS

PPFM Biofilm Production

General PPFM Biofilm Production

Of the nine NJ isolates collected from agricultural soil (see Chapter 1, Materials & Methods, section A), only two produce biofilms: NJ1101 and NJ1104. (For information regarding the isolation and genus identification of NJ1101 and NJ1104, see Chapter 1, Materials & Methods, sections A, B, and C.) Initially, the biofilm-producing capabilities of NJ1101 and NJ1104 were compared through the observation of biofilm development in AMS liquid medium in shaken flasks. This was done for the purpose of documenting *Methylobacterium* isolate biofilm construction by qualitatively observing color, thickness, three-dimensional structure, adhesion and other visual characteristics.

Isolate NJ1104 was selected for more in-depth studies concerning biofilm development because it produced thick, robust, strongly-adhered biofilms under laboratory conditions. Isolate NJ1104 is most closely related to *M. populi* and *M. thiocyanatum* based upon 16S rRNA and *mxaF* gene sequence homology with GenBank reference sequence (see Chapter 1, Figs. 5.1-5.4).

Simulated Multi-Species PPFM Biofilm

To simulate the formation of a multispecies PPFM biofilm, NJ1101 and NJ1104 were grown together or in spent medium from either NJ1101 or NJ1104 culture that had been filtered through a 0.2 μm filter. Spent medium was used in case growth factors or metabolites from one isolate would aid the biofilm production of another. Because NJ1101 biofilm production was weaker and less developed than that of NJ1104, it was hypothesized that other growth factors were needed by the isolates. Details about the setup of the treatments in this experiment can be found in Table 1. By way of comparison, NJ1104 was also grown in filtered spent medium from NJ1101 culture as well. In addition to the spent medium treatments, treatments were included that contained more NJ1101 than NJ1104 and vice versa. The resulting biofilm production was compared to single-species biofilms of each individual isolate.

Culture medium as well as biofilm material was plated onto AMS agar plates to observe colony color and morphology to determine if both organisms were still present and if both had participated in biofilm production. Biofilms were washed three times in distilled water before plating by smearing of the material onto the agar surface and streaking outwardly with a loop.

Effects Substrate Variation on PPFM Growth and Biofilm Development

Methanol, Ethanol, Ethanol/Methanol in Combination as the Growth Substrates

Because the methanol utilization capabilities of the nine NJ agricultural isolates introduced in Chapter 1 varied, it was necessary to determine the limits of growth were for these isolates in elevated methanol. Concentrations of methanol tested using these isolates in AMS liquid medium are detailed in Table 2. (The formulae for AMS medium

Table 1. Treatments for Multispecies Biofilm Experiment. To simulate the formation of a multispecies PPFM biofilm, NJ1101 and NJ1104 were grown together in spent medium from NJ1104 culture that had been filtered through a 0.2 μm filter. The resulting biofilm production was then compared to single-species biofilms of each individual isolate. By way of comparison, NJ1104 was also grown in filtered spent medium from NJ1101 culture as well. In addition to the spent medium treatments, treatments were included that contained more NJ1101 than NJ1104 and vice versa. Each isolate inoculum was at a density of approximately 10^6 CFU/mL and taken during exponential phase. Large inoculum volumes were used to achieve high initial cell densities and ensure biofilm growth.

Treatment	NJ1101 Inoculum (mL)	NJ1101 Spent Medium (mL)	NJ1104 Inoculum (mL)	NJ1104 Spent Medium (mL)	Sterile AMS Medium (mL)
NJ1101 = NJ1104	1.5	-	1.5	-	57
NJ1101 > NJ1104	2.0	-	1.0	-	57
NJ1101 < NJ1104	1.0	-	2.0	-	57
NJ1101 + NJ1104 Spent Medium	3.0	-	-	57	-
NJ1104 + NJ1101 Spent Medium	-	57	3.0	-	-

Table 2. Concentrations of Methanol, Ethanol, Formaldehyde and Sodium Acetate Tested on Nine NJ PPFM Soil Isolates in Liquid AMS Medium. The table below details both the total carbon source amounts (in % of total medium volume or weight) and the individual concentrations of methanol, ethanol, formaldehyde or sodium acetate used to achieve those total carbon source amounts. Treatments with only one substrate include that substrate at the same percentage as the total carbon source. Treatments with substrates in equal amounts (denoted with "=") contain each substrate at half of the percentage total carbon source. Treatments with one substrate concentration higher than another (denoted with ">") include one substrate at two-thirds the total percentage carbon source and one substrate at one-third the total percentage carbon source. All substrates were added to AMS liquid medium in liquid form (percent volume to volume, v/v) except for sodium acetate, for which the percentage was calculated by weight.

Treatment	Total Carbon Source Conc. (%, v/v)	Methanol Conc. (%, v/v)	Ethanol Conc. (%, v/v)	Formaldehyde Conc. (%, v/v)	Sodium Acetate Conc. (% by weight)
Methanol Only	0.10	0.10	-	-	-
	0.50	0.50	-	-	-
	1.00	1.00	-	-	-
	1.25	1.25	-	-	-
	1.50	1.50	-	-	-
	1.75	1.75	-	-	-
	2.00	2.00	-	-	-
	2.50	2.50	-	-	-
	3.00	3.00	-	-	-
	4.00	4.00	-	-	-
	5.00	5.00	-	-	-
	7.50	7.50	-	-	-
	10.00	10.00	-	-	-
Ethanol Only	0.50	-	0.50	-	-
	1.00	-	1.00	-	-

	1.50	-	1.50	-	-
Methanol = Ethanol	0.50	0.25	0.25	-	-
	1.00	0.50	0.50	-	-
	1.50	0.75	0.75	-	-
Methanol > Ethanol	0.50	0.33	0.17	-	-
	1.00	0.67	0.33	-	-
	1.50	1.00	0.50	-	-
Methanol < Ethanol	0.50	0.17	0.33	-	-
	1.00	0.33	0.67	-	-
	1.50	0.50	1.00	-	-
Formaldehyde	0.10	-	-	0.10	-
	0.20	-	-	0.20	-
	0.30	-	-	0.30	-
Formaldehyde = Methanol	0.10	0.05	-	0.05	-
	0.20	0.10	-	0.10	-
	0.30	0.15	-	0.15	-
Sodium Acetate	0.10	-	-	-	0.10
	0.20	-	-	-	0.20
	0.30	-	-	-	0.30
Sodium Acetate = Methanol	0.10	0.05	-	-	0.05
	0.20	0.10	-	-	0.10
	0.30	0.15	-	-	0.15

can be found in Chapter 1, Table 4.) Only isolate NJ1104 was tested in concentrations of 5.0 % and above, due to thresholds for other isolates being reached at lower concentrations. While only liquid culture growth was observed for most of the isolates, colonies on AMS agar were also observed for NJ1104 for methanol concentrations of 5.0, 7.5, and 10.0 % to observe any changes in colony color or morphology due to methanol toxicity.

The toxic effects on *Methylobacterium* species of both ethanol and methanol in combination are unknown. To observe the combined effects of these two substrates on PPFM biofilm production as well as simulate multi-contaminant conditions, NJ1104 was exposed to both ethanol and methanol simultaneously. Concentrations of methanol and ethanol used in this experiment can be found in Table 2. The reason for including treatments where one alcohol concentration was greater than the other was to either enhance or attempt to compensate for the increased toxicity of ethanol due to MDH having a higher affinity for ethanol. The dry weight of biofilms was used to quantify the impact of carbon substrate variation on biofilm mass.

Formaldehyde as a Growth Substrate

Since NJ1104 had shown versatility with methanol, ethanol, and a combination of the two as growth substrates, it was explored further for its ability to utilize formaldehyde. The ability of isolate NJ1104 to grow on formaldehyde concentrations of 0.1 %, 0.2 % and 0.3 % formaldehyde was tested. In addition, ethanol/formaldehyde and methanol/formaldehyde combinations were also investigated. Concentrations of ethanol, methanol and formaldehyde used in this experiment can be found in Table 2.

Sodium Acetate as a Growth Substrate

Concentrations of 0.1%, 0.2% and 0.3% sodium acetate in AMS liquid medium were tested with isolate NJ1104. As with the formaldehyde trials, sodium acetate was also fed simultaneously with methanol or ethanol to observe the effects of multiple carbon sources and potential contaminants on PPFM growth and biofilm production. Concentrations of methanol, ethanol and sodium acetate used in this experiment can be found in Table 2.

Effect of Elevated Zn on PPFM Biofilm Development

Isolate NJ1104 was used to evaluate biofilm development in the presence of elevated Zn. Three experiments were designed to observe what effects elevated $ZnSO_4$ would have on NJ1104 biofilm production. For each experiment, OD (at 625 nm wavelength) was used as a proxy for biofilm development. Details of each of these experiments are found below.

During a preliminary experiment, AMS medium was amended to a concentration of 0.1 mM $ZnSO_4$ for experimental treatments. For control treatments, no Zn was added. Because bacterial biofilms are inhibited or killed time-dependently by the presence of metal ions even at low concentrations (Harrison et al., 2007), amendment time was included as a variable in this experiment. Treatments received amendments at various time points: 0, 24, 48, 72 and 96 hours. This resulted in some cultures receiving the Zn amendment before biofilm growth had begun and others receiving it after biofilms were already established. The OD of each flask was read at 625 nm every 24 hours, up to 96 hours. Although this preliminary study did not contain replicates within treatments, the

experiment itself was repeated multiple times. Data presented here is representative of those repeated experiments.

The next experimental design included $ZnSO_4$ amendments and OD measurements occurring at 12-hour time points instead of once per day. The concentration of $ZnSO_4$ was increased to 1.0 mM $ZnSO_4$ for the purpose of presenting the isolate with a higher level of Zn stress. This experiment was done with replicates of three flasks per treatment. Control flasks received no additional Zn other than what is already present in AMS medium. Cultures were amended with up to 1.0 mM $ZnSO_4$ at 0, 12, 24, 36 and 48 hr time points. OD was measured at 625 nm every 12 hours up to 96 hours. In addition to an OD measurement, biofilms were harvested after the 96-hour reading and DNA was extracted using the MOBIO Power Biofilm™ DNA Extraction Kit. This was done in an attempt to quantify the relative amount of DNA/g of biofilm material because the biofilms were visually comparable in thickness, color, and structural appearance. DNA was visualized and quantified using agarose gel electrophoresis in reference to a Lambda HindIII DNA ladder. Using the weights of each biofilm, approximate DNA amounts per biofilm were calculated.

The last experiment included light and perturbation as variables in addition to $ZnSO_4$ amendments, with an otherwise similar setup to the previous experiments. Details about the setup of treatments in this experiment can be found in Table 3.

RESULTS AND DISCUSSION

PPFM Biofilm Production

Table 3. Third biofilm disruption experiment treatments. Treatments received $ZnSO_4$ amendments at 0, 24, 36, and 48 hours and OD was read daily at 625 nm daily up to 96 hours. Each of these treatments consisted of four flasks under the following conditions: light/unshaken; light/shaken; dark/unshaken; light/unshaken. There were 16 flasks in total. "Light" referred to flasks left on the shaker or bench top to experience ambient lighting, whereas "dark" flasks were covered with aluminum foil to ensure no light entered the flask. "Shaken" flasks were placed on the shaker at 120 rpm, whereas "unshaken" flasks were left on the bench top.

	Shaken (120 rpm)	Unshaken (Table Top)
Light (Ambient)	0 hr 24 hr 36 hr 48 hr	0 hr 24 hr 36 hr 48 hr
Dark (Covered with Aluminum Foil)	0 hr 24 hr 36 hr 48 hr	0 hr 24 hr 36 hr 48 hr

General PPFM Biofilm Production

Two isolates (NJ1101 and NJ1104) were capable of producing biofilms. These isolates were the only two of the nine from NJ to display wrinkled textures in the surfaces of their colonies on AMS agar plates (Fig. 1), indicating that wrinkles in colony construction on solid medium and the ability to produce biofilms in liquid culture are linked. While colonies were always textured, the pattern of texturing changed depending upon the stress level of the isolate (see section B for more details on NJ1104 "flower-shaped" colonies in methanol concentrations between 5.0 and 10.0 %).

Detailed information regarding the color, health, age, and stress level of NJ1104 biofilms (and to a lesser extent, those of the weaker biofilm producer, NJ1101) can be found in Table 4. The information contained in that table is a summation of observations from all of the experiments listed in this chapter. It contains information regarding color, thickness, three-dimensional structure, etc. for NJ1104 and provides information and suggestions as to what those different qualitative and observational designations might mean for the health and age of the biofilm.

Isolate NJ1101 presents as round, "rosy pink" (Pantone 217C), wrinkled colonies on AMS agar plates with 0.5 % methanol added. Colonies often displayed concentric ring patterns and "starburst" like striations between the rings. In AMS liquid culture with 0.5 % methanol that is shaken at 120 rpm, NJ1101 produces "rosy pink" (Pantone 217C) or "pale pink" (Pantone 196C) biofilms that are weakly adhered to themselves and the glass. These biofilms break apart if the flask is shaken vigorously and may even detach during steady shaking at 120 rpm. NJ1101 only produces a biofilm at the bottoms of flasks and partially up the sides of the glass.

Table 4. Biofilm Development Chart for Biofilm-Producing Isolates NJ1104 and NJ1101 in AMS liquid medium.

Biofilm Characteristic	Observation Category	Criteria for Fitting Observational Categories	Significance of Observation	Score
Presence or Absence	No biofilm growth	The glass is clear of colonies to the naked eye.	No biofilm developed or biofilm production has not yet begun.	0
	Biofilm growth	Some attachment has begun in the form of microcolonies or a complete biofilm is present.	Biofilm growth is present, regardless of development stage. Can include initial, basic attachment of microcolonies.	1
Pigment Color	"Colorless"	No color assignment.	Represents an early stage of biofilm growth at which microcolonies on glass are too small or thin to see color.	0
	"White"	Pantone Transparent White	Indicates either an early growth stage (not enough cells to have pink color) or failed growth (cells lose color and no further growth occurs, but remain attached).	1
	"Pale Pink"	Pantone 196C	This is the typical growth color of isolate N1104 and ATCC #700647. For others, it indicates attachment that is too thin to be of darker color.	2
	"Rosy Pink"	Pantone 182C, 203C, or 217C	These are the most common colors for typical, healthy PPFM growth.	3
	"Dark Pink"	Pantone 189C or 197C	Depending on the isolate, these colors can represent typical, healthy PPFM growth, or they can indicate the beginning of low oxygen or low nutrient stress in an older biofilm.	4
	"Red"	Pantone 171C or Warm	These colors signify a biofilm that is past its prime or struggling with stressful conditions.	5

		Red C	They are observed in older biofilms when oxygen or nutrients are becoming depleted. They are also seen in stressed biofilms growing in a toxic substrate, such as ethanol or elevated methanol.	
Extent of Attachment	No clear pattern	Some sections are uniform while others are sparse. Entire sections of glass are bare.	Indicates weak attachment or sheer stress.	0
	Sparse or heterogeneous pattern	Patches of growth with large spaces between streamers or microcolonies. Streamers present are of irregular size and unevenly spaced.	Can indicate varying attachment due to sheer stress but most often indicates early attachment that will become more uniform with time.	1
	Uniform pattern	Growth is evenly spaced and of comparable thickness throughout. Streamers are of comparable size and channels are well-defined between them.	This is the typical, thriving biofilm attachment pattern.	2
Structure	Fine	Particulate or granular appearance with streamers small or absent.	This can indicate early or failed biofilm growth. Most often it is the endpoint of failed growth in which development does not proceed past the microcolony level. It is usually correlated with white colored cells.	1
	Three-dimensional	Thick, long streamers and channels are visible. Streamers moving or pressed down against glass.	This is the most mature biofilm structure.	2
Growth Pattern	No clear pattern	No growth or very patchy growth.	Indicative of unhealthy biofilm growth. Can also signify that sheer stress is preventing	0

			uniform attachment.	
Growth Density	Concentric rings	Biofilm growth is present with bare patches in concentric rings.	This is due to variations in the glass flask surface or sheer stress. Indicative of weak attachment and sensitivity to sheer stress.	1
	Complete coverage	Biofilm covers the entire flask bottom and has crept up the sides without any interruption in attachment.	This represents the strongest degree of attachment.	2
	Thin biofilm	Biofilm is thin and glass can be seen through.	Indicative of early or disrupted attachment.	1
	Thick biofilm	Biofilm is thicker and less glass can be seen through. Streamers are moving freely with shaker movement.	This represents typical biofilm quality and maturity.	2
	Thick mat	Biofilm is so thick that multiple layers of streamers are laid down on top of one another, giving it a pressed, fabric-like appearance. Streamers are attached and immobile. No glass can be seen through.	This is the highest degree of robust, mature biofilm growth. Thus far it has only been seen with isolate NJ1104 and ATCC #700647.	3

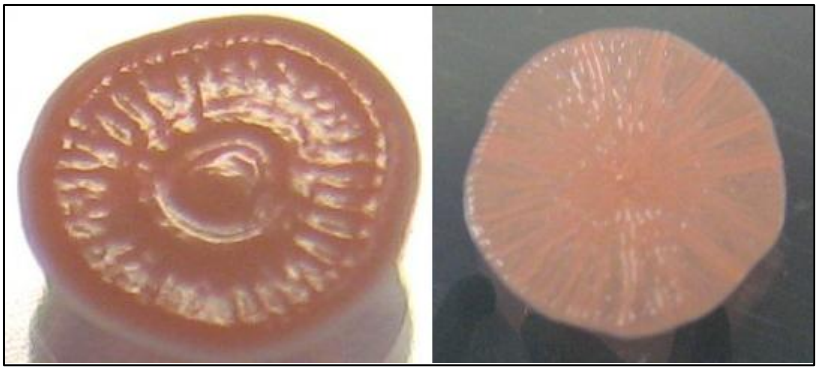

Figure 1. Examples of texture variations in PPFM isolate strains. An NJ1101 colony (left) is characterized by concentric ring patterns with parallel grooves connecting the rings. An NJ1104 colony (right) is characterized by only parallel grooves radiating out from the center in a starburst-like pattern.

Examples of initial NJ1104 biofilm production as well as photos of mature biofilms are in Figs. 2 & 3. Generally, NJ1104 presents as round, "pale pink" (Pantone 196C), wrinkled colonies on AMS agar plates with 0.5 % methanol added. Colonies display either straight-lined, "starburst" patterned striations that radiate out from the center of the colony or a uniform "bumpiness" that sometimes has light striations in it. Sometimes there will also be a thin ring around the outside edge of the colony. Optimal biofilm production occurred for NJ1104 in flasks shaken at 120 rpm at room temperature in AMS medium with 0.5 % methanol as the sole carbon source. Under these conditions NJ1104 produced biofilms that are "rosy pink" in color (Pantone 217C) and are strongly adhered to themselves and the glass. They display a three-dimensional structure of "streamers," or long chain-like threads of biomass that move loosely in the medium, and "channels," clear passage between areas of thick biomass, presumably to allow more surface area for nutrients and oxygen to reach biofilm cells. Three-dimensional structures within single-species biofilms produced by soil-derived bacteria have been described in *Pseudomonas fluorescens* (Baum et al., 2009), but this type of observation has never before been performed for *Methylobacterium* biofilms.

NJ1104 biofilm production is not limited to the bottoms and side of flasks. Examples of initial and mature NJ1104 meniscus biofilms (Figs. 4 & 5) as well as "sticky ring" structures (Fig. 6) can be found in the appendix. NJ1104 produced a thin, almost leathery textured biofilm at the liquid-water interface. This biofilm was usually dark pink (Pantone 189C or 197C) and remained intact when harvested. Similar structures have been noted for other bacteria, and research suggests that some bacteria are able to guide themselves to build certain types of biofilms in different locations (air-water interface vs.

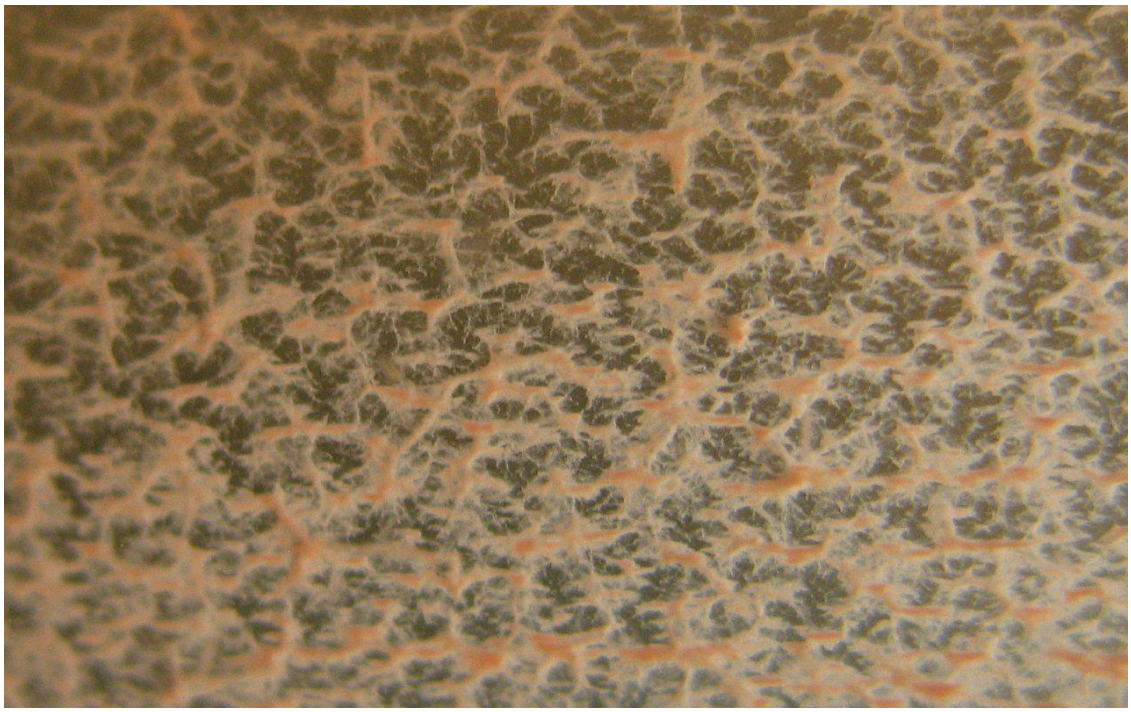

Figure 2. Early developing NJ1104 biofilm attached to glass. This example was taken from an AMS culture with 0.5 % methanol in a flask shaken at 120 rpm.

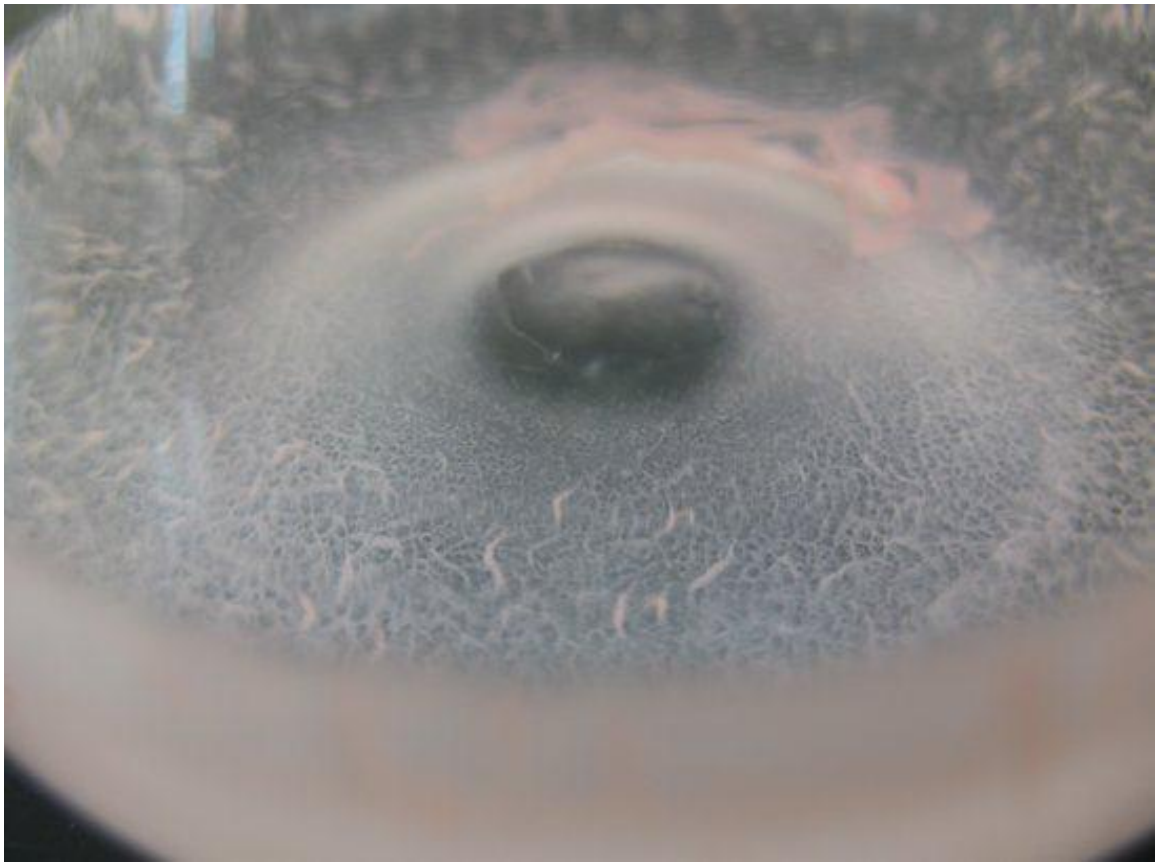

Figure 3. Mature NJ1104 biofilm attached to glass. This example was taken from an AMS culture with 0.5 % methanol in a flask shaken at 120 rpm.

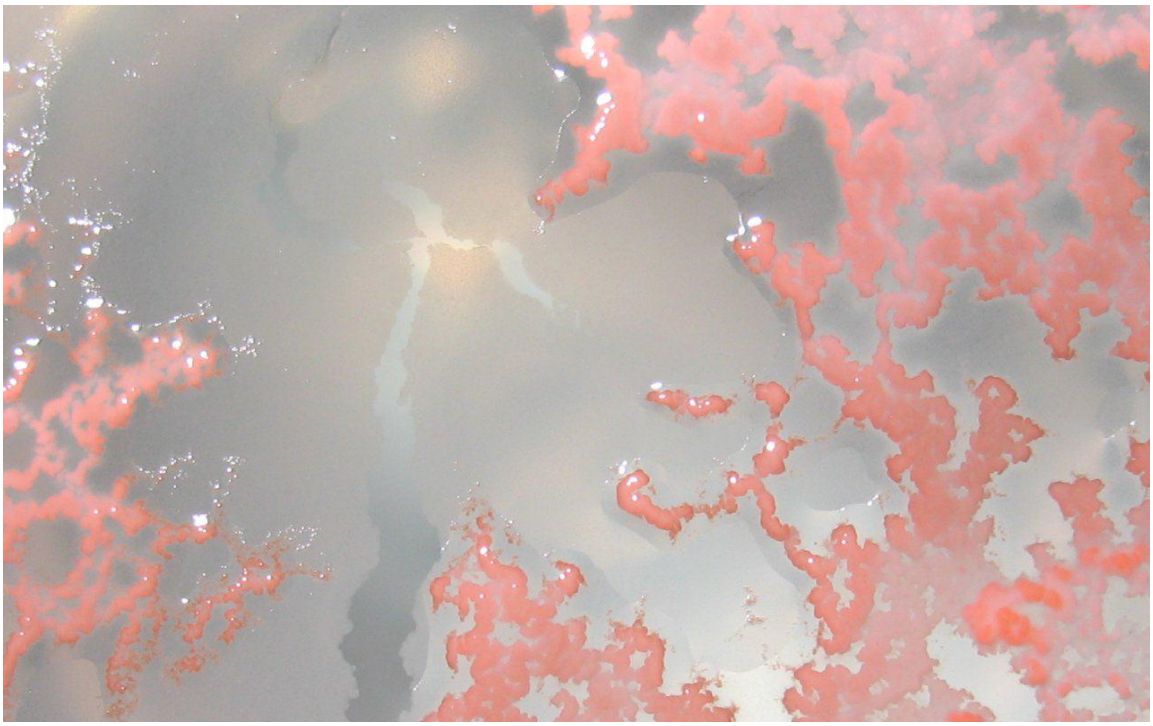

Figure 4. Early developing NJ1104 meniscus biofilm. This example of an NJ1104 meniscus biofilm was taken from a culture grown in AMS medium with 0.5 % methanol and left undisturbed for 1 week.

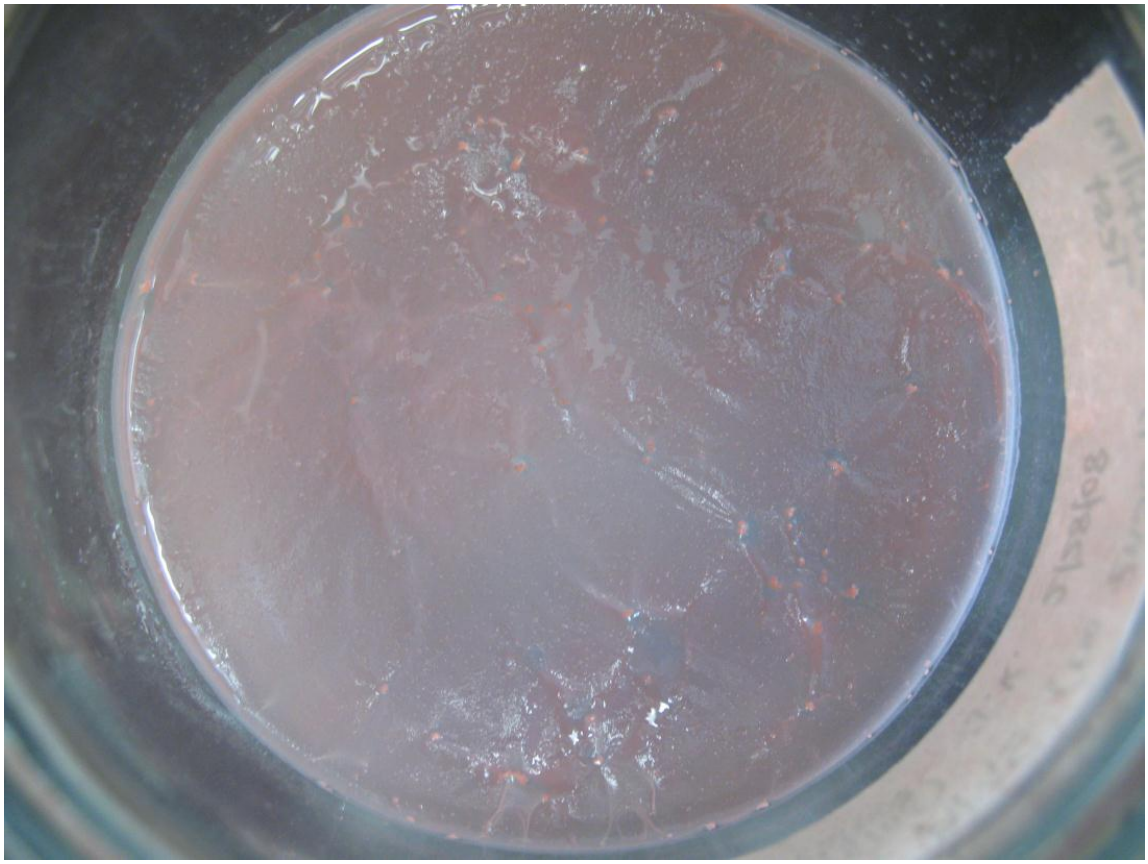

Figure 5. Mature NJ1104 meniscus biofilm. This example of an NJ1104 meniscus biofilm was taken from a culture grown in AMS medium with 0.5 % methanol and left undisturbed for two weeks.

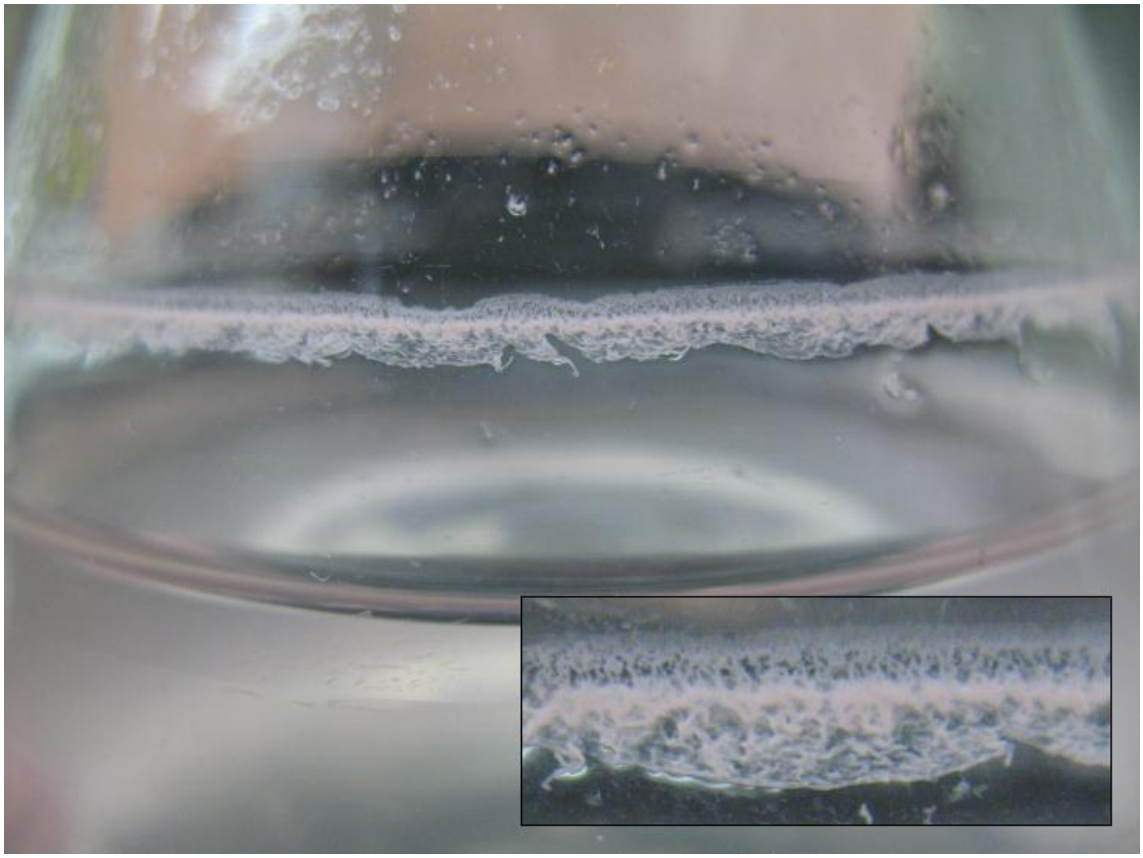

Figure 6. NJ1104 "Sticky Ring." This is an example of a "sticky ring" from an NJ1104 culture grown in AMS medium with 0.5 % methanol and shaken at 120 rpm.

surface-liquid interface) (Davey and O'Toole, 2000), but this has never been demonstrated for PPFMs. In addition to a meniscus biofilm, NJ1104 also produced a "sticky ring" that resulted from the buildup of cells on the side of the flask at the water line with the flask shaking at 120 rpm. Over time, however, it took on a three dimensional structure not unlike that of the biofilm that forms at the bottom of the flask. It displayed the "streamers and channels" three-dimensional structure pattern, it was strongly attached to the glass, and it often remained intact when harvested.

An example of a stationary biofilm growing in a glass jar with labeled structures mentioned here can be found in the appendix (Fig. 7). When left to grow in a stationary container for one month, NJ1104 produced both glass-adhered and meniscus biofilms as well as additional structures. "Fibrous" or "filamentous" bridges formed that connected the aerobic meniscus biofilm to the microaerophilic bottom biofilm in the glass jar. These structures are fragile and soft, collapsing and breaking up during harvesting. In addition to these structures, microcolonies were visible forming on the glass both above the water line and below. These colonies were densest just above the water line and thinned out as the distance between the colonies and water line increased. Below the water line, colonies were densest around anchor points for the filamentous materials, in other words, they were localized around areas where filaments had attached to the glass.

The presence of the filamentous bridges between the top and bottom biofilms and the presence of microcolonies around filament anchors on the glass suggests that diversification of cell function as well as communication between cells is taking place in NJ1104 biofilms. These bridges might be a means of moving nutrients from one biofilm to the other, perhaps because of the difference in oxygen between meniscus and

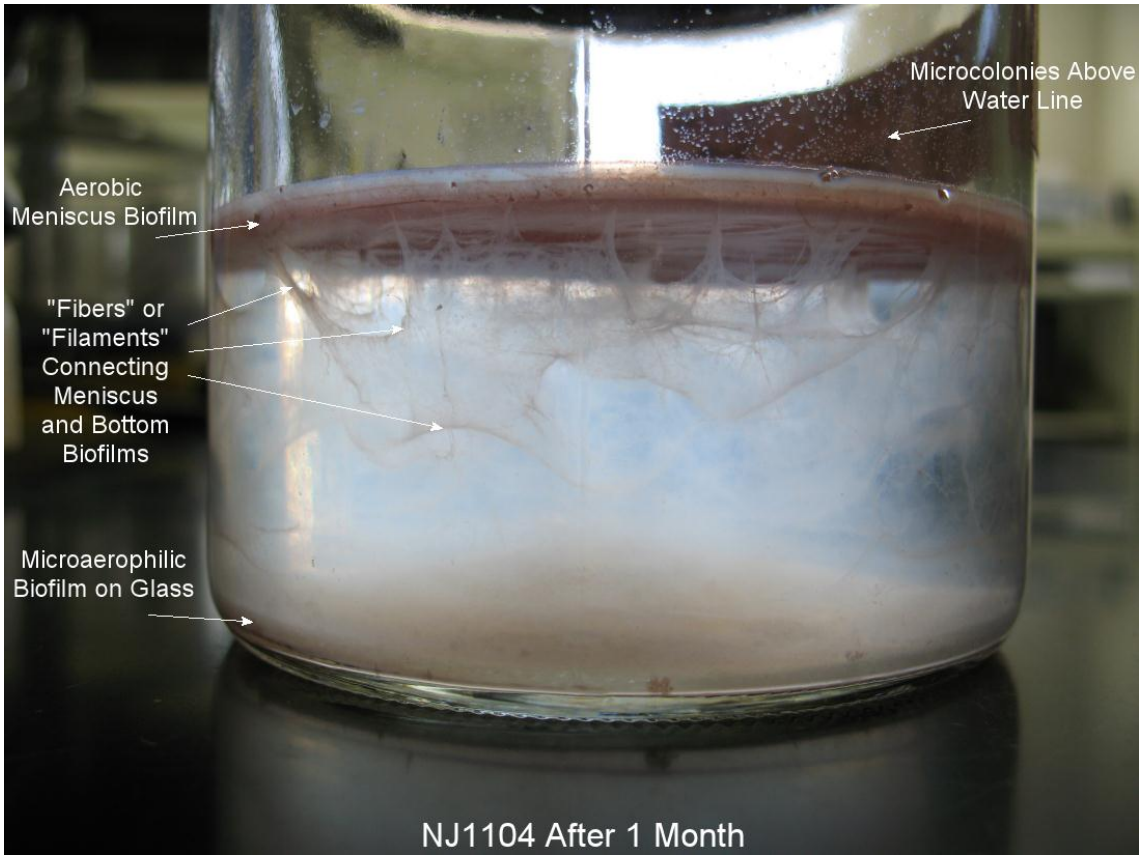

Figure 7. NJ1104 in a Stationary Glass Jar of AMS Medium. Structure produced after one month of growth include: a thick, watery, loosely attached, microaerophilic biofilm at the bottom of the jar; an aerophilic, thin, dense, sticky biofilm at the meniscus; “fibrous” or “filamentous” material joining the meniscus and bottom biofilms together; microcolonies on glass, both above and below the water line.

submerged stationary biofilms. These filaments may also be acting as literal bridges to transport cells to other areas, as is suggested by the movement of cells from filament anchors outwards onto the glass in the form of microcolonies. As they are too fragile to be true anchors that might hold the biofilms in place and can be broken easily by the slightest perturbation, for now it can only be assumed that this filamentous material arises out of a need to transport cells and nutrients from one area of growth to another.

NJ1104 cells diversified when forming biofilms. Not only are multiple types of biofilms formed within one culture but other structures such as filaments and microcolonies occur once biofilm development reached a mature level. An observation in support of diversification within a single biofilm was made with a 45 day-old NJ1104 meniscus biofilm displaying color variations. Photos of the biofilm at 45 days displaying these red spot variations can be found in the appendix (Figs. 8a and 8b). Once growth had occurred for this long, the leathery film starts to wrinkle. Interspersed within these wrinkles emerged color variations that ranged from pale pink to rosy pink to almost red. The most notable of these color variations were red spots that emerged on the biofilm between 30 and 45 days of growth. The reason and purpose for these color variations and spots are unclear, however, this phenotypic variation could also hint at functional variation as well.

These phenotypic color variations may also be indicative of functional or metabolic diversity. Phenotypic diversity and the potential for division of labor between cells within a biofilm have been studied in other species, such as *Bacillis subtilis*, but not in PPFMs. In one such study, it was found that epigenetics (the use of extracellular signaling molecules to induce changes within cells) is employed by *B. subtilis* to give rise

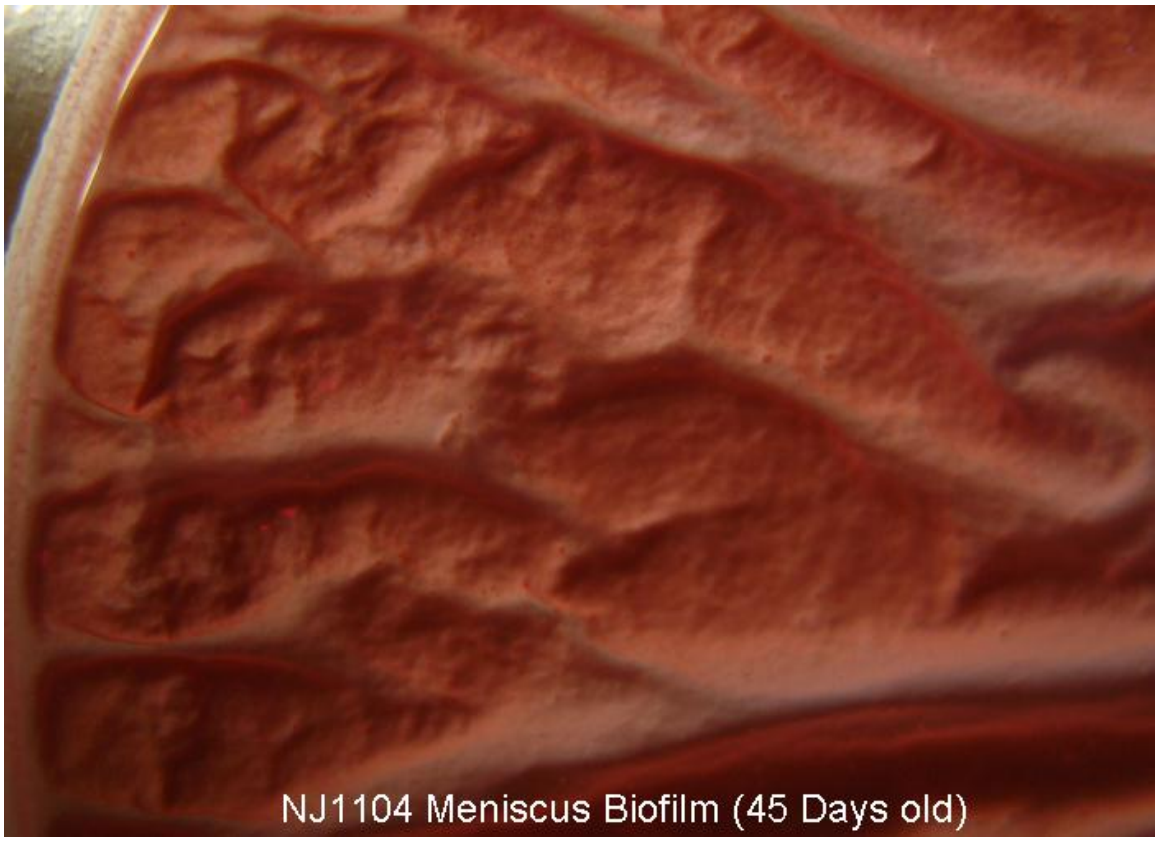

Figure 8a. A 45 Day-Old NJ1104 Meniscus Biofilms Showing Color Variations.

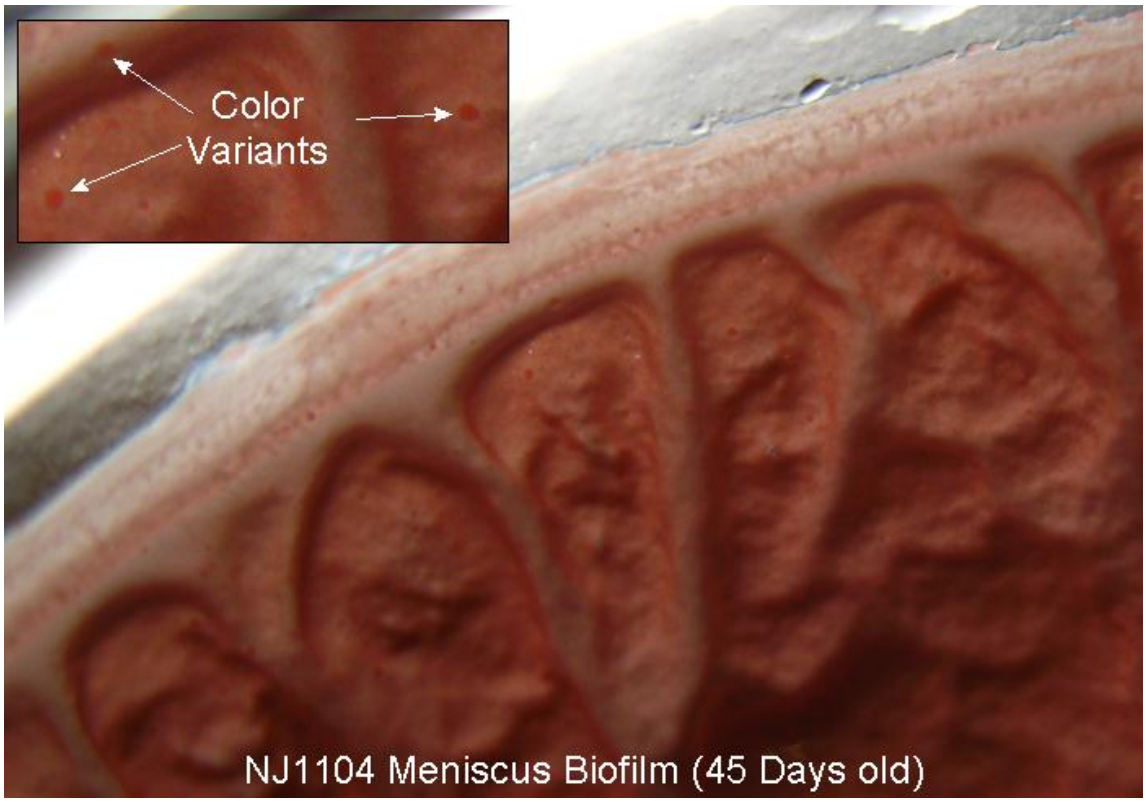

Figure 8b. A 45 Day-Old NJ1104 Meniscus Biofilms Showing Color Variations.

to functional diversity within biofilms (Chai et al., 2008; Kearns, 2008). Perhaps the filamentous "bridges" mentioned earlier are established for the purpose of transporting such signal molecules along with nutrients to distant cells and color variations are the result of functional diversity within NJ1104 biofilms. This is only speculation at this time, but further research may elucidate whether the complex division of labor seen within other biofilms occurs within PPFM biofilms and if the mechanisms and deciding factors for the inhibition or onset of such diversity are similar.

When NJ1104 was growing in stressful conditions, the color, thickness, and texture of its biofilms changed. Stressful conditions included oxygen and nutrients becoming limiting, the biofilm becoming very old, sheer stress, or the presence of toxic levels of substrates or Zn. Under these conditions, the biofilm covered less area on the glass, was very thin and stringy, was not as strongly adhered to the glass, and was generally darker in color. As they aged and oxygen and nutrients became limiting, biofilms were characterized by a foul odor and a "dark pink" (Pantone 189C or 197C) or "red" (Pantone 171C or Warm Red C) color.

Simulated Multi-Species PPFM Biofilm

Because NJ1101's biofilm production differed from that of NJ1104 in that it was thinner and weakly attached, it was hypothesized that NJ1101 needed something else to produce stronger biofilms under laboratory conditions. A qualitative summary of the results of this experiment can be found in Table 5, as well as photographical examples of NJ1101, NJ1104 in Fig. 9. When grown together with equal inoculum densities of NJ1101 and NJ1104 to start, biofilms were produced that resembled NJ1104 single-

Table 5. Qualitative Observational Summary of NJ1101 and NJ1104 biofilm production. Below are the results when NJ1101 and NJ1104 are grown separately, together with equal inoculum densities, together with disparate inoculum densities, and finally with each in the spent medium of the other.

Inoculum & Culture Conditions	Results After 10 Days
NJ1101 Only	Clear medium; one large pink floc; minimal attachment to bottom of flask in a ring pattern (random chunk formations); no odor; no "sticky ring."
NJ1104 Only	Turbid, pink medium; one large pink floc; thin, pale pink "sticky ring" at top; foul odor; uneven attachment on bottom of flask in a ring pattern (medium and long streamers scattered between larger spaces)
NJ1101 = NJ1104	Turbid, pink medium; one medium pink floc; thick, dark pink "sticky ring" at top; foul odor; very even biofilm matrix of "streamers" and "channels" (no long streamers, only medium sized)
NJ1101 > NJ1104	Clear medium; one large pink floc; minimal attachment to bottom of flask in a ring pattern with a few small "streamers"
NJ1101 < NJ1104	Clear medium; even biofilm matrix of long "streamers" and "channels;" dark pink "sticky ring" at top
NJ1101 in Spent NJ1104 Medium	Clear medium; one large pink floc; minimal attachment to bottom of flask in a ring pattern with a few small "streamers"
NJ1104 in Spent NJ1101 Medium	Clear medium; even biofilm matrix of long "streamers" and "channels;" dark pink "sticky ring" at top

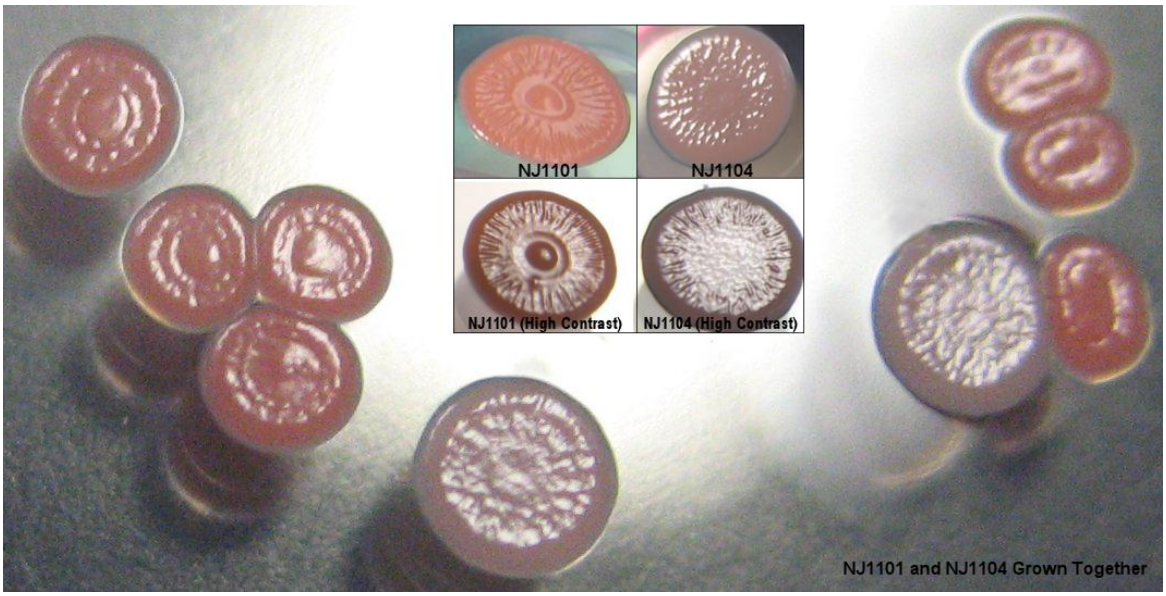

Figure 9. Examples of NJ1101 and NJ1104 Colonies when Growth Together. Colonies were plated from culture containing equal numbers of cells in NJ1101 and NJ1104 inoculum. Inset: Top two colonies are taken in natural lighting and the bottom two were taken at high contrast to make textures more visible.

species biofilms. The same was true for cultures in which twice the number of NJ1104 cells was added compared to NJ1101. When twice the number of NJ1101 cells were added compared to NJ1104, the resulting biofilms resembled that of NJ1104 biofilms, but were weakly attached and detached from the glass after just four days of growth. This experiment suggests that NJ1104 is more dominant in a laboratory setting than NJ1101, and that mixed-species biofilms that consist of these two isolates tend to resemble NJ1104 rather than NJ1101. Both bacterial isolates were present in both the liquid medium and the biofilms, however. Washed biofilms smeared on agar plates gave rise to colonies that resembled both isolates, as did the plating of the spent liquid medium. Growth of either NJ1101 or NJ1104 in the spent medium of the other did not appear to effect biofilm growth.

An interesting result from the plating of liquid medium and biofilm materials from cultures containing both NJ1101 and NJ01104 was the emergence of "hybrid" colonies displaying colors and textures that were a combination or a midpoint between those of the individual isolates. Colonies with hybrid color and texture can be found in Fig. 10. For example, colonies that were the color of typical NJ1101 colonies ("rosy pink") sometimes displayed the radial texture pattern of typical NJ1104 colonies. Similarly, colonies displaying typical NJ1104 "pale pink" pigmentation sometimes exhibited the concentric ring pattern of typical NJ1101 colonies. This may indicate a communication between the two isolates and perhaps a sharing of either DNA or growth materials that might cause such variations to occur.

This type of qualitative analysis of PPFM biofilms provides information about isolate behavior and three-dimensional structure that molecular data cannot provide.

Figure 10. Examples of normal and "hybrid" colony colors and textures for NJ1101 and NJ1104. Colonies were plated from culture containing equal numbers of cells in NJ1101 and NJ1104 inoculum.

While it is known that PPFMs have extensive quorum-sensing systems (Poonguzhali et al., 2007), this has not yet been linked to biofilm production. Information regarding PPFMs and biofilms is limited to PPFM participating in multispecies biofilms and does not examine the abilities of the *Methylobacterium* genus alone (Barbeau et al., 1996; Väisänen et al., 1998; Kelley et al., 2004; Simões et al., 2007). The biofilms described here follow patterns described in the literature for other bacteria but that have never been described in PPFMs. It is known that shear stress affects the adhesion and thickness of *Pseudomonas aeruginosa* biofilms (Fonseca and Sousa, 2007) and that there can be diversified physiological and functional niches within both single and multispecies biofilms (Chai et al., 2008; Stewart and Franklin, 2008). Whereas organisms like *Pseudomonas aeruginosa* and *Bacillus subtilis* have been studied in detail with regard to their biofilm production capabilities, this research now provides evidence of biofilm production in PPFMs indicating that complex and potentially functionally diverse biofilm formation is possible within this genus.

Effects of Substrate Variation on PPFM Growth and Biofilm Development

Growth in different methanol concentrations varied between the nine NJ PPFM isolates. Eight of nine tested grew in concentrations between 0.10 to 0.50 % methanol (Table 6). Viable plate counts were between 10^7 and 10^9 CFU/mL and isolates were rosy pink (Pantone 203C, 217C, or 182C) to dark pink (Pantone 189C, 197C) in color. Growth was slower and yield was lower in concentrations between 1.00 and 1.50 % methanol. Viable plate counts ranged from 10^2 to 10^4 CFU/mL. At concentrations between 1.75 and 2.00 % methanol, no CFU were detected (Table 6).

Table 6. Growth and biofilm production of nine NJ agricultural isolates in varying methanol concentrations. CFU/mL was determined on 10^{-6} dilution plates for concentrations between 0.10 % and 2.00 % methanol. Above 2.00 % and up to 10.00%, cultures were plated directly onto plates without dilution.

NJ Isolate	0.10 %	0.50 %	1.00-1.75 %	2.00-4.00 %	5.00 %	7.50 %	10.00 %
NJ1101 *(biofilm producer)*	• Typical growth* • Weak biofilm**	• Typical growth • Weak biofilm	• Low yield	ND	*nt*	*nt*	*nt*
NJ1104 *(biofilm producer)*	• Low yield+	• Typical growth (Pantone 196C) • Strong biofilm	• Typical growth (Pantone 196C) • Strong biofilm	• Low yield Weak biofilm	2.5×10^3 • 1 mm diameter • Rosy pink (Pantone 217C) 1.0×10^2 • 3-5 mm diameter • Red (Pantone 171C)	4.0×10^3 • 1 mm diameter • Rosy pink (Pantone 217C) 4.0×10^2 • 3-5 mm diameter • Red (Pantone 171C)	9.0×10^2 • 1 mm diameter • Rosy pink (Pantone 217C) 4.0×10^2 • 3-5 mm diameter • Red (Pantone 171C)
NJ1106 & NJ1107	• Typical growth	• Typical growth	• Low yield	ND	*nt*	*nt*	*nt*
NJ1103, NJ1105, NJ1108, NJ1126 & NJ1128	• Typical growth	• Typical growth	• Low yield	ND	*nt*	*nt*	*nt*

ND = Not detected; *nt* = Not tested.

*Typical growth refers to that observed in the laboratory during non-stressful conditions.

Typical CFU/mL counts are between 10^8 and 10^{10} for NJ1104 and between 10^7 to 10^9 for the remaining eight isolates.

**A weak biofilm is one that does not fully form and has weak attachments resulting in the peeling away of the biofilm from the flask as it develops. A strong biofilm is one that develops a mature three-dimensional structure and that is strongly adhered to the glass.

$^{+}$Low yield is relative to typical growth. Low CFU/mL for NJ1104 is between 10^6 to 10^7 and 10^5 for the remaining eight isolates.

With regard to biofilm production in varying concentrations of methanol, isolate NJ1104 did not produce a biofilm at 0.10 % methanol and yield was low (10^6 -10^7 CFU/mL). Between concentrations of 0.50 and 2.00 %, NJ1104 grew well and produced a biofilm. Biofilm development of NJ1104 with methanol concentrations of between 0.25 % and 2.00 % is shown in Figure 10. Between 2.50 and 4.00% growth was slower and yield was lower. These data suggest that at 0.10 % concentration, NJ1104 was not inhibited but neither was growth stimulated by the methanol. Between 0.50 and 2.00 %, this data indicates that the growth of NJ1104 was stimulated by these concentrations of methanol and biofilm production proceeds well. Above 4.00 %, however, the methanol became inhibitory, limiting growth and halting biofilm production.

At 5.00 and 7.50 %, viable plate counts were the lowest and two variant colony types emerged on agar plates (Table 4). One variant was “rosy pink” (Pantone 217C) and 1 mm in diameter, while the other was “red” (Pantone 171C) and 3-5 mm in diameter. In 10.0% methanol concentration, NJ1104 was equally low and two variants emerged again (Table 6). The “rosy pink” (Pantone 217C) variant of 1 mm diameter was seen in 10.0 % methanol, but the second variant of 4-5 mm diameter was a darker shade of red (Pantone Warm Red C). Higher concentrations have not been tested.

The NJ1104 variants plated from concentrations of 7.50 and 10.0 % methanol also displayed an abnormal morphology. NJ1104 is usually pale pink in color, round in shape, and possesses a concentric ring and starburst texture. The small variants from 5.00, 7.50 and 10.0 % methanol concentrations, however, were smooth. The larger variants were “flower”-shaped had a bumpy, uneven, wrinkled texture and were red (Pantone 171C or Warm Red C). The change in morphology and color of NJ1104 colonies challenged with

elevated methanol may indicate a stress response. Variants capable of surviving under those conditions are being selected for. Pictures of the larger, red, "flower-shaped" NJ1104 colonies discussed here can be found in Fig. 11.

Eight of nine NJ isolates do not grow on ethanol alone or with an ethanol/methanol combination. The biofilm development chart (Table 4) was used to measure NJ1104 biofilm development over time, with each culture being scored daily based upon the observational data corresponding to each chart section. The scores were then used as a means to plot biofilm development over time. NJ1104 biofilms are slower to develop in ethanol and do not mature to the same degree as in methanol. For both the methanol and ethanol treatments, cultures exposed to higher concentrations were slower to develop than those exposed to lower concentrations, indicating toxicity at higher concentrations (Figs. 11 & 12). This effect is more pronounced in ethanol, however, suggesting that ethanol is more toxic to NJ1104 at these concentrations than is methanol. Cultures given high methanol concentrations developed nearly to the same level as those given lower concentrations, which was not true for ethanol (Figs. 13 & 14).

While the ability of PPFMs to grow and produce biofilms in relatively high concentrations of methanol and formaldehyde has been investigated (Fletcher, 1983; Bormann et al., 1997; Chongcharoen et al., 2005), growth in ethanol has not been looked at in detail. Isolate NJ1104 grows with ethanol and with an ethanol/methanol combination, but with varying results for the dry weight of the biofilms (Fig. 14). In up to 1.00 % ethanol a typical biofilm formed; at concentrations from 1.00 up to 2.00 % there was slight growth inhibition indicated by decreased viable plate counts and a visible

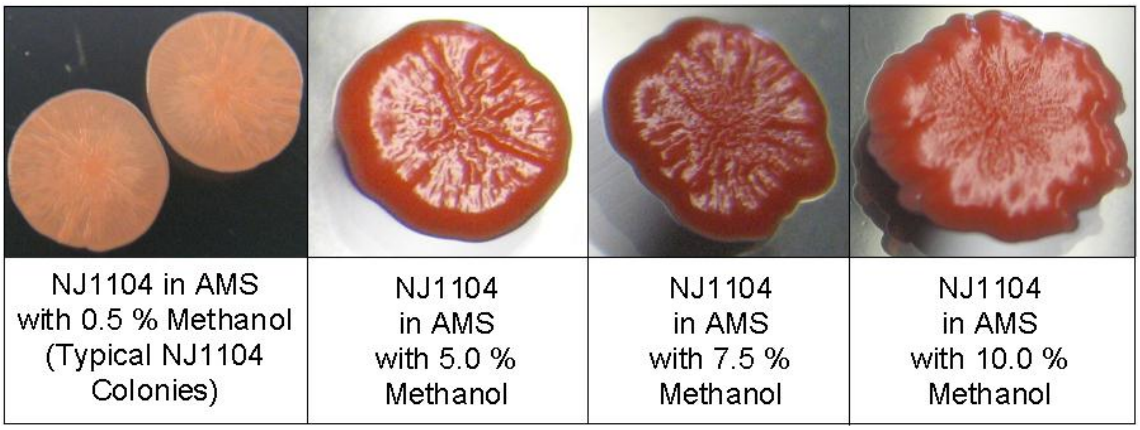

Figure 11. Examples of NJ1104 Colonies Grown with Varying Methanol Concentrations. From left: Normal pale pink, striated NJ1104 colonies from culture with 0.5 % methanol; slightly amorphous, unevenly wrinkled red variant from culture with 5.0 % methanol; amorphous, unevenly wrinkled red variant from culture with 7.5 % methanol; amorphous, only slightly wrinkled variant in from culture with 10.0 % methanol.

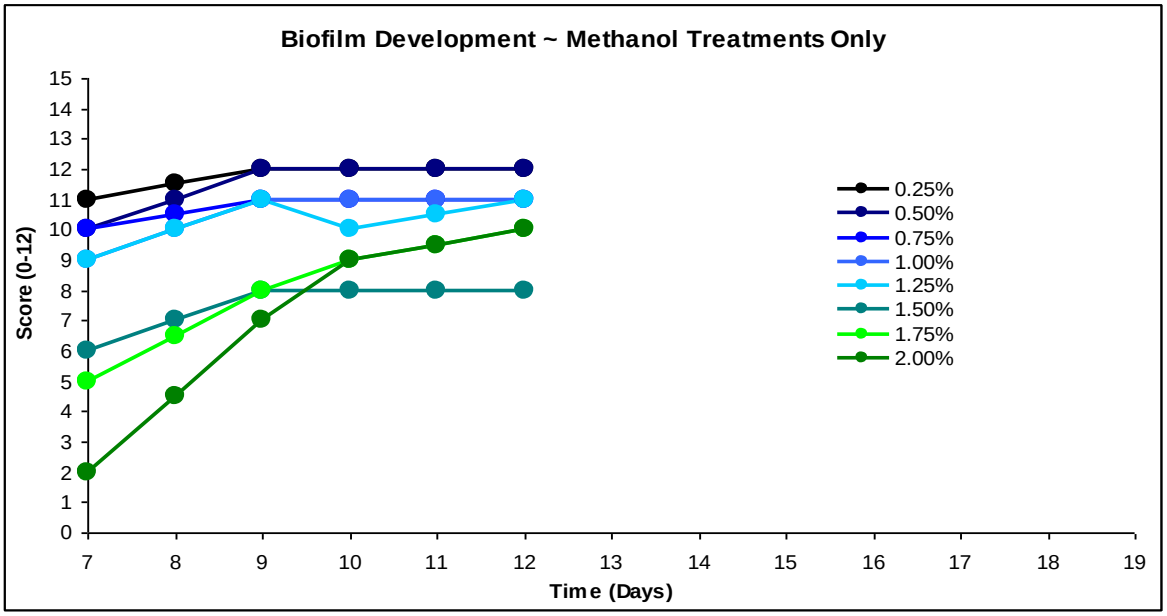

Figure 12. Methanol Treatments Only. Biofilm development was measured qualitatively using the Biofilm Development Key in Table 2 and converted to numerical scores which could then be plotted for visual representation. Below is shown the development of eight biofilms (one flask per methanol concentration) over the course of approximately two weeks. (Note: Data points for days 13 and later not shown as they were identical to day 12.) Biofilm production occurred for all methanol concentrations with a general trend of lower scores for higher concentrations. This was mostly due to changes in color and alterations in structure, indicating methanol toxicity.

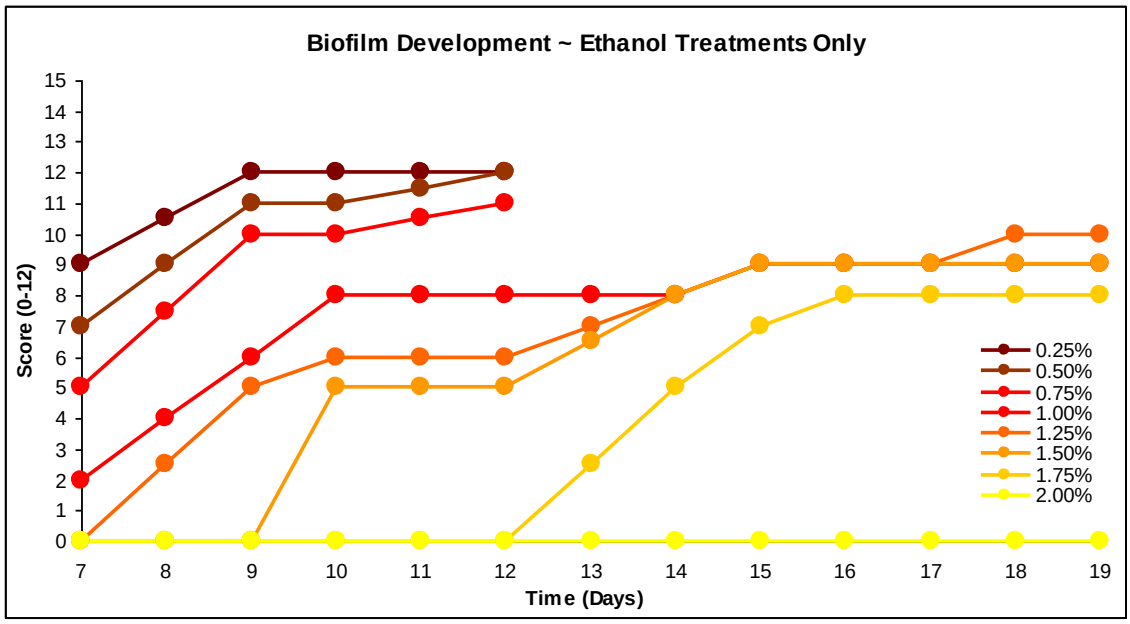

Figure 13. Ethanol Treatments Only. Biofilm development was measured qualitatively using the Biofilm Development Key in Table 2 and converted to numerical scores which could then be plotted for visual representation. Below is shown the development of eight biofilms (one flask per ethanol concentration) over the course of approximately two weeks. Biofilm production occurred for ethanol concentrations between 0.25 % and 1.75 %, but not for 2.00 %, showing a loss of the ability above a certain level of ethanol toxicity. While biofilms did arise at ethanol concentrations of between 1.25 % and 1.75 %, the quality and health of these biofilms was considerably less than other treatments, indicated by lower scores. Like with methanol, there was a general trend of lower scores for higher ethanol concentrations, but the effect is more pronounced with ethanol than with methanol. This was most likely due to the higher affinity of the MDH enzyme for ethanol vs. methanol.

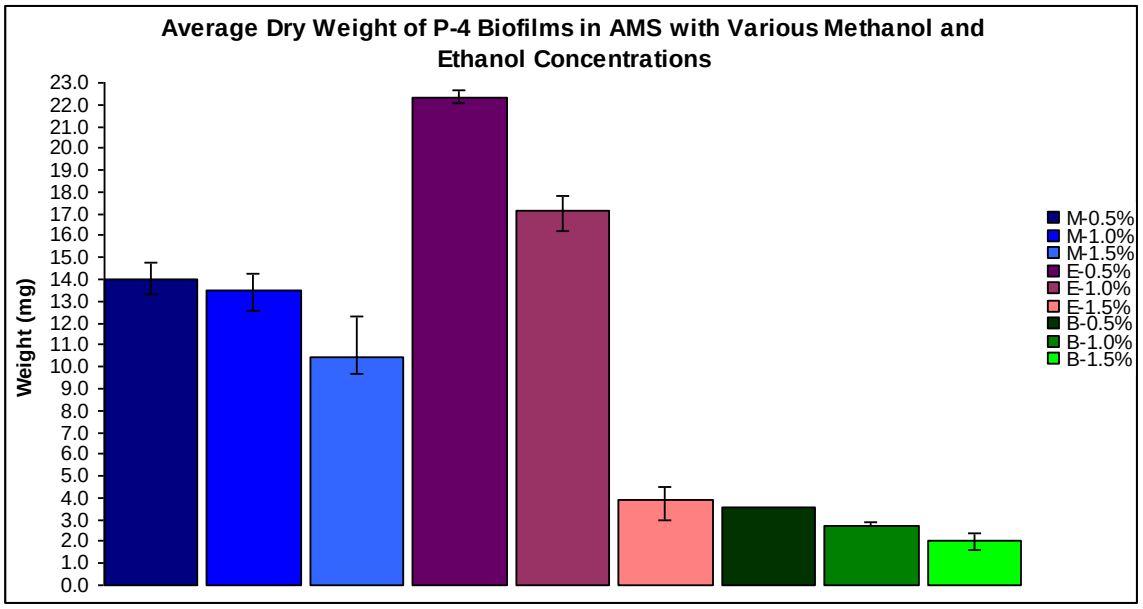

Figure 14. Dry Weight of NJ1104 Biofilms After Growth for Three Weeks with Varying Amounts of Methanol (M), Ethanol (E) or Both (B) as Substrates. Each bar represents the average dry weight (in mg) of biofilms from three flasks with the exception of B-0.5%, which had only one flask, and B-1.5%, which had only two. This was due to instability in biofilm growth that made the film unable to be harvested in its entirety for measurement. Concentration affected the cultures more drastically for ethanol. There was a significant drop in biofilm weight only between 1.0 % and 1.5 % for methanol concentrations, whereas ethanol-fed biofilms experienced significant drops in weight between 0.5 % and 1.0 % as well as between 1.0 % and 1.5%. This may be due to NJ1104 producing high EPS to protect the growing biofilm against ethanol stress at lower concentrations, but at high concentrations or with two alcohols present, the stress becomes enough to significantly inhibit biofilm development. Or it may be that NJ1104 is receiving twice the amount of carbon from ethanol as a substrate and is able to produce more cells.

reduction in biofilm thickness, but biofilm production still occurred (Fig. 4). Above 2.50 % ethanol concentration, NJ1104 was killed.

NJ1104 did not grow in any liquid medium that contained formaldehyde, whether alone or in combination with ethanol or methanol, even at 0.1 % concentration. Isolate NJ1104 also did not grow on sodium acetate at any concentration, whether alone or in combination with ethanol or methanol. These results are interesting considering that methanol is converted to formaldehyde in the first step of assimilation (Chistoserdova *et al.*, 2003) and that it has been cited in the literature that some species of *Methylobacterium* can tolerate very high concentrations of formaldehyde (Chongcharoen *et al.*, 2005). In addition, sodium acetate is a common root exudate and a readily decomposable carbon source that PPFMs would encounter in the rhizosphere. It may be, however, that NJ1104 is simply not able to use these substrates or that it may require something else from soil or other bacteria to utilize it that it does not have in a laboratory setting. These results also are not meant to imply that all PPFM species are incapable of utilizing sodium acetate as a carbon source, but they do suggest that metabolic versatility regarding some substrates (methanol and ethanol) does not translate to versatility with others (sodium acetate and formaldehyde).

Effect of Elevated Zn on PPFM Biofilm Development

During growth of NJ1104 in a shaken flask, OD rose initially, usually over the first 48 hours, and then rapidly declined. The fall in OD signified the start of biofilm production because the culture medium became clearer as cells transition from a planktonic to a sessile lifestyle. Thus, OD measurement can act as an indirect way of

quantitatively measuring the onset of biofilm formation as well as signify the extent to which cells have participated in the biofilm. A gradual decline in OD after biofilm production (in which the medium remains turbid for 24 hours or more after biofilm growth as begun) indicates a slow switch from planktonic to sessile life. Conversely, a sharp decrease in OD (in which the medium becomes clear and colorless within 12 hours after biofilm growth has been initiated) indicates a more complete and abrupt shift. Disruption of biofilm production was characterized by higher than normal OD late in the growth cycle, a lag in the OD drop, or an actual visual difference in biofilm appearance.

When examining the 0 hr and 24 hr amendment time points for the preliminary experiment, OD was lower than the control and subsequent amendment time points until day 2 (Fig. 15). This indicates slower growth until day 2. After day 2, the OD of these two time points increased dramatically and peaks at values much higher than the control and subsequent amendment time points (Figure 14). This may indicate a usage of the Zn as a nutrient. After the peak in OD at day 3, however, biofilm production began in all treatments except 0 and 24 hr (not shown in graph). This was indirectly measured as a drop in OD as cells moved from planktonic to biofilm growth. The sharpest drop in OD was observed in the 24 hr treatment, but biofilm production was not immediately visible as it was in other treatments. The 0 hr treatment experienced the most gradual decline in OD after day 3 out of all treatments (Fig.15). Biofilm production was not visible until day 4. This treatment also experienced a high medium turbidity and pink color after biofilm production had begun.

Whereas the liquid medium was clear in treatments with later amendment times, the medium in the 24 hr treatment was cloudy and pink. This color and turbidity

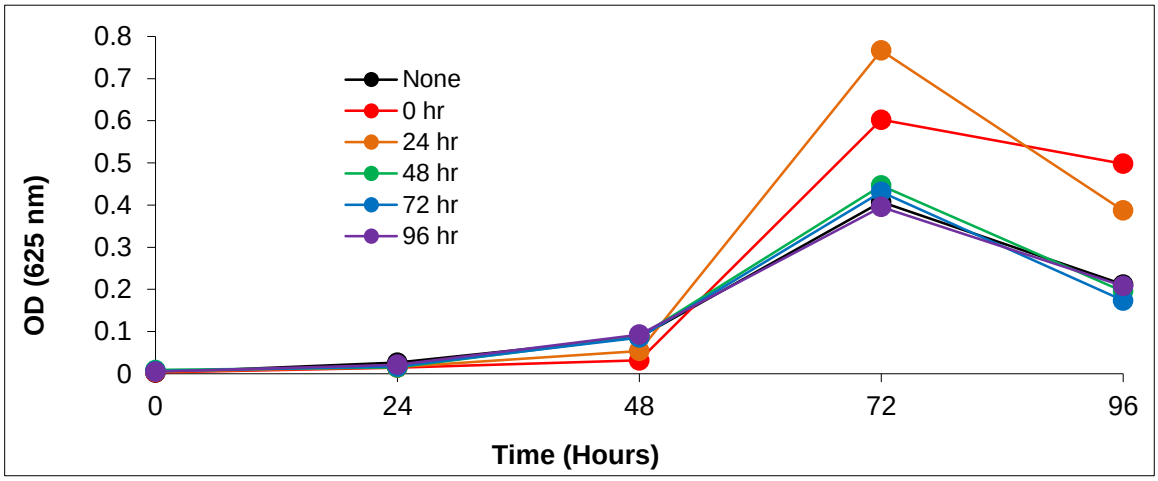

Figure 15. Biofilm Production of Isolate NJ1104 in 0.1mM $ZnSO_4$-Amended Liquid AMS Agar Over Four Days. The 0.1 mM $ZnSO_4$ amendment was added at the following time points: No amendment (solid square), 0 hrs (solid diamond), 24 hrs (solid circle), 48 hrs (hollow square), 72 hrs (hollow diamond) and 96 hrs (hollow circle). Onset of biofilm production was indirectly measured using optical density (OD) at 625 nm wavelength. Planktonic growth is designated by an increase in OD and biofilm production is indicated by a decrease in OD. Growth and biofilm production was observed in all treatments after 48 hrs and after 72 hrs, respectively.

continued even after biofilm production was well underway. This suggests either a reluctance of cells to commit to biofilm production or that the elevated Zn was chemically disrupting production. Both copper and zinc ions have been known to negatively impact the transition from the yeast way of life to a hyphal phenotype in fungal cultures (Harrison et al., 2007), and so this may also translate to the transition in bacteria from planktonic to biofilm lifestyle.

The presence of 1.0 mM $ZnSO_4$ had a different effect on OD measurements. Treatments that received elevated Zn at the 0, 12 or 24 hr time points experienced lower OD readings early on than treatments that received it at 36 or 48 hrs (Fig. 16). After biofilm production was initiated, this pattern reversed, with treatments that received Zn at 24 hrs or sooner exhibiting higher OD than those that received it 36 hrs or later (Fig. 16). When considering these two experiments together, 0.1 mM concentration may be at a level where Zn served as a nutrient whereas at 1.0 mM Zn may be toxic for NJ1104. This concentration was not high enough to kill NJ1104 entirely or halt biofilm growth, but it does cause a slight lag in growth when NJ1104 is exposed 24 hrs or earlier. The OD of the culture medium after onset of biofilm growth was increased for those treatments after 48 hours, perhaps indicating a stress response of cells that are reverting back to the planktonic lifestyle. It is important to note, however, that these observed differences in OD were not statistically significant, and when error bars are introduced, all treatments had comparable OD throughout the time course.

To gain a more specific indication of cell growth, DNA within the biofilms was quantified. While all biofilms were comparable in size, color and three-dimensional structure, the amount of DNA contained within them varied. The most DNA was found in

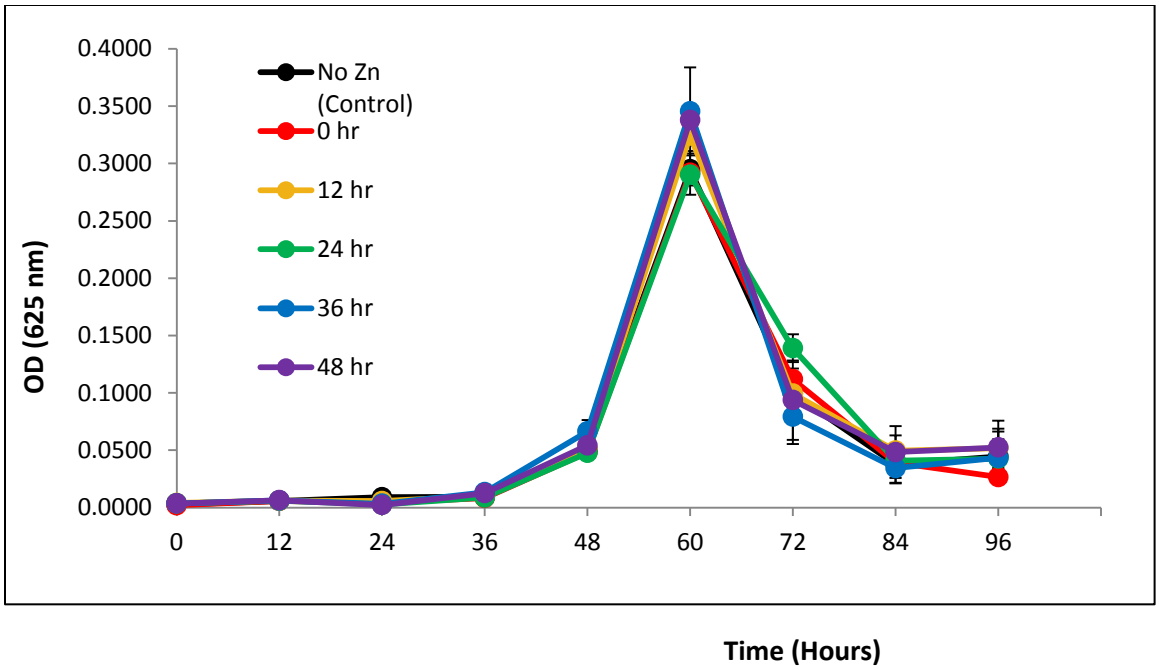

Figure 16. Biofilm Production of Isolate NJ1104 in 1 mM $ZnSO_4$-Amended Liquid AMS Agar Over Four Days (96 Hours). Comparable OD readings are seen over time for replicate flasks in each of six treatments with $ZnSO_4$ amendments as various time points**.** Onset of biofilm production was indirectly measured using optical density (OD) at 625 nm wavelength. Each point represents an average of three OD readings per treatment.

biofilms exposed to 1.0 mM Zn at the 24, 36 and 48-hr time points (Fig. 17). These treatments contained between 28 and 36 ng DNA/mg biofilm mass. The treatments with 0, 12 and 24-hr amendment times had 10-15 ng less DNA per mg biofilm mass (Fig. 17). The weights of the biofilms were comparable to each other (between 0.116 g and 0.167 g), with the exception of one 0 hr replicate and one 12 hr replicate that were noticeably lighter (0.076 g and 0.085 g, respectively) and one 48 hr replicate that was noticeably higher (0.215 g). These results suggest that biofilm growth was inhibited when the $ZnSO_4$ was added at time points of 12 hr or sooner, resulting in fewer cells and thus less DNA. Comparable biofilm weights with lower DNA yield for treatments with earlier amendment time points suggest an increase in EPS production as a defense mechanism. This may have given the biofilms a larger appearance comparable to those of later time points when in fact they contained fewer cells. Similarly, these data suggest that biofilms exposed to 1 mM $ZnSO_4$ at 24 hrs or later are more resilient to the toxic effects of the metal or are better able to use the excess Zn as a nutrient. This may be true, since the biofilm matrix protects the bacteria both physical and chemically from metal toxicity better than if they were living in a planktonic state. Other metals such as iron have been known to not only affect biofilm development, but also the amount of extracellular DNA that is released from biofilm cells, for example in *Pseudomonas aeruginosa* biofilms (Yang et al., 2007).

During the last experiment, perturbation was found to have a greater influence on biofilm production than the availability of light (Fig. 18). While NJ1104 does produce biofilms regardless of whether the flask is shaken or not, the resulting biofilms vary dramatically in color, size, adhesion strength to glass, and three-dimensional structure.

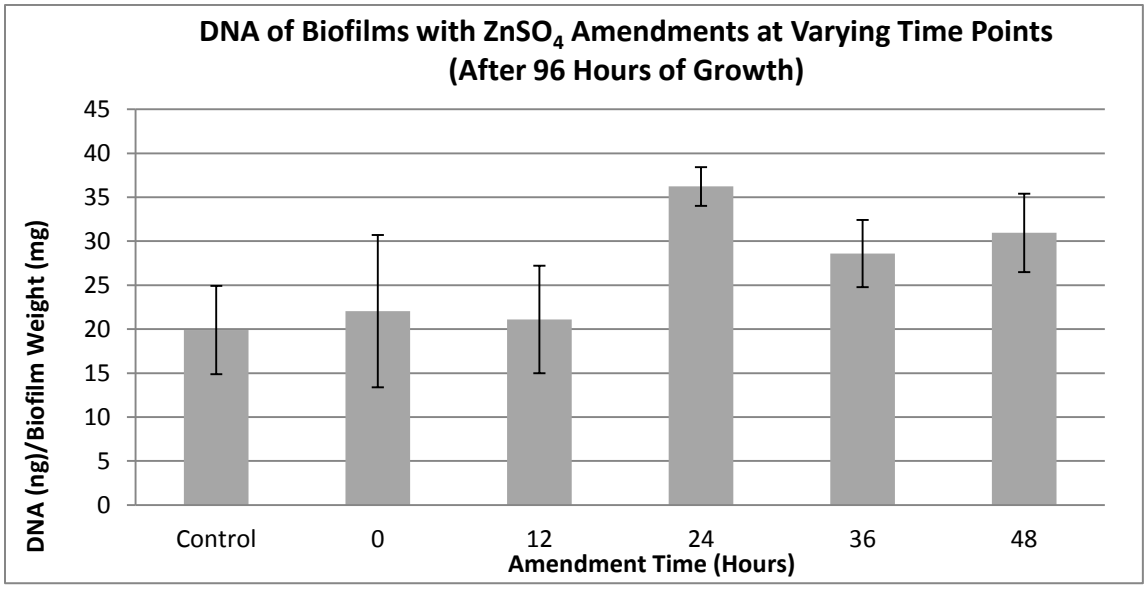

Figure 17. Amount of DNA in Biofilm Material. The data shown below are the amounts of DNA (weight in ng) per weight of biofilm material (mg) found in biofilms from treatments amended with 1 mM $ZnSO_4$ at varying time points (from Figure 5).

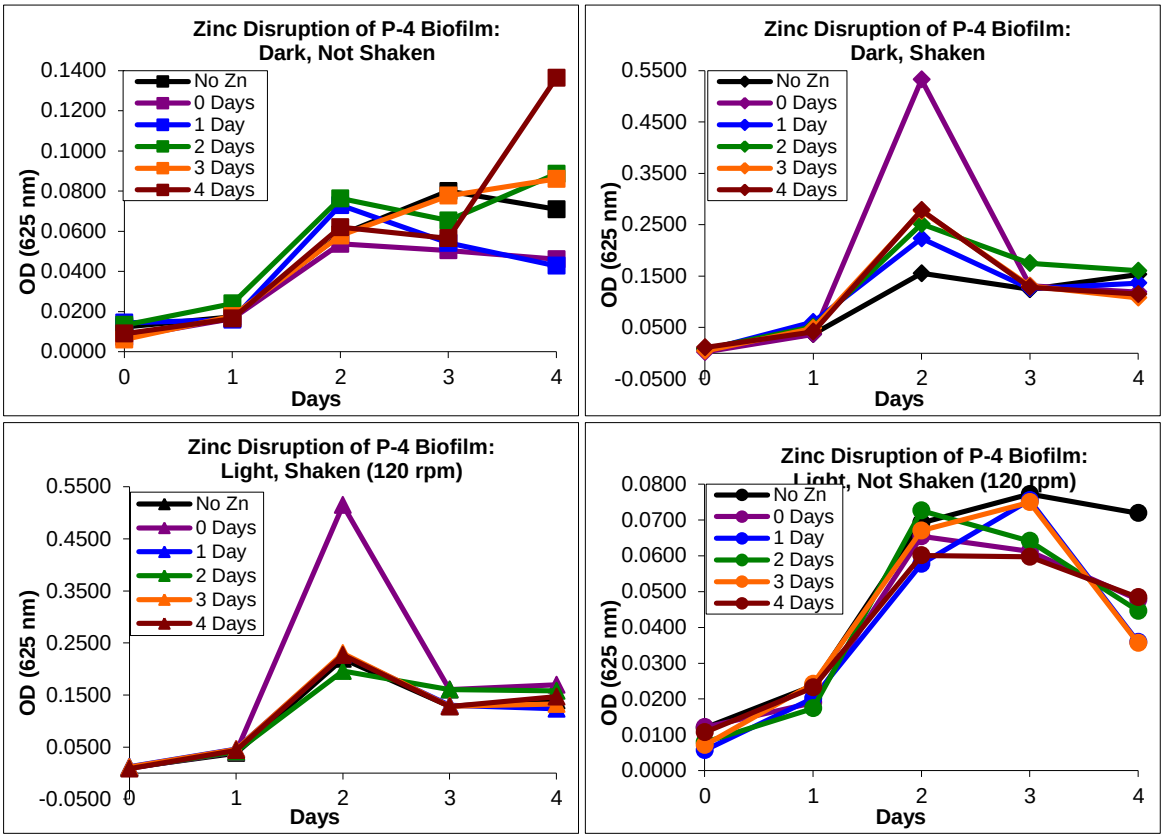

Figure 18. Biofilm Production of Isolate NJ1104 in 0.1mM $ZnSO_4$-Amended Liquid AMS Agar Over Four Days with Varying Light and Perturbation Conditions. Two leftmost graphs show cultures grown shaken at 120 rpm, while the rightmost graphs were left undisturbed. The cultures shown in the two top graphs were grown in the dark, whereas those grown in natural and laboratory lighting are at the bottom. Whether cultures were grown in light or darkness, the OD for shaken cultures followed a pattern different from undisturbed culture OD. Undisturbed biofilms were thick, almost fluffy, and very loosely attached to the glass. These biofilms were loosely attached to themselves, falling apart with perturbation. This growth was characterized by a slow increase in OD steadily throughout the experiment, with a large influx of planktonic cells entering the biofilm not being seen. In shaken flasks, thinner biofilms developed that

were strongly adhered to themselves and the glass. These biofilms had well-defined three-dimensional structure. OD over time showed an increase early on followed by a decline, indicating a switch from planktonic to sessile lifestyle.

CONCLUSIONS

Some, but not all, *Methylobacterium* species engage in biofilm production and the nature of those biofilms vary by isolate. Either PPFMs exhibit varied ability to produce biofilms, which is plausible considering that not all isolates do under laboratory conditions, or it may indicate the need for growth factors or similar materials from the soil environment that it does not receive in a laboratory setting. Colonies from multispecies cultures were not only visually identifiable as either NJ1101 or NJ1104, but the presence of "hybrid" colonies possessing some visual characteristics of both isolates were observed. This suggests communication between the two species and might hint at horizontal gene transfer events or other exchange of growth materials.

At 1.0 mM concentration, biofilm production proceeds, but there is a noticeable disruption in the number of cells (and amount of DNA) left in the biofilms at the end of the experiment. At higher concentrations, it seems as though cells revert back to planktonic lifestyle more often than at lower concentrations. This suggests that biofilm production in the rhizosphere will not be inhibited by everyday solubilization of Zn from soil by cells, but that biofilm production in Zn-contaminated soils may be inhibited by higher concentrations. If PPFMs were to be used for metal phytoremediation efforts in soil, it would be necessary to first find the upper limits of tolerance for strains in question concerning biofilm production in the presence of elevated metals. Inhibition of biofilm production may result in a decrease in PPFM-plant symbioses, thus lessening the beneficial effects of PPFM colonization in contaminated soils.

The information presented here sheds light on the capabilities of individual PPFM isolates to produce biofilms and delves into the nature and construction of those biofilms.

These data also suggest that biofilm production in PPFMs is versatile and continues even under stressful conditions, albeit proceeding in a different manner. This ability to maintain biofilm production under various stressful conditions might give insight into how PPFMs are able to colonize plants so successfully as biofilm production is a mode of phylloplane colonization for bacteria. This is only speculation, however, as environmental biofilms, both aqueous and on plant surfaces, are nearly always multispecies biofilms. It has been proposed that rhizosphere colonization by plant-associated bacteria is largely accomplished through biofilms, but research on environmental biofilms (as opposed to clinical biofilms) is limited and has only been explored in detail in recent years (Fujishige et al., 2006). More research into environmental biofilm production in rhizosphere and phyllosphere settings will continue to shed light on PPFM biofilm production and how it might proceed outside of a strictly aquatic and laboratory environment.

Isolates of the genus *Methylobacterium* have varying capabilities as far as substrate utilization, even with C_1 substrates commonly used to sustain them in laboratory settings. Isolate NJ1104 displayed a unique ability among the nine isolates to grown in 10.0 % methanol, something that has not been noted for PPFMs in the literature. Though the literature suggests that some species are capable of utilizing high concentrations of formaldehyde, it was not observed with these isolates. Sodium acetate, a common root exudate, was also not utilized by isolate NJ1104, which is surprising considering PPFM association with plants. NJ1104 did display the ability to utilize concentrations of both ethanol and methanol that were higher than the remaining eight NJ isolates. This indicates either an actual ability of NJ1104 to thrive in higher

concentrations of these alcohols or that biofilm production allows NJ1104 to protect itself against the toxic effects of elevated alcohol concentration.

The affinity of the MDH enzyme is twenty-five times higher for ethanol than for methanol (Bruice, 2001). This results in higher toxicity earlier on in biofilm development, which may at first result in higher EPS production for protective purposes. Indeed, higher EPS was observed at the two lowest ethanol concentrations. Further research is needed to elucidate whether this is indeed a stress response to the ethanol.

With the appearance of filamentous bridges and color variations of biofilms grown in stationary conditions, evidence exists for multi-functionality and communication among cells in growing PPFM biofilms. This was also evident when two isolates (NJ1101 and NJ1104) were grown together, producing biofilms that contained both organisms. The resulting plated colonies not only followed color and texture patterns for each isolate but also showed color and texture "hybrids." While these results are extremely interesting and insightful, they only scratch the surface of PPFM cellular communication and what its implications might be for colony morphology and pigmentation, functional diversification within biofilms, and communication between phenotypically disparate biofilms. More research in this area that combines these phenotypic and qualitative observations with molecular analyses would enable researchers to further understand the intricate and complex world of PPFM single and multi-species biofilms. With this knowledge, everything from how PPFMs competitively colonize plants to how to rid showers in everyday households of persistent PPFM colonization will be better understood.

The experimental data presented in this chapter further clarifies the capabilities of PPFM isolates to grow and produce biofilms when challenged with varying and potentially toxic substrates as well as elevated Zn concentrations. These data reveal that biofilm production varies between *Methylobacterium* species, with some species being able to create biofilms even in the face of substrate toxicity (elevated methanol and ethanol), low oxygen concentration (undisturbed, unshaken cultures), sheer stress (shaken cultures), and elevated metal salt concentration ($ZnSO_4$). While biofilm production varied greatly with differing culture conditions, these data attest to the versatility of substrate utilization and other tolerances that some PPFMs possess. *Methylobacterium* ability to compete successfully in a phyllosphere setting may be due in part to the adaptability of biofilm production and the flexibility in the range of utilizable substrates PPFMs possess. However, NJ1104, arguably the most versatility and resilient isolate in these studies, was not able to utilize formaldehyde or sodium acetate as growth substrates. This was contrary to the literature for formaldehyde and counterintuitive for a soil PPFM with regard to sodium acetate, and indicates the need for further research regarding the capabilities of isolates in this genus. Understanding what species can utilize which substrates or can withstand which types of stresses will be of use when determining which species best compete in the environment and have the opportunity to stimulate crop growth.

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Chapter 4

Zinc Tolerance Strategies and Solubilization of Hopeite by Soil-Derived *Methylobacterium* Species

ABSTRACT

The presence of bioavailable metal nutrients is paramount to the ability of a soil system to sustain plant, animal, and microbial life. Bacteria can participate in the cycling of metals, influencing their solubility and potentially increasing the bioavailable fraction in soil for plant uptake. This research investigates the Zinc (Zn) tolerance strategies of a plant growth-promoting genus of bacteria, *Methylobacterium*, and investigates the ability of select species to solubilize the Zn phosphate compound, hopeite. Zn is an important micronutrient for all forms of life. It is essential to the composition of DNA binding proteins and alcohol dehydrogenase enzymes, but can become toxic at elevated concentrations. Here we present our findings that *Methylobacterium* species can both solubilize and precipitate Zn salts, and can solubilize Zn phosphate, thus converting an otherwise water insoluble and solid form of Zn into a water soluble and bioavailable form. Elucidating the metal tolerance strategies and metal cycling abilities of plant-associated bacterial genera such as *Methylobacterium* will expand our understanding of how these bacteria might be useful for further agricultural and bioremediation applications.

INTRODUCTION

The genus *Methylobacterium* consists of pink-pigmented, facultatively methylotrophic bacteria, or PPFMs. They are plant growth promoters capable of utilizing methanol, a common chemical release by plants, as a sole carbon and energy source. As plant-associated bacteria native to soil as well, *Methylobacterium* spp. may have the potential to influence the chemical cycles of metal nutrients and alter their bioavailability to plants. There have been a limited number of studies regarding tolerance strategies regarding zinc (Zn) in the *Methylobacterium* genus. One study found that Zn-resistance was a characteristic of the *Methylobacterium* genus (Kunito et al., 1997), while another found fault with the methods of the other study, noting that an isolate highly similar to *M. radiotolerans* was merely tolerant if not fully susceptible to Zn (Zarnowski et al., 2002). The Zn tolerance or resistance of this genus has not been investigated further.

Although individual PPFM species are themselves not ubiquitous in soil, the genus *Methylobacterium* is. While their plant colonizing activities are vastly supported in the literature, there is some debate as to the presence of Zn resistance or even tolerance within the genus *Methylobacterium*. The presence of these plant growth-promoting bacteria has never been linked to increases in Zn solubilization or improvement in its bioavailability to plants. The effects of *Methylobacterium* growth on the Zn chemical cycle and the potential for these bacteria to increase its bioavailability to plants remain unknown.

Zn is an important micronutrient for plants, animals, and microorganisms alike. In bacteria, Zn is incorporated into Zn-finger proteins. Zinc fingers are DNA binding motifs that have protein backbones in association with Zn ions and amino acid residues (Weaver, 2002). They associate with DNA molecules by fitting into the major groove of

the helix to facilitate the binding of transcription factors to the DNA (Weaver, 2002). The Zn ions help stabilize the protein structure to ensure that it has the proper shape for fitting into the major groove of the DNA helix, and thus are extremely important for DNA transcription and maintenance (Weaver, 2002). Natural inputs of Zn into soil systems come from erosion of parent rock materials. In contaminated systems, anthropogenic sources such as industrial wastes or acid mine drainage serve as inputs into the system. At low concentrations Zn serves as a component in many enzymes and influences essential cell processes. At high concentrations, however, Zn can be toxic to microorganisms.

Zn is a transition metal with valence electrons on two of its outer shells, existing most often in the Zn^{2+} cationic state in soil. It is a reactive metal that binds to other metals and organic matter, often resulting in the formation of insoluble Zn salts. Its chemical forms, bioavailability, and mobility in soil and groundwater will vary with soil texture, pH, temperature, and moisture (Han et al., 2001). This can render bioavailable Zn scarce for plants and microorganisms, and makes contaminated soil very difficult to remediate.

Bacteria can mobilize Zn for uptake by plants. This is accomplished through the production of organic acids or chelators in the rhizosphere of the plant (Whiting et al, 2001). Symbiotic bacteria colonizing the roots of plants have an affect on Zn chemistry, increasing the bioavailable pool of Zn for use by the plant as a nutrient. In return, bacteria receive growth substrates and growth factors in the form of root exudates. The plant produces its own acids and chelators, creating an environment in the rhizosphere that contains increased heavy metal concentration compared to bulk soil. As a result, Zn tolerant bacteria can be found in the rhizosphere.

Bacteria living in association with plants, particularly in the rhizosphere, can alter the bioavailability of metal nutrients, increasing the amounts that are available to both microbes and plants (Whiting et al, 2001; Lodewyckx et al, 2002). Plants can only utilize the soluble fraction of metals as nutrients, as only the soluble forms can be chemically extracted from soil by plant root cells. Through the production of organic acids and chelating compounds, bacteria can solubilize insoluble metal salts and increase the bioavailable fraction of metals in soil. For zinc (Zn), a common metal in flux in a rhizosphere setting, solubility is greatly decreased by the formation of zinc phosphate compounds. While the effects of metals like iron on biofilm production have been investigated for other bacteria such as *Pseudomonas aeruginosa* (Yang et al., 2007), the effect of elevated Zn on biofilm production has not been looked at in as much detail and certainly not with regard to PPFMs. Zinc phosphate solubilization has been documented in *Pseudomonas fluorescens*, for example (Di Simine et al., 1998), but not yet in *Methylobacterium* species. We hypothesize that PPFMs can participate in the cycling of nutrients in a rhizosphere setting, increasing the bioavailable pool of certain metals for uptake by plants. Through biofilm production, PPFMs have a competitive advantage when colonizing plant roots and increase the potential for plant-bacteria nutrient transfer due to extensive surface contact with plant cells. To test this, we set out to observe the behavior of several PPFM isolates on solid media as well as in liquid medium. Specific objectives were:

1) to observe behaviors resulting in the alteration of Zn bioavailability, namely precipitation or solubilization;

2) to identify a white precipitate (Zn salt) being solubilized by PPFM isolates;

and 3) to investigate organic acid production as a means of solubilizing zinc phosphate.

MATERIALS AND METHODS

Isolation of Methylobacterium Species

Enrichment cultures were set up using Holmdel sandy loam soil from the Adelphia Farm in New Jersey (plot 306) and soil extract medium (SEM). A total of 10 g of soil and 200 mL of SEM medium were placed into 500 mL Erlenmeyer flasks. The flasks were shaken at 110 rpm for 24 hours. Subcultures were made by taking 20 mL of medium from each flask and placing it into new flasks containing 180 mL of SEM with 5 mM $ZnSO_4$ also added. After 24 hours, subcultures were made with SEM medium containing 10 mM $ZnSO_4$. This process was repeated once daily in 5 mM increments until the concentration of $ZnSO_4$ added to the SEM was 25 mM. The medium was then dilution-plated onto agar plates using 0.1 mL aliquots per plate. Agar for the plate was made as per ATCC #784 Medium (Ammonium Mineral Salts Medium or AMS), which contains 0.5% methanol (v/v) as the sole carbon source. The medium contained 5 mM $ZnSO_4$, resulting in cloudy agar due to the production of a white, fine-grained precipitate that forms when Zn is added to AMS medium. The agar was modified from the original recipe to include only ten percent of the original amount of K_2HPO_4. This was done to facilitate the solidification of the agar, which is hampered by the addition of the $ZnSO_4$. After 1, 2, and 3 weeks of growth in the dark at 30°C, the plates were screened for potential isolates. The reason for three screenings is to harvest the fast-growing colonies at 1 week and harvest slow-growing colonies at 2 or 3 weeks. Because colonies become

very numerous and dense at three weeks and colonies may overlap, earlier screenings ensured some isolates would be obtained even if colonies could no longer be harvested after three weeks. A total of nine pink bacterial colonies were selected and grown on unamended AMS agar plates with 0.5% methanol as the sole carbon source. Culture inoculum was obtained from AMS streak plates of each isolate.

Identification of Bacterial Isolates

DNA was extracted three times from liquid cultures of each of the nine isolates and combined during cleanup to concentrate the DNA. Fragments of the 16S rRNA gene were obtained by PCR amplification using 27F and 519R primers. Fragments of the *mxaF* gene were also obtained using 1003F and 1561R primers. The *mxaF* gene was chosen to compliment 16S rRNA species relatedness because it is a highly conserved functional gene. The resulting enzyme (methanol dehydrogenase, MDH) is related to methanol metabolism, which is the sole carbon source for these isolates. Gene fragments were sequenced and used in conjunction with the BLAST online search tool and GenBank online database to determine species relatedness and genus.

Screening of Isolates on Zn-Amended Agar to Assess Tolerance to Zn

Initially, each of the nine NJ PPFM isolates were screened on $ZnSO_4$-amended solid AMS (Ammonium Mineral Salts) agar and TSA (Trypticase Soy Agar) to observe the limits of growth in the presence of Zn. Isolates were also tested in $ZnSO_4$-amended AMS liquid medium. $ZnSO_4$ was chosen because it is a common form of Zn found in soil systems. Concentrations in liquid medium tested were 1, 5, 10, 15, 20, and 25 mM

$ZnSO_4$. Concentrations in solid AMS agar were limited to only 5 and 10 mM $ZnSO_4$, as concentrations above that precipitated too heavily to be considered. Isolates were tested on 5, 10, 15 and 20 mM concentrations on TSA plates. Concentrations above 25 mM have not been tested due to solubility constraints in the AMS minimal medium.

Each of the nine isolates was grown on $ZnSO_4$-amended AMS agar. The agar had a translucent or fine-grainy, whitish appearance, as opposed to unamended AMS agar, which is clear. Each isolate was evaluated after 3 weeks of growth for any visible signs of chemical alteration of the Zn amendment within the agar (precipitation or solubilization). Areas where the cloudiness of the agar disappeared after 3 weeks were designated as clearing zones. The width of resulting clearing zones was measured in millimeters (mm). Since the clearing effect was not uniform surrounding all colony growth, multiple measurements were taken for each bacterial isolate to obtain a diameter range.

Identification of Solid Precipitate in AMS Medium

To identify the precipitate causing the cloudiness in the Zn-Amended AMS agar, 5 g of the white, grainy solid was collected by pulling 6 L of AMS medium through a vacuum filter apparatus. This large volume of medium was necessary to obtain enough precipitate for X-Ray Diffraction (XRD) analysis. It was also necessary to homogenize and mix the precipitate. XRD analysis was performed on the solid and the diffraction pattern compared to those of known Zn salts to determine its identity.

Measurement of pH of Isolate Cultures

The pH of the $ZnSO_4$-amended AMS medium was adjusted to 6.50 before

autoclaving. After autoclaving and the addition of 0.5% methanol as a carbon source, the pH was measure once again in each separate flask prior to the addition of the inoculum. The pH of each isolate culture was then measured after ten days of growth.

RESULTS AND DISCUSSION

Identification of Bacterial Isolates

A total of nine bacterial strains were isolated from the NJ Adelphia Farm soil. The resulting 16S rRNA and *mxaF* gene fragment sequences were compared. Isolates were most closely related to species in the genus *Methylobacterium* and were given the designations: NJ1101, NJ1103, NJ1104, NJ1105, NJ1106, NJ1107, NJ1108, NJ1126 and NJ1128 (see Chapter 1, Tables 5.1 through 5.4 for BLAST identifications of *mxaF* and 16S gene sequences). Isolates N1103, NJ1105, NJ1106, NJ1107, NJ1108, NJ1126 and NJ1128 were most similar to *M. fujisawaense*, *M. oryzae* and *M. phyllosphaerae* species, with varying homologies of between 96% and 99% to sequences in the GenBank database depending upon the isolate. Isolate NJ1101 was most similar to *M. radiotolerans* species and P-4 was most similar to *M. populi* and *M. thiocyanatum* species.

Screening of Isolates on Zn-Amended Agar to Asses Tolerance to Zn

Isolate growth on AMS liquid medium and on AMS agar plates amended with $ZnSO_4$ can be found in Table 1. All isolates were able to grow on concentrations of up to 10 mM $ZnSO_4$ on AMS agar plates and up to 20 mM $ZnSO_4$ TSA plates, with the exception of NJ1106 and NJ1107 which could withstand 5 mM ZnSO4 AMS agar plates.

Table 1. The growth of nine New Jersey *Methylobacterium* soil isolates in $ZnSO_4$-amended AMS liquid medium and on amended AMS agar plates.

Isolate	Highest Concentration of $ZnSO_4$ Tolerated in AMS Liquid Medium (mM)[1]	Highest Concentration of $ZnSO_4$ Tolerated on AMS Agar (mM)[2]	Highest Concentration of $ZnSO_4$ Tolerated on TSA Agar (mM)[3]
NJ1101	5	10	20
NJ1103	5	10	20
NJ1104	10	10	20
NJ1105	5	10	20
NJ1106	5	5	ND*
NJ1107	5	5	ND
NJ1108	20	10	20
NJ1126	5	10	20
NJ1128	20	10	20

*ND = Growth not detected

[1] Maximum $ZnSO_4$ tested in Ammonium Mineral Salts (AMS) medium = 25 mM

[2] Maximum $ZnSO_4$ tested on AMS agar = 10 mM

[3]Maximum $ZnSO_4$ tested on 1% Trypticase Soy Agar (TSA) = 20 mM

NJ1106 and NJ1107 did not grow on TSA plates and therefore concentrations higher than 10 mM could not be tested. In liquid medium, most isolates grew in 10 mM $ZnSO_4$, but NJ1106 and NJ1107 again were limited to growth in only 5 mM $ZnSO_4$. Thus, NJ1106 and NJ1107 appear to not only have different growth requirements than the other seven isolates, but they are also not Zn tolerant to the same degree.

The differences in thresholds of Zn tolerance between the agar plates and the liquid medium may indicate a reduction of apparent metal concentration in the plates due to it becoming bound to the agar. The presence of a haze or cloudiness in the AMS agar plates confirms the presence of a precipitate and suggests a lowering of the soluble Zn concentration. Thus, while it may appear as if the isolates could withstand a higher concentration on the agar plates than in liquid medium, this may only be an artifact of the Zn precipitating out of solution or becoming bound to the agar matrix. In addition, TSA contains more components for Zn to bind to (peptone, etc.), and so the perceived higher Zn resistance on TSA as compared to AMS may only be due to the binding of Zn to these medium components.

The presence of a precipitate in the liquid AMS medium also indicates a lowering of Zn solubility. AMS medium contains both monobasic and dibasic potassium phosphate (K_2HPO_4 and KH_2PO_4, respectively) (see Table 4 in Chapter 1). In order to allow for the solubilization of the $ZnSO_4$ amendment into AMS liquid medium or agar, the concentration of K_2HPO_4 must be reduced to 1/10 of the required amount. This alteration of these components does not appear to drastically effect the growth of the PPFM isolates, but it does serve to ensure that the precipitate does not occur in AMS medium when the effective concentration of the Zn is of concern.

Even though the effective Zn concentrations in both the liquid and solid mediums may be lower than desired due to the binding of the Zn to medium components or the reduction of Zn solubility, the fact that the isolate growth thresholds in Zn were lower in the liquid AMS medium suggests that as the better medium for testing isolate Zn tolerance. The concentrations in AMS liquid cultures were most likely closest to the intended amounts and therefore gave the most accurate measurement of the isolates' Zn threshold concentrations due to it being a minimal medium and having no agar or other complex constituents to bind to the Zn.

These concentrations, even if they may have been lower than intended, were still influential as far as bacterial resistance is concerned. Total Zn concentrations of 1 mM and 5 mM in AMS liquid medium did not inhibit any of the nine isolates and in some cases (NJ1101 and NJ1104) these amendments resulted in increased growth. Yet, during the isolation of these isolates from soil, a large reduction of total soil cultivable bacterial counts was observed when comparing unamended agar plates to 1 and 5 mM $ZnSO_4$-amended agar plates. Unamended agar plates yielded bacteria in numbers greater than 10^7 CFU/mL, compared with 5 mM amended plates which only produced counts of 10^2 and 10^3 CFU/g. This can indicate that the nine PPFM isolates are Zn tolerant relevant to the overall soil bacterial community.

As shown in Table 2, two of the nine isolates (NJ1101 and NJ1104) formed neither clearing zones nor precipitates on 5 mM $ZnSO_4$-amended AMS agar after 3 weeks. Growth of other isolates (NJ1106 and NJ1107) resulted in the production of a white, heterogeneous halo near colony growth that consisted of white, fine, fibrous crystals embedded in the agar, indicative of a precipitate being formed.

Table 2. Characterization of nine New Jersey *Methylobacterium* soil isolates on $ZnSO_4$-amended AMS agar plates. Isolate behavior regarding Zn was observed, with three distinct behaviors resulting from growth on Zn-amended agar: growth with no visible change in the agar; growth accompanied by the formation of a white precipitate within the agar; and growth accompanied by the appearance of clearing zones in the agar. Only two isolates were found to form a precipitate, while the majority of isolates were capable of forming clearing zones. NJ1101 and NJ1104 were the only isolates to exhibit tolerant growth on Zn-amended medium without the production of clearing zones or a precipitate.

Isolate Name	Precipitation of Unknown Zn Salt	Solubilization of Zn Phosphate (Hopeite)
NJ1101	-†	-
NJ1103	-	+*
NJ1104	-	-
NJ1105	-	++**
NJ1106	+	-
NJ1107	+	-
NJ1108	-	+
NJ1126	-	+
NJ1128	-	++

†No effect observed.

*Slight effect; halo or clearing zone with thickness < 3 mm observed.

**Moderate effect; halo or clearing zone with thickness > 3 mm observed.

Five of the isolates (NJ1103, NJ1105, NJ1108, NJ1126 and NJ1128) produced clearing

zones in the agar, indicative of the solubilization of the fine precipitate that originally caused the agar to become cloudy during creation. Isolates NJ1101, NJ1104, NJ1106 and NJ1107 never produced clearing zones, even after multiple transfers onto fresh, Zn-amended AMS agar. These results show that while all nine soil isolates were able to grow in 5 to10 mM $ZnSO_4$-amended AMS medium during isolation and culturing in the lab and on 5 mM-amended AMS agar, the behavior exhibited varied between them. This reveals that *Methylobacterium* species are capable of both precipitation (NJ1106 and NJ1107) and solubilization (NJ1103, NJ1105, NJ1108, NJ1126 and NJ1128) of Zn salts, but do not require either activity to be sustained on amended agar (NJ1101 and NJ1104). These data suggest that multiple tolerance mechanisms are employed by *Methylobacterium* species in the presence of elevated zinc.

The diameters of the clearing zones observed around streaks and colonies of five of the nine isolates (NJ1103, NJ1105, NJ1108, NJ1126 and NJ1128) ranged from 1 to 5 mm in width (Figs. 1 & 2). The size and occurrence of visible clearing zones appeared to be directly related to the number of bacterial cells, as the widest clearing zones surrounded areas of thickest bacterial growth. Since all isolates were streak-plated for this analysis, both bacterial growth and clearing zone width was thickest around the initial streaks and thinnest around single colonies.

Identification of the Medium Precipitate

XRD analysis was performed to identify the white precipitate formed when $ZnSO_4$ is added to AMS medium. The diffraction pattern of the unidentified solid was

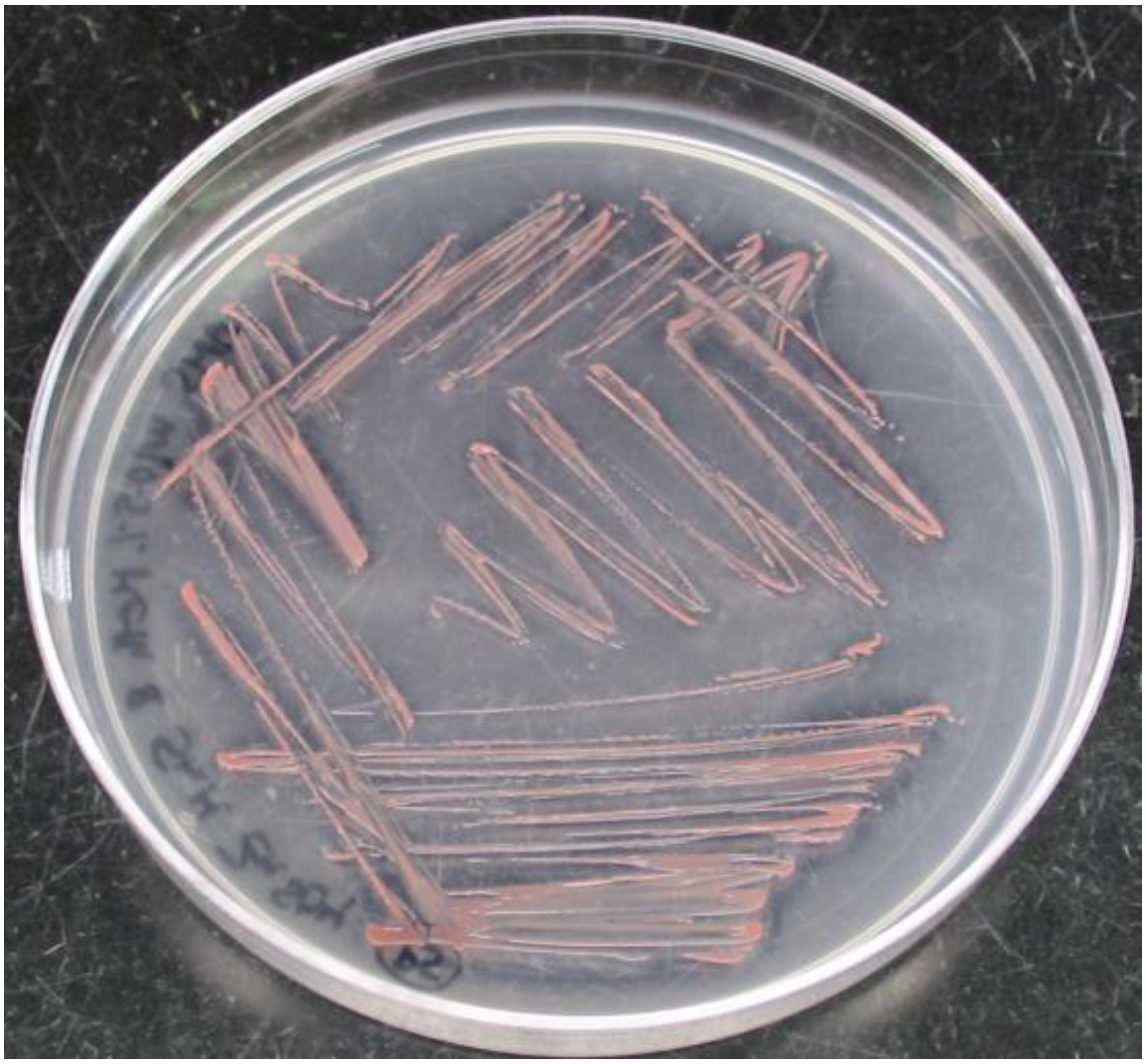

Figure 1. Isolate NJ1105 Growing on AMS with 0.5 % Methanol and 5 mM $ZnSO_4$. The agar becomes cloudy due to a white precipitate, formed when the excess $ZnSO_4$ reacts with the phosphates contained in the AMS medium. Note the whitish, cloudy color of the agar compared to the black counter top. The dark halo around areas of the most NJ1105 growth is actually the counter top showing through where the bacteria have formed clearing zones in the agar.

Figure 2. Detailed Close-up of Isolate NJ1128 Growing on AMS with 0.5 % Methanol and 5 mM $ZnSO_4$. This photo shows clearing zones made around areas of thickest growth of isolate NJ1128, a strong producer of clearing zones compared to other isolates. As in Figure 1, note that the thickest and most pronounced clearing zones are located around areas of thickest isolate growth.

compared to those of various Zn salts to identify the compound. The pattern matched that of hopeite, a zinc phosphate hydrate compound [$Zn_3(PO_4)_2 \cdot 4H_2O$] (Figs. 3 & 4). Of the 77 peaks recognized in the diffraction pattern for the unknown precipitate, 67 were successfully matched with those of hopeite. All remaining peaks that could not be identified were recorded as being below 8.00 % relative intensity, and all but two of those peaks were below 4.00 % relative intensity. These minor discrepancies in background peaks can be attributed to impurities in the form of other media components collected along with the precipitate and present during the analysis and can be ignored for the purposes of this analysis.

The production of clearing zones in AMS agar made hazy by a hopeite, zinc phosphate precipitate, confirms the ability of *Methylobacterium* species to solubilize Zn phosphate compounds. These are compounds that would otherwise be part of the insoluble fraction in soil and would therefore not often be bioavailable to plants. Because of the close relationship between *Methylobacterium* species and plants, the ability to solubilize otherwise insoluble Zn phosphate may suggest a role for these bacteria in the chemical cycling of Zn in soil, but also that the chemical forms of Zn that arise from interactions with PPFMs may lead to an increase in the bioavailability of this nutrient to plants.

Measurement of pH of Isolate Cultures

Bacteria have been noted in the literature for producing organic acids that can solubilize insoluble metal salts, especially in rhizosphere communities (Whiting et al., 2001; Carlot et al., 2002) To determine a mechanism by which the *Methylobacterium*

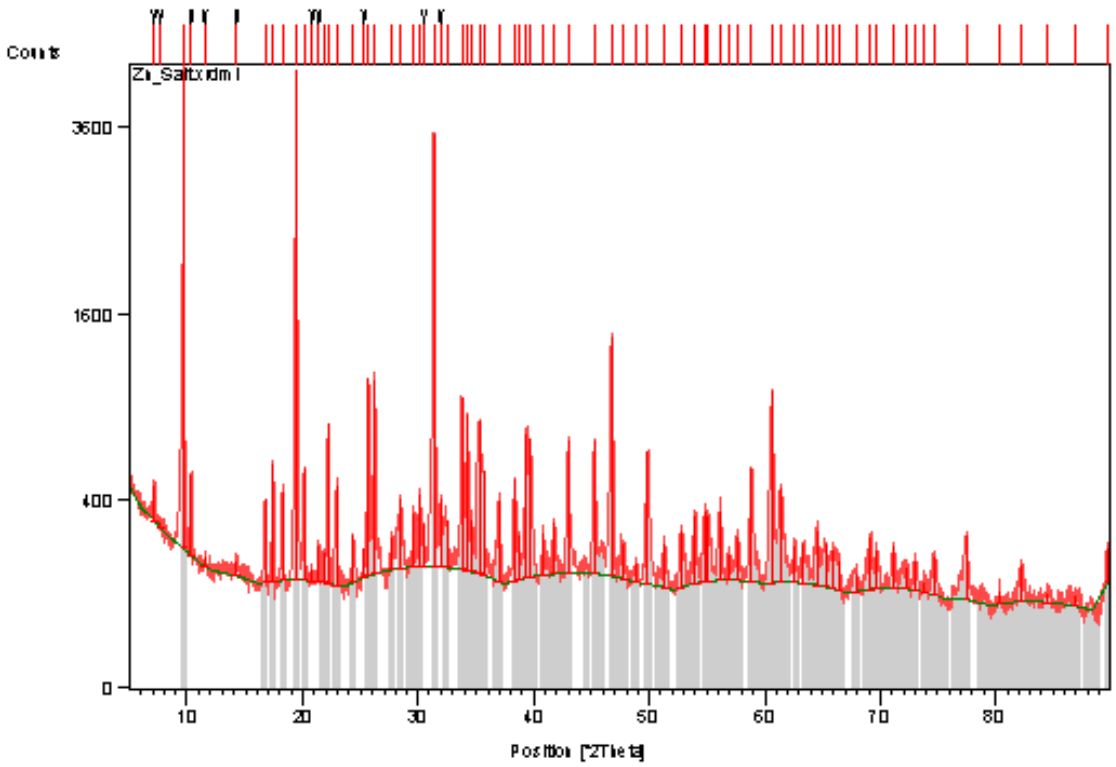

Figure 3. The Standard X-Ray Diffraction Pattern of the Zn Phosphate Hydrate Compound, Hopeite.

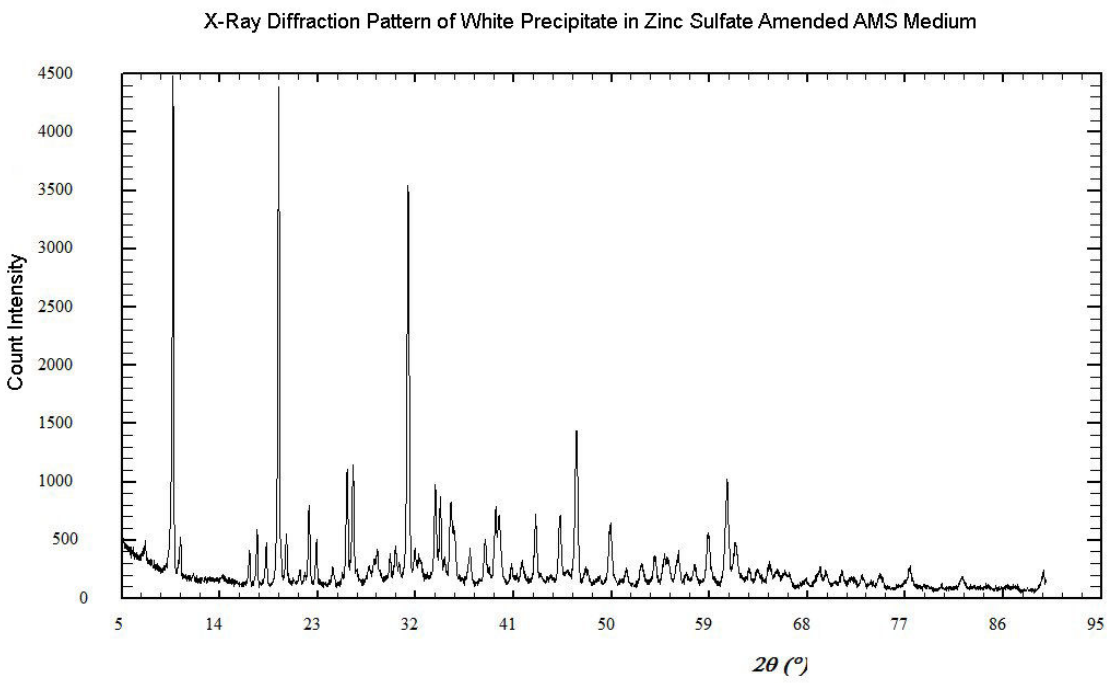

Figure 4. The X-Ray Diffraction Pattern of the Unidentified Precipitate. When compared with Figure 3, the diffraction pattern of the unidentified precipitate is very similar to that of hopeite (zinc phosphate hydrate) and was therefore identified as such. Of the 77 peaks recognized in this diffraction pattern, 67 were matched with those of hopeite. All of the peaks that could not be identified were below 8.00 % relative intensity, and all but two of those peaks were below 4.00 % relative intensity. Discrepancies in these minor background peaks can be attributed to impurities obtained during collection of the precipitate and are due to other media components being present along with the precipitate during the analysis.

isolates solubilize hopeite, the pH of active cultures was measured after ten days of growth. All isolates acidified the culture medium (Fig. 5). The acidification rate, however, and the final degree of acidification varied significantly by isolate, especially early in the incubation period. Isolates NJ1103, NJ1105, and NJ1128 were slower to acidify the medium by two days, but brought about a lower pH than other isolates (NJ1106 and NJ1107) that were faster in acidifying the medium. No pattern observed between medium acidification and the formation of clearing zones, although it is worthy of note that the only two isolates found to precipitate Zn were also responsible for the two highest resulting pH values after 11 days. This combined with a lower tolerance of those isolates for Zn seems to suggest that they may be exhibiting an avoidance strategy compared to other isolates.

While some of the isolates found to solubilize hopeite did in fact acidify the culture medium (NJ1103, NJ1126 and NJ1128), the pH also dropped during the growth of another isolate, NJ1104, that neither solubilizes or precipitates Zn on solid media. Conversely, other isolates found to solubilize hopeite (NJ1105 and NJ1108) did not acidify the culture medium. Therefore, no correlation was seen between the ability to solubilize hopeite and a drop in the pH of the culture medium for any isolate. This analysis suggests that while organic acid production may be a mechanism for some species, it may be that *Methylobacterium* species employ other methods not investigated here, such as the use of chelating compounds.

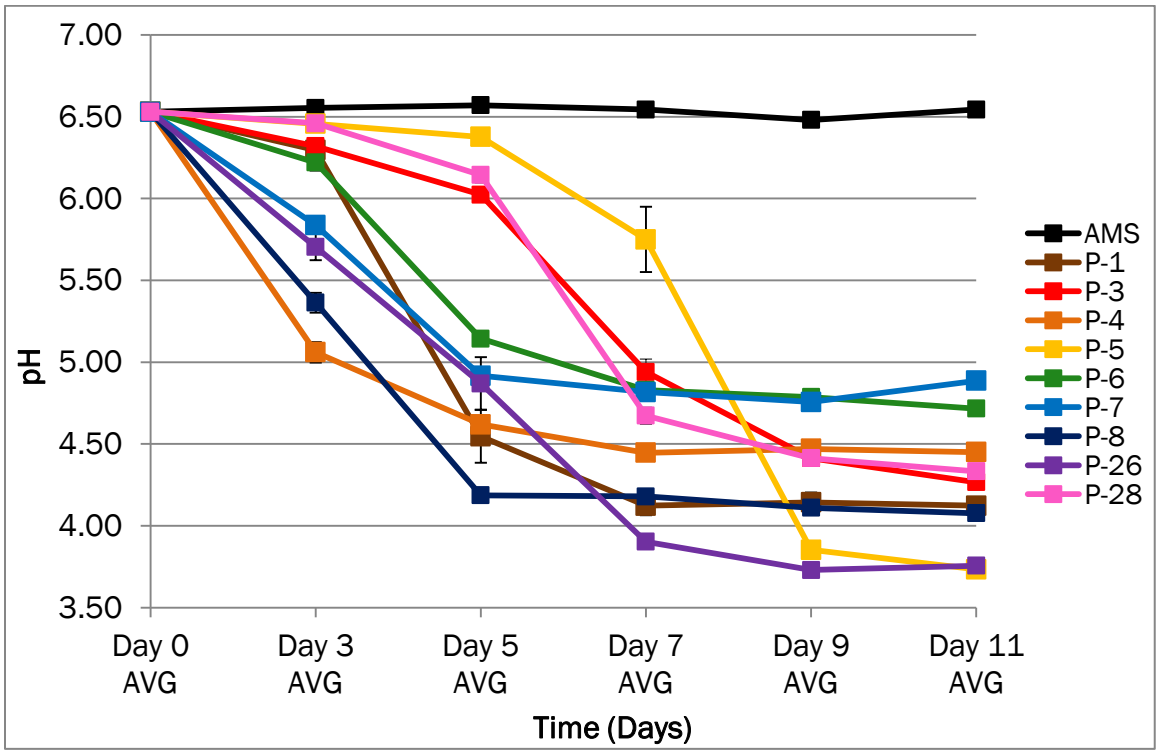

Figure 5. The pH of isolate liquid culture in AMS medium over time. While all isolates eventually acidified the culture medium, they did so to varying degrees and experienced a significantly different rate of acidification early on. Isolates NJ1103, NJ1105, and NJ1128 were slower to acidify the medium early in the experiment, but brought about a lower pH than other isolates (NJ1106 and NJ1107) that were faster in acidifying the medium. Because all isolates acidified their medium, there was no pattern observed between that and the formation of clearing zones, although the two isolates found to precipitate Zn (NJ1106 and NJ1107) were also responsible for the two highest resulting pH values after 11 days. This combined with a lower tolerance of those isolates for Zn seems to suggest that they may be exhibiting an avoidance strategy as compared to other isolates.

CONCLUSIONS

Methylobacterium species vary in their ability to alter Zn chemistry in soil. Some solubilize Zn salts, some precipitate them, and others do neither. Those that solubilize Zn have the ability to increase the bioavailable pool of Zn to plants in soil, increasing the amount that plants can take up for nutrient purposes and during phytoextraction in contaminated soils. Some species were found to solubilize Zn phosphate, specifically hopeite, via a mechanism which may include but is not limited to organic acid production. While the exact mechanism remains unknown, acidification of the culture medium did not always coincide with the presence of clearing zones, hinting at multiple mechanisms by which these species solubilize Zn phosphate compounds. It may be possible that some *Methylobacterium* species do solubilize Zn salts through the production of organic acids, but if that is true, it is not so for all species. Isolates that solubilized Zn phosphate without acid production may be employing other chemical chelators. The clearing zones produced in solid medium indicate the diffusion of the solubilizing agent into the medium from the sites of PPFM growth. The fact that clearing zones are thicker and more translucent around areas of denser PPFM growth might indicate that the production of solubilizing agents is density dependent and induced when more cells are present, or it could simply be that the effect is more noticeable when higher numbers of cells are present. It is clear from these results that *Methylobacterium* have multiple Zn tolerance level and therefore varying tolerance strategies.

When considered in conjunction with the ubiquity of these bacteria in soil environment, the ability to produce biofilms in the presence of elevated Zn, the tendency towards plant colonization and their plant growth-promoting abilities, the solubilization

of an otherwise immobile pool of Zn in soil has far-reaching implications for plant health and the remediation of Zn-contaminated soil. Present naturally around the roots of plants in uncontaminated soil, *Methylobacterium* species may be able to increase the bioavailable fraction of Zn in soil, placing this nutrient in a form conducive to uptake by plants. In Zn-contaminated soil, these bacteria could potentially be seen as facilitators of Zn phytoextraction, increasing not only the uptake rate of Zn but also the storage capacity of the plant due to Zn mobilization. From an agricultural perspective, PPFMs could be of interest in improving nutrient uptake in crop plants.

The mechanisms by which *Methylobacterium* species alter Zn chemistry in soil as well as aspects of potential Zn tolerance mechanisms, is therefore certainly an area that deserves more attention in the future. Understanding not only what *Methylobacterium* species are capable of regarding Zn mobilization in soil but also the mechanisms involved will provide valuable insight into the relationship between these bacteria and plants in their shared soil environment.

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Chapter 5

Zn Tolerance and Ability of *Methylobacterium* spp. to Promote the Growth of Red Clover (*Trifolium pretense* L.) Seedlings Varies by Species and Proximity to Plant

ABSTRACT

Methylobacterium species have the potential for use in agricultural and bioremediation applications such as the improvement of crop nutrition and yield and the remediation of Zn-contaminated soils. Before the potential of this genus can be realized and practically applied to help solve these problems, it is important to understand the limitations of PPFM plant growth promotion (PGP). PGP is supposedly widespread in the genus, as plant colonization and the metabolism of methanol on plant surfaces are common ways of life for environmental PPFMs. This research examines not only the extent to which soil-derived PPFM isolates can improve the germination, yield, and disease susceptibility of red clover (*Trifolium pratense*) seeds and seedlings, but also explores the varying PGP capabilities of PPFM species. While some isolates were found to lower germination rates and increase the instance of common nutrient deficiencies and fungal diseases, other isolates improved upon these aspects. These results stress the importance of elucidating the individual characteristics of PPFM strains so as to avoid the use of ones that hinder plant growth and learn to stimulate those that promote plant growth.

INTRODUCTION

Methylobacterium species are known plant colonizers that specialize in but are not limited to the utilization of C_1 compounds. They are known as pink-pigmented, facultatively methylotrophic (PPFM) bacteria. PPFMs have been isolated from more than 70 species of plants, including *Arabidopsis*, tobacco, barley, clover, rice, wheat, *Lotus*, soybean, Scotch pine, and maize (Holland and Polacco, 1992; Lidstrom and Chistoserdova, 2002; Romanovskaya et al., 2001). In a phyllosphere or rhizosphere environment, *Methylobacterium* species have an advantage over other potential bacterial and fungal colonizers in that they have the ability to metabolize methanol, a plant-derived and readily available growth substrate in those environments. The PPFMs' ability to utilize methanol allows them to take advantage of the methanol that is emitted from plant leaves, while their facultative nature helps them to be competitive with other phyllosphere microorganisms when methanol concentrations on the leaves fluctuate seasonally or in relation to plant health (Sy et al., 2005; Madhaiyan, et al., 2006a).

Well-adapted to life on plant surfaces, both above-ground and rhizosphere *Methylobacterium* species can positively affect the growth and health of plants, making them of interest in the agricultural industry. Symbiotic associations between plants and PPFMs have been shown to improve the germination rate, growth, flavor, and systemic immunity of certain plant species through bacterial production of vitamins, phytohormones, and caroteinoids (Zabetakis, 1997; Lidstrom and Chistoserdova, 2002; Madhaiyan, et al., 2004; Madhaiyan, et al., 2005; Madhaiyan, et al., 2006b; Abanda-Nkpwatt et al., 2006; Hornschuh et al., 2006). Because they occupy specialized physical and metabolic niches in the environment, PPFMs fill functional niches that may be exploited to increase crop yields, protect against insect and microbial plant pathogens,

and perhaps even for bioremediation purposes.

Before the environmental role and potential of this genus can be assessed and these beneficial properties improved upon for agricultural purposes, studies are needed to determine how widespread plant growth-promotion is in the genus and what the nature of those benefits might be.

The objectives of this section of research were as follows:

1) to observe the development of a native PPFM population in a NJ soil when planted with red clover (*Trifolium pratense* L.);

2) to observe the impact of red clover seedling presence or absence on PPFM zinc (Zn) tolerance in a $ZnSO_4$-amended NJ soil; and

3) to compare PPFM isolates from a NJ soil for their plant growth-promotion abilities when colonizing red clover.

MATERIALS AND METHODS

Observation of Native Soil PPFM Populations in Presence of Red Clover Seedlings

For the completion of the first objective, soil was collected from the Horticulture Farm II on Ryders Lane in New Brunswick, NJ. (See Chapter 1, Tables 1, 2 and 3 for additional information about this soil source, designated as site NJ-2.) The soil was taken from a collection pile (soil had been moved from another location on the farm to where the pile was located) that had been left standing for three years. An established plant community consisting of grasses including perennial ryegrass (*Lolium perenne*), moonflower (*Ipomoea alba*) and wild (ox-eye) daisy (*Leucanthemum vulgare*) had overtaken the pile. Surface soil was collected, taken only from the first 10 cm with the

goal of collecting rhizosphere bacterial species. Only the main bodies of the plants, leaves and large roots were removed on site. Smaller debris such as thin roots was not removed prior to sieving at the laboratory to ensure that much the rhizosphere community was being collected. Soil was sieved through a 2 mm mesh size sieve and mixed before placing into 4-in. diameter pots.

Red clover (*Trifolium pratense*) seeds were planted singly in the center of the pots and allowed to grow for one week. Red clover was chosen because it is an agriculturally relevant plant due to its use as a fallow field cover during crop rotations. Also, it is a fast-growing plant that *Methylobacterium* species avidly colonize. One gram of rhizosphere soil and 1 g of non-rhizosphere soil were collected from ten pots for a total of ten rhizosphere and ten unplanted samples each. Rhizosphere soil was defined as soil within 1 cm of the red clover roots and was collected by loosening the soil with a sterilized metal scoopula, removing the seedlings and shaking the roots into a test tube for ease of collection and weighing. Non-rhizosphere soil was defined as soil located greater than 1 cm distance from any root and was collected with a sterilized metal scoopula.

Each sample of rhizosphere or non-rhizosphere soil was immersed in 9 mL of 15.76 g/L BisTris buffer (pH = 6.5) and shaken in test tubes for ten minutes. Samples were allowed to settle for 1 minute to reduce large particulates in the supernatant and floating plant debris was skimmed off. Samples were not centrifuged, however, as this might have caused a reduction in PPFM numbers due to the settling out of bacteria attached to fine particulate material. Methanol was used not only as a carbon source for growing PPFM colonies, but also to verify that colonies were *Methylobacterium* spp. and rule out other pink-pigmented, non-methylotrophic soil natives such as *Serratia* and

Roseomonas species. PPFM colonies were counted after two weeks in order to compare the number of *Methylobacterium* colony-forming units (CFU) in the rhizosphere and non-rhizosphere samples.

Impact of Seedling Presence on PPFM Zn Tolerance in Soil

From the above experiment in section A, 47 colonies were cultivated further in order to observe their tolerance to Zn, including 23 non-rhizosphere and 24 rhizosphere PPFMs. Isolates were sustained as pure cultures on Ammonium Minerals Salts (AMS) agar plates with 0.5 % methanol as a carbon source. (See Chapter 1, Table 4 for AMS components.) Agar was amended with 5 and 10 mM $ZnSO_4$ to test for Zn tolerance. After two weeks, presence or absence of growth on these two concentrations was recorded for each of the 47 isolates and percentages were obtained.

Growth Promotion of Red Clover Seeds and Seedlings by PPFMs

A series of bench top experiments were conducted in response to numerous claims in the literature that plant growth-promotion was a ubiquitous trait within the genus *Methylobacterium*. These experiments were conducted to assess the ability of PPFM isolates to promote seed germination, seedling growth and innate systemic resistance to microbial plant pathogens in red clover.

Determining Effects of Seed Sterilization and PPFM Inoculation on Seed Germination and Seedling Growth

Soaking the seeds in PPFM culture was used as the mode of PPFM innoculation because literature suggests that seed-inoculation is the major method of both above- and below-ground plant colonization (Romanovskaya *et al*., 2001). PPFMs associate with the seed coats of plants in soil and, as the plant grows, they colonize the roots and follow the plant as it pushes up out of the soil, resulting in stem and leaf colonization (Romanovskaya *et al*., 2001).

In a preliminary experiment, NJ1104 inoculation and the seed sterilization methods were investigated as variables. NJ1104 was selected for its biofilm production, a major mode of plant colonization (Morris and Monier, 2003). Both sterilization and seed inoculation can be stressful for the seeds, and therefore it was important to determine if one or both procedures had a negative impact on germination or fresh weight of the seedlings. Four treatments consisting of 100 seeds (10 seeds per petri dish) were implemented: non-inoculated and non-sterilized; non-inoculated and sterilized; inoculated and non-sterilized; and inoculated and sterilized seeds. Percent germination and average seedling fresh weight was determined for each treatment.

Effect of NJ1104 colonization and $ZnSO_4$ Amendment on red Clover Growth

NJ1104 was tested for its ability to improve red clover germination and growth in $ZnSO_4$-amended conditions. NJ1104 was chosen because it had extensively colonized red clover seedling during preliminary experiments. It was chosen over isolates that highly acidify their growth medium (e.g. NJ1128) so acidification would not be an added cause of mortality for the red clover. Red clover seeds were grown on Petri dishes containing filter paper (9.0 cm diameter Whatman 40 paper) moistened with either sterile distilled

(DI) water or water containing either 2.5 or 5 mM dissolved $ZnSO_4$. Red clover seeds were surface-sterilized with 70 % ethanol and 10% bleach treatments prior to inoculation. Red clover seeds were inoculated with isolate cultures after sterilization and washing with sterile DI water. The Petri dishes were sealed with Parafilm to prevent desiccation. Seedlings were grown for one week including seed germination time before data was recorded. Germination, seedling fresh weight and the occurrence of growth ailments (stunted growth, branched roots) and fungal disease (root rot caused by *Fusarium oxysporum*) were recorded. Multivariate analysis of variance (MANOVA) was used to determine whether the effects of both NJ1104 inoculation and $ZnSO_4$ amendment on red clover growth were significant.

Effects of Four PPFM Isolates (NJ1103, NJ1107, NJ1108 and NJ1126) on Red Clover Growth

The ability of four isolates (NJ1103, NJ1107, NJ1108 and NJ1126) to promote the growth of red clover seeds and seedlings was investigated. Seeds were sterilized as before and 10 seeds per petri dish (total 100 per treatment) were inoculated with one of the four cultures or with no culture for the control treatment. Data regarding seed germination and seedling fresh weight as well as the instance of nutrient deficiencies and disease were collected.

The occurrence of three types of red clover diseases and indications of poor growth were also used to determine whether the PPFM isolates had the ability to reduce disease. The three ailments recorded (see photos in Figure 1) were as follows:

Figure 1. Examples of normal, diseased, and nutrient-deficient red clover seedlings. **A.)** A normal, 3 week-old red clover seedling displaying a single tap root. Some mycorrhizal associations are visible near the bend in the root, but largely the root is smooth. **B.)** A seedling exhibiting the branched root deformity, in this case resulting in bifurcation into two roots and then a second bifurcation of one of those branches into two more roots. Curling of the root is also apparent at the site of the first bifurcation, as is the stunting of the overall seedling height. For data purposes, this seedling was counted as

being both stunted and having the branched root deformity. **C.)** A seedling afflicted with root rot, a disease caused by the fungal pathogen *Fusarium oxysporum*. Of note is the short termination of the root as well as the brown discoloration where the fungus has pinched and rotted the root. **D.)** A seedling exhibiting both the root rot disease and the branched root deformity. For data purposes, such seedlings were counted as having both ailments.

- *Stunting*: Seedlings with short roots and stems were recorded as stunted. These seedlings' leaves hung very close to the soil and the overall size of the seedling was, on average, about one-third of normal height. Red clover possesses single, long tap roots usually devoid of root hairs. Short roots that terminate just beneath the soil surface were considered stunted.
- *Root-Rot*: This is a disease caused by the fungal pathogen *Fusarium oxysporum*. It results in the pinching and eventual rotting away of roots very close to the soil surface. Roots afflicted by this pathogen are characterized by short termination with a brown discoloration at their tip where the rot has occurred.
- *Branched Roots*: Because seedlings typically have one long tap root devoid of root hairs, presence of extensive mycorrhizal colonization (root hair-like extensions) or a branching of the root into two or three shorter roots is recorded as a developmental deformity. In nutrient-deficient or ailing seedlings, the roots become forked and mychorrhizal fungi colonize in an attempt to acquire more nutrients for the plant as well as the rhizosphere microbial community. Mycorrhizal associations in this case most likely resulted from endophytic fungi present during seed sterilization that were not able to be removed from the seeds via these sterilization methods.

Statistical significance was determined using MANOVA. Data was reported either in average mg for seedling fresh weight or as percentages for germination and disease instance.

RESULTS AND DISCUSSION

Observation of Native Soil PPFM Populations in Presence of Red Clover Seedlings

The results of the observation of native rhizosphere and non-rhizosphere PPFMs can be found in Table 1. PPFM CFUs were ten-fold higher in rhizosphere soil, indicating that PPFMs proliferated to higher degrees around the roots of this host plant than in the surrounding, non-rhizosphere soil. This shows that the presence of red clover does influence PPFM growth and results in an increase in PPFM population in the vicinity of the roots. This is most likely due not only to plant-derived methanol supporting PPFM growth in rhizosphere soil but also to root exudates and other materials being released by the plant.

Impact of Seedling Presence or Absence on PPFM Zn tolerance in Soil

Table 2 shows the percentages of rhizosphere and non-rhizosphere PPFM isolates obtained from the data in Table 1 that were capable of growing on 5 and 10 mM $ZnSO_4$-amended AMS agar plates. The percentage of isolates capable of living on 5 mM and 10 mM $ZnSO_4$ was identical regardless of isolate origin. However, there was a nearly five-fold decrease in the ability of non-rhizosphere isolates to survive 10 mM $ZnSO_4$. This suggests that the rhizosphere soil environment allows for the selection of Zn-tolerant PPFMs. For processes like phytoremediation, for which PPFMs may be exploitable, the ability of these species to adapt to their environment is crucial. If rhizsophere environments select for metal-tolerant PPFMs in uncontaminated soils, it could be theorized that species highly resistant to metals like Zn could emerge and be sustained within the plant rhizosphere in contaminated soil settings, provided that the plants are able to survive higher concentrations of Zn around their roots. This symbiosis may be

Table 1. PPFM CFU per gram of soil from rhizosphere and non-rhizosphere soil. CFU per gram of both rhizosphere soil (within 1 cm of red clover roots) and non-rhizosphere soil (>1 cm from red clover roots) is shown. There was a ten-fold higher amount of PPFMs detected in rhizosphere soil than in non-rhizosphere soil, indicating a stimulation of the native PPFM population by the host red clover plants.

Red Clover Seedling	PPFM CFU/g from Rhizosphere Soil	PPFM CFU/g from Non-Rhizosphere Soil
1	4.8×10^3	2.0×10^2
2	7.3×10^3	5.0×10^2
3	8.7×10^3	1.0×10^2
4	8.3×10^3	2.0×10^2
5	6.1×10^3	2.0×10^2
6	7.5×10^3	ND*
7	6.4×10^3	4.0×10^2
8	5.1×10^3	7.0×10^2
9	5.9×10^3	2.0×10^2
10	5.9×10^3	4.0×10^2
AVERAGE	**6.6×10^3**	**2.9×10^2**

* ND = Not detected.

Table 2. Percentage growth of PPFMs on $ZnSO_4$-amended AMS agar plates. Agar used to make these plates was amended to concentrations of 5 mM and 10 mM $ZnSO_4$. The same percentage of isolates was capable of living on 5 mM and 10 mM $ZnSO_4$ regardless of whether they originated from rhizosphere or non-rhisosphere soil. There was an almost five-fold decrease in the ability of non-rhizosphere isolates to grow successfully on 10 mM $ZnSO_4$. These data indicate that rhizosphere soil environment allowed for the selection of Zn-tolerant PPFMs. have a much higher tolerance and storage capacity with regard to Zn than other plant species.

Soil Type	Growth on 5 mM $ZnSO_4$ AMS Agar	Growth on 10 mM $ZnSO_4$ AMS Agar
Non-Rhizosphere	19 of 23 (82.61 %)	4 of 23 (17.39 %)
Rhizosphere	22 of 24 (91.67 %)	22 of 24 (91.67 %)

possible for bacteria living in the rhizosphere of Zn-hyperaccumulator species, such as *Thlaspi caerulescens*, which have higher tolerance and storage capacity with regard to Zn than other plant species.

Growth Promotion of Red Clover Seeds and Seedlings by PPFMs

Determining Effects of Seed Sterilization and PPFM Inoculation on Seed Germination and Seedling Growth

In this preliminary study, germination rate of red clover seeds that were both non-inoculated and sterilized was the lowest, (Table 3) but these results were not statistically significant ($p>0.05$). Therefore, neither sterilization nor inoculation had a significant effect on germination rate.

NJ1104 association resulted in an increase in seedling fresh weight, regardless of whether or not the seeds were sterilized prior to inoculation (Table 3). It was difficult to ascertain significance of the results due to data variability. Some pots had far more seedlings to measure than others due to loss of seeds that never germinated, and seedling weights varied drastically within the same treatment and Petri dish. Despite this, the results of this experiment seemed to indicate that NJ1104 might have a positive effect on seedling fresh weight if not on germination. Because of these results, NJ1104 was used again to determine if inoculation had an effect on Zn-challenged seedlings.

Effect of NJ1104 colonization and $ZnSO_4$ Amendment on red Clover Growth

NJ1104 had a significant ($p<0.05$) effect on germination rate, but a negative effect on the seed (Table 4). Because NJ1104 produces acid when growing in AMS medium, it

Table 3. Results of a seed inoculation experiment in which sterilization and NJ1104 inoculation were investigated as variables potentially affecting red clover growth. This experiment included both sterilization and PPFM inoculation as variables. Seed germination rate and fresh seedling weight were both used to assess the success of resulting seedlings. Each treatment represents ten Petri dishes with ten seeds per dish. Germination rate was lower for seeds that were not inoculated with NJ1104 and were surface sterilized than for any of those in other treatments, but the results were not statistically significant ($p>0.05$) (Table 3). Seedling fresh weight was higher regardless of seed sterilization, but this was also not statistically significant ($p>0.05$).

Treatment	Germination Rate	Average Fresh Weight of Seedling (mg)
Non-Inoculated, Surface-Sterilized Seeds	0.45	11.2
Non-Inoculated, Non-Sterilized Seeds	0.61	10.3
NJ1104-Inoculated, Surface-Sterilized Seeds	0.64	15.2
NJ1104-Inoculated, Non-Sterilized Seeds	0.61	14.3

Table 4. Results of a seed inoculation experiment in which NJ1104 inoculation and 5 mM $ZnSO_4$ amendment were investigated as variables potentially affecting red clover growth. This experiment included PPFM inoculation and $ZnSO_4$ concentration added in a liquid amendment as variables. Seed germination rate, fresh seedling weight, and incidence of disease were used to assess the success of resulting seedlings. Zn presence significantly and negatively affected disease incidence ($p<0.05$). Only the presence of $ZnSO_4$, however, and not concentration, was a significant and negative factor for seed germination. NJ1104 significantly ($p<0.05$) and negatively affected germination rate, possible due to the combined effects of both NJ1104's acid production and Zn toxicity.

Inoculum	$ZnSO_4$ Concentration (mM)	Germination Rate	Seedling Fresh Weight (mg)	Disease Incidence
None	0.0	0.56	13.98	0.32
None	2.5	0.66	7.79	0.93
None	5.0	0.70	11.90	0.91
N1104	0.0	0.44	11.49	0.64
N1104	2.5	0.56	8.09	1.0
N1104	5.0	0.52	6.89	1.0

is possible that NJ1104 may also have been growing and producing acid in the presence of the methanol produced by the seeds as they attempted to germinate. If so, these results may suggest a decrease in germination of red clover caused by either low pH or $ZnSO_4$ kept in solution by the low pH. The combination of pH stress and metal toxicity may have been a factor as well, whereas without the added stress of the Zn being present, NJ1104's acid production might not have had a detrimental effect. AMS is buffered at 6.5, but NJ1104 reduces the pH of this medium to approximately 5.5 during growth. But because the Zn amendment was already in solution when added to the filter paper, it is unlikely that lower pH would raise the concentration, as the Zn was already dissolved.

Zn presence significantly increase disease incidence ($p<0.05$). That is, the presence of $ZnSO_4$ in amended treatments increased the instance of diseased seedlings compared to unamended treatments (Table 4). The concentration of $ZnSO_4$ was not a significant factor in that the effect on the rate of disease was the same for both concentrations of $ZnSO_4$ and only its presence or absence mattered.

Because of these results, Zn was removed as a variable for the final experiment and the use of NJ1104 was discontinued. Isolates NJ1103, NJ1107, NJ1108 and NJ1126 were used in further experiments.

Effects of Four PPFM Isolates (NJ1103, NJ1107, NJ1108 and NJ1126) on Red Clover Growth

Because NJ1104 appeared to lack obvious plant growth promoting abilities, there was cause to question the ubiquity of plant growth promotion in the genus *Methylobacterium*. Further experiments with isolates NJ1103, NJ1107, NJ1108 and

NJ1126 were conducted to ascertain whether other isolates might display more noticeable growth promotion properties. Overall disease and deficiency (stunting, branching roots, root rot) significantly correlated with lower red clover germination ($p<0.1$) (Table 5). The term "disease" encompassed fungal ailments as well as nutrient deficiencies. The number of stunted seedlings correlated significantly with lower germination rate ($p<0.1$) while branching roots and root rot alone did not significantly correlate. These data indicate that both disease and nutrient deficiency were the main cause of failed germination in these experiments.

PPFM inoculation had a significant and positive impact on disease incidence ($p<0.05$). That is, on average, PPFM inoculation resulted in fewer diseased seedlings after germination. This was especially apparent with isolates NJ1103 and NJ1126. The incidence of disease for NJ1103-inoculated seeds was significantly different from those of NJ1107 and NJ1108 ($p<0.05$). In NJ1103-inoculated treatments, disease rate was the lowest, whereas NJ1107 and NJ1108 had some of the highest incidences of disease in the experiment. NJ1107 and NJ1108 were statistically identical as far as overall disease occurrence but significantly different in terms of germination rate ($p<0.1$).

Table 5. Effects of NJ1103, NJ1107, NJ1108, and NJ1126 inoculation on seed germination, seedling fresh weight and disease instance in red clover. This experiment included four PPFM inoculation treatments and one control treatment. Percent seed germination, fresh weight, and incidence of specific diseases were measured to assess the success of resulting seedlings. Disease (stunting, branching roots, root rot) correlated significantly with lower red clover germination ($p<0.1$), as did stunted seedlings with lower germination rate ($p<0.1$). Branched roots and root rot effects did not significantly correlate with each other. PPFM inoculation significantly and positively impacted disease incidence ($p<0.05$), especially with isolates NJ1103 and NJ1126. Disease incidence was significantly different for NJ1103-inoculated seeds than from those inoculated with NJ1107 and NJ1108 ($p<0.05$). Disease was lowest for NJ1103-inoculated seeds, and highest with NJ1107 and NJ1108. The effects of NJ1107 and NJ1108 were not significantly different for overall disease occurrence but they were for germination rate ($p<0.1$).

Inoculum	Average Seedling Fresh Weight	Germination	Branched Root Seedlings	Seedlings with Root Rot	Stunted Seedlings
None	13.5	0.64	0.029	0.086	0.232
NJ1103	14.2	0.56	0.000	0.040	0.140
NJ1107	12.9	0.54	0.000	0.090	0.247
NJ1108	14.7	0.72	0.079	0.069	0.282
NJ1126	12.6	0.56	0.000	0.011	0.249

CONCLUSIONS

An enriching effect is seen for Zn-tolerant *Methylobacterium* species in the rhizosphere of red clover seedlings vs. in bulk soil > 1 cm from the seedlings. More *Methylobacterium* species were found within <1 cm of red clover seedlings than > 1cm away, in unamended soil. When some of these isolates were screened on AMS agar containing 5 and 10 mM $ZnSO_4$, a higher percentage of rhizosphere isolates were able to grow than bulk soil isolates. This suggests that rhizosphere *Methylobacterium* species are better adapted for growing in elevated Zn than bulk soil species. This is probably due rhizosphere cells experiencing elevated Zn around roots due to chelation and solubilization effects by root exudates but also receiving the benefits of nutrients in those extracts and from plant-released methanol.

In Zn-contaminated soils, concentrations of Zn are so high as to cause toxicity for both microbes and plants alike. Studies like this indicate that selection for Zn-tolerance species does occur in the plant rhizosphere. It is possible to assume, then, that resistance species able to tolerant contaminant-level concentrations of Zn in soil might be selected for by the presence of plants. If hyperaccumulator and metal tolerant plants are instituted at contaminated sites, the data presented here indicate the possibility that Zn-resistant PPFM species might be selected for in the rhizosphere. This, in combination with other selection methods such as application of methanol, may indicate a way in which PPFMs might be exploitable for the phytoremediation of Zn-contaminated soil.

Through the course of this research, it was found that not all PPFMs possess plant growth-promoting properties. Abilities vary with species, and thus it is not sufficient to say that the genus is capable of plant growth-promotion, as not all possess this ability. In

addition, it is not sufficient to cite that a species merely improves growth, as methods for this vary with species and some methods may be more desirable for some plants than with others. While one species may improve germination rate, another may lower disease. Both would be considered plant growth-promoting but have very different effects on the plant. This is extremely important to note when characterizing the genus *Methylobacterium* and considering species beneficial to phytoextraction as there is likely no "magic bullet" that will possess all desired capabilities under all conditions. It is also relevant to the agricultural industry. Not all PPFMs will be beneficial. It may be important to select not only which species are capable of growth-promotion, but those capable of it while in association with the host plant of interest and which accomplish the desired effect or overcome a target ailment.

These experiments clearly demonstrate that PPFM species vary widely in their interactions with plants and the affects that those interactions have on germination rate and incidence of disease. It cannot be said, therefore, that plant growth promotion is a ubiquitous trait of the genus *Methylobacterium*. It also cannot be said that if a species is plant growth-promoting in one aspect, it will be in all other aspects as well. Plant colonization does not always ensure growth-promotion. This must be taken into account when selecting species either for remediation purposes or for the improvement of yield or mortality of crop plants in the agricultural industry.

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DISCUSSION

Many of the findings contained in this research challenged conventions, assumptions, and conclusions found in literature pertaining to the genus *Methylobacterium* while other results have not been previously discussed before. This indicates a need for further research regarding this genus and hints at the incomplete nature of our understanding of PPFM lifestyle and role within the environment. Highlighting areas of research where more information and data are needed will help fuel future studies involving *Methylobacterium* species to not only piece together in a laboratory setting what this genus is capable of, but also what further potential exists for application of these bacteria to real-world agricultural and bioremediation problems and limitations.

The elucidation and application of a functional gene capable of serving as a genetic marker for the *Methylobacterium* genus has been accomplished in this research. Chapters 1 and 2 both address the potential for *mxaF* gene sequences to not only characterize new environmental PPFM isolates from soil and plants but also to begin to provide information regarding the evolution of the *mxa* methylotrophy system in bacteria. *mxaF* sequence and PPFM geographic distribution from New Jersey (NJ), California (CA) and South Carolina (SC) soils as well as from the skin of a blueberry of NJ origin formed no pattern when environmental pH, plant cover or type, soil type (excluding blueberry PPFMs), and annual rainfall (excluding blueberry PPFMs) were considered. These results were important for a number of reasons. Firstly, the finding that plant cover/type did not affect the selection of *mxaF* sequence of PPFM species was in

contention with research that suggested plant species as a strong selective factor for PPFM species distribution in the environment (Knief et al., 2010). Secondly, it was hypothesized that pH might have an effect on *mxaF* sequence, as some bacteria such as *E. coli* change induce the production of different periplasmic enzymes when in different pH conditions. Methanol dehydrogenase (MDH) is a periplasmic enzyme and changes in its sequence can drastically affect enzyme efficiency. Thus, it was thought that different external pH would affect the types of *mxaF* gene sequences that would result in optimal MDH functions at those different pH values. This is feasible given that only a permeable barrier exists between PPFM MDH enzymes and their external environment. But the results in Chapter 1 indicate that pH did not affect *mxaF* sequence or PPFM distribution. Combined with plant cover/type not affecting *mxaF* sequence or PPFM distribution either, these results would seem to indicate a reason for why PPFMs are so resilient and widespread in the environment. If influential environmental parameters such as pH and the influence of root exudates and other plant materials PPFMs come into contact with regularly do not select for different *mxaF* sequences even though each species has its own slightly different sequence indicates robustness and versatility in the sequences and functionality of this gene in fluctuating environmental settings. While this knowledge may be biologically counterintuitive, it supports even further the use of this gene as a biomarker for the *Methylobacterium* genus by virtue of its robustness and ubiquity in PPFMs.

The reliability and reproducibility of this method of characterizing PPFMs using *mxaF* sequence continued when *mxaF* sequence was compared with reference sequences in GenBank from other methylotrophy genes with similar sequences and functions. *xoxF*

and other generically annotated pyrroloquinoline-quinone (PQQ) methanol/ethanol dehydrogenase genes were used to present challenges when examining phylogeny using *mxaF*, as the presence of highly similar sequences can sometimes cause phylogenetic analyses to become less reliable and more difficult to discern. But even with a much larger sample set involving many bacterial genera and multiple methylotrophy genes, *mxaF* sequences in PPFMs continued to tightly cluster. *mxaF* is therefore a functional gene conserved enough to be useful as a genetic marker but variable enough to show differences in single species that will still not be easily confused with *xoxF* or similar genes when analyzing PCR products from bacterial isolates of many genera.

When examining the construction of operons within the *mxa* family of methylotrophy genes, it quickly became clear that the genus *Methylobacterium* display a pattern that is nearly identical between species. This was not true of other genera, save for *Bradyrhizobium* spp., which were missing nearly all of the genes except for three. *Xanthomonas* spp. were also missing many essential genes for the transcription of *mxaF*, the acquisition of calcium ions, the association of PQQ with the main enzyme, etc. No other bacteria had the exact pattern of *mxa* genes on their chromosomes that PPFMs did. This combined with the lack of many essential genes in other genera and the occurrence of *mxa* genes on a plasmid of a strain of *M. nodulans* was enough to suggest horizontal gene transfer (HGT). This is plausible when considering that *Bradyrhizobium* spp. are common rhizsophere bacteria and the *Xanthomonas* genus is best characterized by plant pathogens. *Methylobacterium* spp. would therefore encounter at least those two genera of bacteria on a regular basis in the environment.

HGT may have taken place from *Methylobacterium* species to others, resulting in only a few genes being transferred, providing a justification for the presence of these genes on PPFM plasmids as well as implying that genes found in *Bradyrhizobium* and *Xanthomonas* species may not be functional without the remaining *mxa* genes. It may also be, however, that PPFMs have acquired genes from other bacterial genera and developed subsequent genes later on. This seems less likely, but HGT could not be confirmed and the direction ascertained from the presence of mobile genetic elements in the vicinity of suspected HGT sites or analysis of G+C content. It may be that HGT events occurred so long ago that genes and genomes of these bacteria have since acclimated to each other and erased much of the evidence. Or, it could be that because these bacteria are all soil or plant bacteria that experience UV damage regularly, their G+G content is already very high and HGT events cannot be visualized by looking at G+C content alone. Therefore, until more completed genomes with searchable gene maps are available, data remains limited and it can only be surmised that HGT has taken place within this genetic system. The direction of transfer and what implications this might have for the evolution of *mxa*, the largest methylotrophy system in bacteria, remains unknown.

There is a host of literature that examines PPFM capabilities to promote the growth of certain plant species and to utilize various substrates for growth and energy acquisition. While a strain of *Methylobacterium extorquens* was found to be able to survive and grow in high concentrations of formaldehyde (Chongcharoen et al., 2005), none of the isolates tested in Chapter 3 were able to grow in even a 0.1 mM concentration of formaldehyde. Not only does this indicate clearly that not all strains are utilizable for

all bioremediation applications, in this case the remediation of soil contaminated with formaldehyde, but it also raises an interesting point regarding the toxicity of intermediates of methanol assimilation into biomass. During the assimilation of the carbon contained in methanol, an oxidation reaction takes place that coverts the substrate to formaldehyde. From there, it is taken inside the cell and the carbon is incorporated into cell biomass. It is counterintuitive, then, to realize that very low concentrations of formaldehyde are inhibitory and cause the extinction of cell cultures in the laboratory. These findings illustrate that we are far from understanding the mechanisms by which PPFMs deal with formaldehyde toxicity while will maintaining the ability to convert methanol and take the resulting formaldehyde into their cells. This could indicate differences either in laboratory conditions compared to a soil or phyllosphere environment or in the timing and manner in which formaldehyde is applied in the laboratory verses being converted by the bacteria themselves that results in an altered ability to tolerate toxicity.

Another counterintuitive result was the inability of any isolates tested in Chapter 3 to utilize sodium acetate as a carbon source. Sodium acetate is a readily utilizable substrate found in plant root exudates that many species of bacteria can utilize. However, it is clear from the results presented in Chapter 3 that a number of PPFM soil isolates cannot utilize sodium acetate as a growth substrate, yet many of those same isolates were avid colonizers of red clover seeds and seedlings. When considering PPFMs for applications in the agricultural industry, it is important to keep the findings of this research in mind. Not all substances provided by plants will be utilizable and the ability to colonize plants and even promote plant growth is not indication of the diversity of

substrates a species can use. It is therefore necessary to determine what substrates will stimulate PPFM species of interest on desired plant hosts.

Isolate NJ1104 grew in concentrations of methanol up to 4.0 % and was still alive if not growing in up to 10.0 %. These findings are substantial when considering the work of other research groups touting PPFM survival and biofilm production in elevated methanol. One study found that alcohols decreased the ability of bacteria to attach to various surface types (Fletcher, 1983) while another noted that a strain of *M. rhodesianum* was capable of growing and producing intermediate compounds valuable to industry at 25 g/L methanol or 2.5 % (Bormann et al., 1997). Not only was NJ1104 capable in surviving in concentrations much higher than 2.5 %, but biofilm production still occurred in up to 4.0 %. While NJ1104 did not itself display any plant growth-promoting abilities on red clover, strains that can withstand high amounts of methanol and have a positive effect on crop plant development are of use in the agricultural industry. Foliar-applied methanol has been suggested by researchers as a way of stimulating PPFM growth on plants to then facilitate their plant growth promoting abilities (Madhaiyan et al., 2006). For this to become a reality, strains able to promote the growth of crop plants, maintain biofilm production and maintain growth in the high concentrations of methanol that would be applied to the leaves of crops are essential to find.

Biofilm production of individual *Methylobacterium* species has not been previously described in the literature. PPFMs are participants in multi-species biofilms on dental equipment, paper machines, shower curtains, etc. (Barbeau et al., 1996; Väisänen et al., 1998; Kelley et al., 2004; Simões et al., 2007), and quorum sensing systems are

widespread in the genus (Poonguzhali et al., 2007). However, studies of the abilities of individual species and what substrates or other parameters might affect biofilm development have never been conducted. Because biofilm production is a major method by which bacteria colonize plant surfaces (Morris and Monier, 2003) and due to the physical and chemical protection provided by the EPS matrix, research exploring the highlights and limitations of PPFM biofilm production is important when trying to ensure the colonization of plant surfaces. In Chapter 3, it was discovered that only a small subpopulation of PPFM isolates were capable of producing biofilms in a laboratory setting. This could indicate that biofilm production is not a characteristic of the whole genus or that some species require environmental growth factors or signals to begin and maintain biofilm production. In addition to this finding, it was also observed that two biofilm producers, NJ1101 and NJ1104, had varying capabilities and produced starkly different types of biofilms. NJ1101 produced weakly attaches, sparsely coating, thin biofilms that dissociated very easily. NJ1104 produced strongly attached, thick biofilms that extensively covered glass surfaces and had a number of other structures (sticky rings, floating films at the meniscus, filaments joining meniscus and submerged biofilms together) associated with them. Again, this might indicate either a differential ability within the genus to produce biofilms or perhaps different mechanisms by which biofilms are produced between species. Or it may be that NJ1101 is missing something it requires to produce biofilms that it may have had in its native soil environment. Whether PPFMs are displaying different ability levels or they require different growth factors to produce biofilms, this information would be of value to the agricultural community when trying to stimulate plant growth-promoting PPFMs to colonize crop plants. If growth factors could

be discovered and employed to facilitate PPFM biofilm growth that would not have the same affect in other bacteria, this might give PPFMs an advantage.

The amount of qualitative and observational data regarding the appearance and structure of PPFM biofilms should also not be ignored. The structure of the advanced, mature biofilms of NJ1104 when left to grow in stationary conditions for over six weeks is not only beautiful and intriguing, but also hints at a high level of cell-to-cell communication and functional diversity within the biofilms of this strain. Whereas NJ1101 biofilms consisted only of a single, uniformly colored film attached to glass bordered by growth medium, NJ1104 biofilms displayed numerous color, structures, and – presumably – functions within the same culture. Contained within Chapter 3 are photos of NJ1104 biofilms that show not only the submerged films that attach to glass, but also the films that develop at the meniscus. Certainly, the oxygen requirements of these two films must differ, and their structures were vastly different. The submerged, attached film in a stationary jar was thick, almost fluffy in appearance, with a loose and uniform structure and a pale uniform pink color. The meniscus film, however, was much darker and varied in color, was thin and leathery in texture, and displayed ridges and valleys of three-dimensional structure. Thus, PPFMs that produce biofilms can alter the structure of the films to suit their oxygen and nutrient needs. In addition, colonies were observed on the glass above the water level, indicating the high level of motility and desiccation tolerance in this strain. Finally, the fibrous filaments that connected not only the two biofilms but also anchored the culture to the glass were particularly intriguing. Their occurrence hints at cellular communication and differentiation into different structures even outside the main biofilms, presumably for the transportation of nutrients and other

substances between the two films. This level of functional diversity has never been revealed in *Methylobacterium* species before, and it is the responsibility of the scientific community now to pursue this type of research with flow-cell and imaging technologies with the goal of augmenting our understanding of PPFM biofilm development and functional diversity. Learning about the types of structures PPFMs form, why they are formed, and what mechanisms – both chemical and genetic – are involved in these processes will help us to better predict and facilitate PPFM biofilm production in the future.

Zinc (Zn) resistance in *Methylobacterium* species has been a subject of debate in the literature. Only two research groups thus far have addressed it thus far. Kunito et al. in 1997 determined that Zn resistance was a trait of the genus, citing results from their agar plate studies using soil dilutions. Almost in direct reply to this, Zarnowski et al. in 2002 cites the Kunito paper and criticizes the methods contained within. This latter study, which was designed to describe radiation resistance in an *M. radiotolerans* strain, also found that this same strain was very much susceptible and not even slightly tolerant to Zn. Indeed, the methods of the earlier paper seem simplistic and leave out many key details of their setup, but the latter study seemed ready to condemn PPFM Zn resistance based on the susceptibility of one strain. In Chapter 4, the resistances of nine isolates to Zn was investigated, with one isolate (NJ1101) being most closely related to *M. radiotolerans* by virtue of its 16S rRNA and *mxaF* gene sequences. Not only was NJ1101 able to withstand concentrations of Zn higher than those found in the Zarnowski paper, but all nine isolates grew in concentrations that had severely reduced or eliminated the

occurrence of other bacteria from the same soil. Thus, it is our contention that some PPFM species are Zn tolerant, and that this trait is worthy of further investigation.

It was clear, however, that the choice of medium played a significant role in how the isolates were exposed to Zn. Solid agar as well as agars and liquid mediums richer in their components served to bind the highly reactive Zn, thereby lowering the effective concentration. This study indicates that tolerance levels for PPFMs should be obtained in minimal liquid medium, such as Ammonium Mineral Salts (AMS) Medium, that contains very little for the Zn to bind to. Even the standard protocol for making AMS contains too much dibasic potassium phosphate and results in the formation of an insoluble Zn phosphate precipitate. Therefore, this research has proven that amendment of AMS with soluble Zn forms, such as Zn sulfate, should coincide with a 1:10 reduction in the amount of disbasic potassium phosphate while continuing to utilize the full amount of monobasic potassium phosphate. This ensures the buffer capacity will remain intact while eliminating the generation of an insoluble Zn precipitate.

When growing on Zn-amended plates, three distinct behaviors were seen, two of which have not been previously documented in the literature, those being the precipitation of an insoluble Zn salt and the solubilization of hopeite. Hopeite, an insoluble and nonbioavailable Zn phosphate compound, can be solubilized by some *Methylobacterium* species. Aside from being an ability not previous characterized in PPFMs, this raises an immediate flag as to the potential of PPFMs to assist in both nutrient acquisition for crop plants as well as phytoremediation processes like the removal of metal contaminants from soil. By solubilizing this insoluble Zn salt, PPFMs make it bioavailable to plants. The significance of this finding is two-fold. Firstly, a pool

of Zn previously unavailable to plants as a source of Zn nutrients is now made so. For crops, this could mean that colonization by certain PPFM species may aid the plants in acquiring Zn nutrients, potentially avoiding ailments and deformities that result from Zn deficiency. Secondly, increased bioavailability to plants can facilitate the removal of Zn from soil by way of plant extraction, useful in bioremediation practices. Hyperaccumulator plants can take up and store far more Zn than can normal plants, but only from the bioavailable fraction. Because Zn is very reactive and has a tendency to bind and form multiple salts and chemical compounds in soil once it is deposited, the solubilization of an insoluble fraction in soil is a very useful trait for PPFMs to possess.

Using a drop in pH as a proxy for organic acid production, an attempt was made to elucidate the mechanism by which PPFMs solubilize hopeite. However, no pattern was observed between those that did or did not produce acid and those that did or did not solubilize the Zn phosphate. Thus, while acid production may be a mechanism for some, it is not the only mechanism according to these results. It may be that chemical chelators, like siderophores for iron, may be employed by PPFMs when solubilizing Zn-containing compounds. But whether or not this is indeed a mechanism, and whether or not hopeite solubilization is passive or intended on the part of the PPFMs, remains unknown. If the solubilization of Zn salts is to be stimulated for applications in the agricultural industry or for bioremediation, it is necessary to understand the mechanisms behind it. In addition, the ability of these isolates to solubilize Zn salts raises questions as to how these bacteria handle other metals in soil. Is it feasible that PPFMs may be able to solubilize other metals, perhaps ones such as copper that behave in a chemically similar fashion to Zn.

This knowledge, if true, would expand even further the potential that *Methylobacterium* species have for application in agricultural and remediation industries.

With regard to biofilms in Chapter 4, low levels (0.1 or 1.0 mM) of Zn sulfate did not inhibit or delay biofilm production in isolate NJ1104. However, an effect on the DNA per gram of biofilm material was seen, with the general trend of more DNA present if the Zn amendment was made at 24 hours or later in the growth of the culture. These results are interesting, although they were not statistically significant. This might be due to variability in the setup, as imperfections and variations in the glass flasks as well as day-to-day variability in spectrophotometer readings might give the illusion of a trend or make a true trend not statistically significant. To improve upon this setup, reduce variability, and continue to assess the effects of Zn toxicity on biofilm production, more controlled conditions, like those found in flow-cell setups, are needed. It is clear that bench top experiments involving shake flasks are not sufficient to determine the subtler effects that lower concentrations of metals have on biofilm development.

Plant growth promotion (PGP) in *Methylobacterium* species is one of the most widely mentioned and fervently pursued topics in PPFM-related literature. It is considered by many research groups to be a characteristic of the genus. However, in Chapter 5 it was observed that not all *Methylobacterium* species are capable of improving the seed germination, seedling fresh weight, or disease resistance of red clover. In fact, some isolates (interestingly enough, the biofilm-producing NJ1104) actually were show to decrease germination and have little effect on disease. This may not be true for other plants, though, as only red clover was used in these studies. Thus, it is important to note that the occurrence of PGP for one plant species does not ensure the bacteria will

improve the growth of another plant species. Indeed, it has been noted that certain *Methylobacterium* species gravitate towards specific plant species, or more accurately, that the seeds and rhizospheres of plants select for certain PPFM species (Knief et al., 2010). Therefore, not all PPFM species may be suitable when attempting to stimulate PGP on specific crop plants, for example.

Of the isolates that did promote plant growth, some improved germination while other lowered the instance of nutrient deficiencies or diseases. Now, not only it is known that all PPFMs do not share the ability to promote the growth of plants, but also one must pay careful attention to the ailment of interest that one wishes to have the plant overcome. If a PPFM culture that stimulates germination is applied to a plant leaves, there may be no effect. If a species that aids in the staving off of root rot is applied but the plant is experiencing a nutrient deficiency, the deficiency will not be overcome. This research indicates that the question of the existence of PGP abilities of the *Methylobacterium* genus and how best to apply them for the improvement of the health and yield of crop plants is more complex than just whether or not any members of the genus will colonize the plant. As more information is deduced regarding the abilities of individual *Methylobacterium* strains, the agricultural industry will be able to move forward with the idea of protecting their crop plants with species capable of overcoming specific ailments and deficiencies.

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CONCLUSIONS

The major conclusions of this research are as follows:

1) *mxaF* sequence is a useful and reliable genetic tool for the characterization of environmental *Methylobacterium* species due to the near exclusivity of PPFM *mxaF* sequences compared to other bacteria genera.

2) pH and plant type do not select for *mxaF* sequence and therefore also do not drive *Methylobacterium* species distribution in the environment.

3) *Methylobacterium mxa* gene family operon construction displays a pattern not found in other bacterial genera.

4) Evidence of HGT exists within the *mxa* methylotrophy family of genes in the form of *mxa* gene presence on *Methylobacterium* plasmids and the presence of minimal and possibly non-functional *mxa* genes in other bacterial genera.

5) Some *Methylobacterium* species are capable of solubilizing the insoluble Zn salt, hopeite, thereby increasing its bioavailability.

6) Biofilm production in the *Methylobacterium* genus varies by species and indicates a high level of functional diversification as a function of biofilm age and various growth conditions.

7) Not all *Methylobacterium* species participate in plant growth promotion, and those that are capable of promoting plant growth do not all do so in the same manner.

The need to improve the health and yield of crop plants and to clean up contaminated soils is felt worldwide. Currently, the use of fertilizers and chemical

pesticides dominate the agricultural industry's attempts to overcome nutrient deficiencies and deter pathogens in order to improve crop yields. Bioremediation methods like phytoextraction are often not considered or are found to be cost effective for the removal of metal pollutants from contaminated soils and other physical or chemical methods are employed. The need is there to improve upon existing practices in the agricultural and bioremediation fields as well as finding new ways of combating these issues without the use of potentially dangerous chemicals or simply moving or postponing the problem. Delving into the capabilities and potential benefits of native soil and plant bacteria such as those of the *Methylobacterium* genus may provide insight into how these bacteria might be exploitable for safer and more environmentally friendly solutions to these problems.

Our understanding of the genus *Methylobacterium* is still limited. This type of research will expand it and should be continued in the future. There is still unexplored potential contained in this genus of which this research has only begun to scrape the surface. If *Methylobacterium* species are capable of participating in the cycling of Zn ions and compounds, altering the bioavailability of the metal in soil systems, it is possible that they may also influence the cycling of other metal nutrients in soil. Altering metal bioavailability has far reaching effects on soil fertility and microbial, animal and plant nutrition. With the ability to raise the solubility of insoluble pools of Zn in soil, *Methylobacterium* species have the ability to aid Zn-deficient crop plants or increase the uptake of Zn contamination into hyperaccumulator plants involved in phytoextraction in soils. But before this potential can be realized, the research presented here must be

expanded upon and continued from bench top studies towards the eventual goal of real-world application.

It is clear from the research presented here that the capabilities of the *Methylobacterium* genus vary greatly between species. Biofilm production and plant growth promotion are species-specific and multiple Zn tolerance levels and toxicity survival strategies were observed. It is necessary to know the capabilities of individual species, since not all will benefit agricultural or bioremediation processes. And, beyond this research, it is important to not only study the capabilities of individual species but now to study the effects and abilities of consortia on plant development, Zn cycling, and biofilm production. PPFMs do not live as pure cultures in the environment, and pure culture studies can only give us an idea of potential, not of what occurs in the environment. With the groundwork laid here, we should now move towards the construction of consortia and field studies involving multiple PPFM species as well as PPFMs growing together with bacteria of other genera. Only then will we begin to truly understand how the abilities of isolates will fare in a mixes-species, open-environment setting.

mxaF has proven to be a useful genetic marker for the *Methylobacterium* genus, and *Methylobacterium mxa* gene family operon construction appears to be conserved. However, data in this research is limited to the use of one database and by the number of completed genomes with searchable gene maps available for the analysis. To continue to answer questions regarding the evolution of the *mxa* system in bacteria and to understand the versatility of *mxaF* sequence and function, more data is needed. If multiple databases were employed and as more completed genomes are made available, this research can be

expanded upon and a clearer picture of horizontal gene transfer among *mxa* methylotrophy genes in bacteria can be resolved.

There are too many aspects that remain "black boxes," too many important problems to address, and too much curiosity to be sated to ignore the potential of the *Methylobacterium* genus. If this type of research is not continued in the future, much of that potential may remain unrealized. There are obstacles to overcome: limited data availability, practicality of environmental application, and the ambiguity and error involved with genetic research and cataloguing of sequences in online databases. If no one seeks to overcome these hurdles for fear of failure, the potential of the *Methylobacterium* genus may remain theoretical indefinitely.

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