Diversity of *Methylobacterium* Species Associated with New Zealand Native Plants

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Abstract

The genus *Methylobacterium* are pink-pigmented facultative methylotrophs (PPFMs), and are abundant colonizers of the phyllosphere, due to the availability of methanol, a waste product of pectin metabolism during plant cell division. Besides methanol cycling, *Methylobacterium* has important effects on plant health. The phyllosphere is an extreme environment with a landscape that is heterogeneous, continuously changing as the plant grows, and is exposed to very high ultra violet irradiation. Geographically, New Zealand has been isolated for over a million years, has a biologically diverse group of species, and is considered a biodiversity hotspot, with most of the native plants being endemic. We hypothesize that NZ native plants harbor diverse groups of *Methylobacterium* species, and to test this we aimed to isolate *Methylobacterium* species from the phyllosphere of native New Zealand plants. A leaf imprinting technique using methanol supplemented AMS agar media was used to isolate bacteria and diversity was determined using a combination of ARDRA, 16S rRNA gene sequencing and phylogenetic analysis. *Methylobacterium* spp. were successfully isolated from 19 of the 21 plant species used in this study. Eleven *Methylobacterium* species have been identified in association with the phyllosphere of native NZ plants: *M. adhaesivum*, *M. brachiatum*, *M. komagatae*, *M. marchantiae*, *M. mesophilicum*, *M. phyllosphaerae*, *M. fujisawaense*, *M. oryzae*, *M. radiotolerans*, *M. tardum* and *M. zatmanii*, with the first six being the most frequently isolated from more plant species. In this study other α, β, γ-proteobacterial species were also isolated: *Hyphomicrobium*, *Methylopila*, *Rhizobium*, *Achromobacter*, *Methylophilus*, *Ramlibacter* and *Xanthomonas*; *Janibacter melonis* (Actinomycetes); *Niastella populi* (Bacteroidetes) and *Paenibacillus lautus* (Firmicutes), highlighting the presence of potential novel methanol utilizer within the ecosystem. Results from this study indicate that *Methylobacterium* are abundant and dominant members of the NZ phyllosphere environment, with species diversity and composition dependent on the host plant species.

Acknowledgements

All praises are to the Almighty Allah who enabled me to complete this thesis. I'd like to express my heartfelt respect and gratitude to my supervisor Associate Professor Ian McDonald for his guidance, constructive suggestions and encouragement during the entire period of research and in preparing this thesis. His door was always open for me for help and advice, many thanks. I have found a warm, friendly and collaborative environment within the Thermophile Research Unit throughout of my research. In particular I'd like to thank Stephen Archer for help with many technical problems, giving good advice and teaching me the basics. Thanks to Chelsea Vickers for her assistance in the laboratory, especially with PCR. Thanks to Charles Lee for giving suggestions when I have presented my research in the TRU lab meeting. Thanks to Craig Herbold for his advice, and Eric Bottos for help with making primers and with technical problems arises during electrophoresis and visualization. I have found Professor Craig Cary is a superb mentor. I can't forget his supportive sentence *"One of us has to guide you to complete your masters and this is your masters, you have to achieve this"* – he replied when I approached him to take me under his wing. Special thanks go to Craig Cary and Ian McDonald for allowing me to conduct my research in the TRU. I have found Professor Hugh Morgan has always been supportive. Special thanks also go to Hugh Morgan for his everyday looking after when Ian McDonald was away for study break. Thanks are extended to Lynne Parker, Colin Monk and Roanna Richards-Babbage for their support and assistance in my every mechanical problem to fix. I can't finish this research without their help. I'd like to thank John Longmore in the Waikato DNA Sequencing unit, who has seen my sad face several times and helped me to successfully complete the sequencing. I am thankful to Andrew Grilli, for allowing me to spend time at his nursery, to know about native plants and take samples of them. I am thankful to my mother, brothers and sisters and special thanks to my children Nazifa and Rayyed for their sacrifices and day to day encouragement at every down moment through the entire period of the research for success.

Dedication

I’d like to dedicate this thesis to my late youngest brother Nasim and late father Alhaj Md Abu Zahed Talukder, who I believe always, expects more from me.

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List of Abbreviations

%	Percentage
µg	Microgram
µL	Microlitre
µM	Micromolar
µm	Micrometer
Acc. No.	Accession number
AFM	Atomic force microscopy
AIDS	Acquired immune deficiency syndrome
AMS	Ammonium mineral salts
ARDRA	Amplified ribosomal DNA restriction analysis
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
C1	Single-carbon compounds
CFU	Colony forming unit
cm	Centimeter
Cn	Multi-carbon compounds
Da	Dalton
DMF	N,N-dimethyl formamide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EPS	Extracellular/Exo polysaccharide
ExoSAP	Exonuclease I Shrimp Alkaline Phosphatase
F	Forward
g	Gram
gdw	Gram dry weight
HGA	Homogalacturonan acid
IAA	Indole-3-acetic acid
kb	Kilo bases

l	Litre
L	Litre
M	Molar
MDH	Methanol dehydrogenase
mg	Milligram
MIDc	Minimal inhibitory dose
min	Minute
ml	Milliliter
mM	Millimolar
mm	Millimeter
mol	Mole
MQ H_2O	Milli-Q water
NCBI	National center for biotechnology information
ng	Nanogram
nm	Nanometer
NZ	New Zealand
^oC	Degree Celsius
^oF	Degree Fahrenheit
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PHB/V	Poly-β-hydroxybutyrate/valerate
PME	Pectin methylesterase
ppbv	Parts per billion by volume
PPFMs	Pink-pigmented facultative methylotrophs
RBP	Ribulose-1,5-bis-phosphate
RDP	Ribosomal database project
RFLP	Restriction fragment length polymorphism
RG-I	Rhamnogalacturonan-I
RG-II	Rhamnogalacturonan-II
RMP	Ribulose-5-mono-phosphate
rpm	Revolutions per minute

rRNA	Ribosomal ribonucleic acid
SAM	S-adenosyl methionine
SDS	Sodium dodecyl/lauryl sulfate
sec	Second
SET	Tris-sucrose-EDTA
sp.	Species
spp.	Plural species
TAE	Tris acetate EDTA
TE	Tris-EDTA
Tg	Teragram (10^{12} grams)
TMAH	Tetramethylammonium hydroxide
tRNA	Transfer RNA
UVR	Ultraviolet radiation
V	Volt
VOCs	Volatile organic compounds
XGA	Xylogalacturonan acid

Chapter 1: Background and Literature Review

In the lower atmosphere, methanol (CH_3OH) is one of the major volatile organic compounds (VOCs). The range of methanol concentrations is 1-10 ppbv (parts per billion) in the lower atmosphere (Heikes et al., 2002; Schade & Goldstein, 2001). In the atmosphere, methanol reacts with photo-chemically produced hydroxyl radicals (OH) to form formaldehyde (CH_2O) and per-hydroxyl radical (HO_2) (Japar et al., 1991). Both products are highly reactive and participate in chemical reactions including ozone depletion and smog formation. The reaction mechanism in the atmosphere is:

$$\mathbf{CH_3OH + \cdot OH \rightarrow H_2O + \cdot CH_2OH \rightarrow H_2O + \cdot CH_3O}$$

$\cdot CH_2OH$ and $\cdot CH_3O$ both react with molecular oxygen, therefore the net reaction of methanol to form formaldehyde and HO_2 is:

$$\mathbf{CH_3OH + \cdot OH \rightarrow H_2O + HO_2 + HCHO}$$

The primary source of methanol in the atmosphere is the terrestrial ecosystem, producing 187 Tg/year (Stavrakou et al., 2011). Plants emit 100-128 Tg of methanol per year, about two-thirds of total global methanol emissions (Galbally & Kirstine, 2002; Jacob et al., 2005). However, 4.9×10^{12} mol of methanol enters the atmosphere per year (Madhaiyan et al., 2011), due to biological methanol oxidation. It is well established that methanol is the key substrate for methanol oxidizing bacteria (Lidstrom, 2006), and that the plant phyllosphere is a major site for methanol production. Therefore the phyllosphere is a promising habitat to study methanol consuming organisms, especially the genus *Methylobacterium*, a common leaf colonizer.

1.1 The Genus *Methylobacterium*

In 1976, the genus *Methylobacterium* was first described as a new genus of facultative methylotrophic bacteria (Patt et al., 1976). Bacteria of the genus *Methylobacterium* are pink-pigmented facultative methylotrophs (PPFMs), Gram-negative, rod shaped, belong to the class Alphaproteobacteria, order Rhizobiales and family Methylobacteriaceae. Methylotrophy is defined as the ability to aerobically use single-carbon (C1) compounds (i.e. reduced organic compounds containing no carbon-carbon bonds) as substrates. These substrates include methane, methanol, methylated amines, halogenated methane and methylated sulfur. *Methylobacterium* are facultative, meaning they are also capable of utilizing multicarbon compounds. Methylotrophs are a diverse group of bacteria, including Gram-positive and Gram-negative genera, and have two subgroups- obligate and facultative. As a result, they play an important role in the global carbon, nitrogen and sulfur cycles.

1.1.1 Species of *Methylobacterium*

Interest first began to study PPFMs extensively in the 1960's and 1970's because of their biotechnological potential, and researchers found one-carbon assimilation pathways are common to methylotrophic bacteria. The first PPFM strain isolated was *M. organophilum*, initially it was able to utilize methane but later lost this ability (Patt et al., 1974). In a detailed biochemical, physiological and morphological study of 149 *Methylobacterium* strains, including *M. organophilum*, it was found that none of the strains utilized methane (Green & Bousfield, 1981; Green & Bousfield, 1982). As a result, besides the type species *M. organophilum* (Patt et al., 1976), four other *Methylobacterium* species were added to the genus, namely *M. rhodinum*, *M. mesophilicum*, *M. radiotolerans*, and *M. extorquens* (Bousfield & Green, 1985; Green & Bousfield, 1983). Prior to renaming three were *Pseudomonas* species, namely *P. rhodos* (Heumann, 1962), *P. mesophilica* (Austin & Goodfellow, 1979) and *P. radiora* (Ito & Iizuka, 1971), and the last one was *Protonomas extorquens* (Urakami & Komagata, 1984). DNA-DNA similarity studies (Hood et al., 1987), and

electrophoretic comparison of total soluble proteins (Hood et al., 1988; Urakami et al., 1993) from representative strains also identified several new species within the similarity groups of the genus *Methylobacterium*. Three new species were proposed, *M. rhodesianum*, *M. zatmanii*, and *M. fujisawaense* (Green et al., 1988). The overall similarities of identified PPFM organisms include, rod shaped, aerobic, Gram-negative, with growth on a wide range of multi-carbon and one-carbon compounds, fatty acid composition (70-90% of $C_{18:1}$) (Urakami & Komagata, 1979) and DNA base composition (68.0-72.4 mol% G+C) (Urakami & Komagata, 1979; Urakami & Komagata, 1986). However these organisms have internal heterogeneity (Hood et al., 1987; Urakami et al., 1993); highlighted by analyses including DNA-DNA similarity (Green & Bousfield, 1983; Urakami & Komagata, 1981). To date 45 species of the genus *Methylobacterium* have been described (http://www.bacterio.cict.fr/m/methylobacterium.html) (Table 1.1).

1.1.2 Habitat

The PPFM bacteria are strictly aerobic and facultative due to the utilization of a wide range of one carbon to multi-carbon substrates to produce their biomass. In nature, members of this genus are ubiquitous, isolated from a wide range of habitats; such as soil, dust, fresh-water, drinking water, sea-water, air, leaf surface, root nodules, rhizosphere, rice grains, and lake sediments (Corpe & Rheem, 1989; Green & Bousfield, 1982; Irvine et al., 2012), cold lands, such as Antarctica (Moosvi et al., 2005), coastal Antarctic ice core (Antony et al., 2012), and the bottom of the Kuroshima Knoll sea in Japan (Inagaki et al., 2004). These bacteria also occur in potable water supplies in hospital environments (Furuhata & Koike, 1990; Furuhata & Koike, 1993; Kaneko & Hiraishi, 1991), bathrooms and washstands (Furuhata & Matsumoto, 1992), and the human foot micro-flora (Anesti et al., 2006). Some members of the genus have been reported to cause opportunistic infections in immunocompromised patients (Houge et al., 2007), including the blood of a patient with AIDS (Holton et al., 1990). The ability to scavenge trace amounts of nitrogen and carbon makes them well adapted to different types of stressful conditions.

Table 1.1: Published species of *Methylobacterium*

Species Name	16S rRNA Acc. No.	Source of Isolation	Reference
M. adhaesivum	AM040156	Drinking water	(Gallego et al., 2006)
M. aerolatum	EF174498	Air samples	(Weon et al., 2008)
M. aminovorans	AB175629	Soil	(Urakami et al., 1993)
M. aquaticum	AJ635303	Drinking water	(Gallego et al., 2005a)
M. brachiatum	AB175649	Fresh water	(Kato et al., 2008)
M. bullatum	**FJ268657**	**Leaf of *F. hygrometrica***	**(Hoppe et al., 2011)**
M. cerastii	**FR733885**	**Leaf of *C. holosteoides***	**(Wellner et al., 2012)**
M. chloromethanicum	CP001298	Soil, polluted environment	(McDonald et al., 2001)
M. dankookense	FJ155589	Drinking water	(Lee et al., 2009)
M. dichloromethanicum	AB175631	Active sludge	(Doronina et al., 2000)
M. extorquens	AB175632	Forest and garden soil	(Urakami & Komagata, 1984)
M. fujisawaense	AJ250801	Soil	(Green et al., 1988)
M. funariae	**FJ157975**	**Phylloids of *F. hygrometrica***	**(Schauer & Kutschera, 2011)**
M. gnaphalii	**AB627071**	**Leaf of *G. spicatum***	**(Tani et al., 2012a)**
M. goesingense	**AY364020**	**Plant *Thlaspi goesingense***	**(Idris et al., 2006)**
M. gossipiicola	**EU912445**	**Leaf of cotton**	**(Madhaiyan et al., 2012)**
M. gregans	AB252200	Fresh water	(Kato et al., 2008)
M. hispanicum	AJ635304	Drinking water	(Gallego et al., 2005a)
M. iners	EF174497	Air samples	(Weon et al., 2008)
M. isbiliense	AJ888239	Drinking water	(Gallego et al., 2005b)
M. jeotgali	DQ471331	Jeotgal, seafood	(Aslam et al., 2007)
M. komagatae	AB252201	Fresh water	(Kato et al., 2008)
M. longum	**FN868949**	**Leaf of *A. thaliana***	**(Knief et al., 2012b)**
M. lusitanum	AB175635	Sewage station	(Doronina et al., 2002b)
M. marchanitae	**FJ157976**	**Thallus of *M. polymorpha***	**(Schauer et al., 2011)**
M. mesophilicum	**AB175636**	**Leaf of *L. perenne***	**(Austin & Goodfellow, 1979)**
M. nodulans	**AF220763**	**Nodules of *Crotalaria***	**(Jourand et al., 2004)**
M. organophilum	AB175638	Lake samples	(Patt et al., 1976)
M. oryzae	**AY683045**	**Stem of *Oryza sativa* L.**	**(Madhaiyan et al., 2007a)**
M. oxalidis	**AB607860**	**Leaf of *Oxalis corniculata***	**(Tani et al., 2012b)**
M. persicinum	AB252202	Fresh water	(Kato et al., 2008)
M. phyllosphaerae	**EF126746**	**Leaf of *Oryza sativa* L.**	**(Madhaiyan et al., 2009)**
M. platani	**EF426729**	**Leaf of *Platanus orientalis***	**(Kang et al., 2007)**
M. podarium	AF514774	Human foot microflora	(Anesti et al., 2006)
M. populi	**CP001029**	**Poplar plantlets**	**(Van Aken et al., 2004a)**
M. radiotolerans	**D32227**	**Rice seeds**	**(Ito & Iizuka, 1971)**
M. rhodesianum	AB175642	Fermentor	(Green et al., 1988)
M. rhodinum	**AB175644**	***Alnus* rhizosphere**	**(Heumann, 1962)**
M. salsuginis	EF015478	Sea water	(Wang et al., 2007)
M. soli	EU860984	Forest soil	(Cao et al., 2011)
M. suomiense	AB175645	Soil	(Doronina et al., 2002b)
M. tardum	AB252208	Fresh water	(Kato et al., 2008)
M. thiocyanatum	**U58018**	**Rhizosphere of *A. aflatunense***	**(Wood et al., 1999)**
M. variabile	AJ851087	Drinking water	(Gallego et al., 2005c)
M. zatmanii	AB175647	Fermentor	(Green et al., 1988)

1.2 The Phyllosphere

The below-ground parts are called the rhizosphere, while the above-ground parts of plants, such as the stem and leaf, are called the phyllosphere, though more typically phyllosphere is used to refer to the surface of the leaf (Lindow & Brandl, 2003). The phyllosphere represents the largest biological surface, estimated to be two times the surface of the earth, and is involved in many processes essential for the global ecosystem including carbon-dioxide fixation, oxygen release, and primary biomass production (Delmotte et al., 2009). This surface is considered a hostile environment for bacterial epiphytes. Morphological feature of leaf surface and its surrounding climate feature both make phyllosphere ecosystem harsh than other habitat. Leaf surface topography changes as they grow, old leaves have much tougher surfaces than young leaves (Mechaber et al., 1996) (Figure 1.1). Bulges and troughs are produced by the epidermal cells of leafs, which in turn determine nutrients availability on the surface of the leaf. Bacterial epiphytic establishment on leaves is not dependent on exogenous sources of nutrients. A healthy leaf is colonized by many bacteria (Hirano et al., 1982; O'Brien & Lindow, 1989), which indicate the presence of carbon, essential for energy and growth, nitrogen, and essential organic and inorganic molecules on leaves. Healthy epidermal plant cells constantly leak organic and inorganic molecules, such as amino acids, sugars (including sucrose, glucose, and fructose), methanol, and various salts to their surfaces (Corpe & Rheem, 1989; Fiala et al., 1990). An uncolonized bean leaf can accumulate an average 2.5 µg of sugar and this amount is adequate to support 10^7 bacteria (Mercier & Lindow, 2000). There is also evidence that a substantial amount of sugar is not depleted by colonizers, indicating that bacterial accessibility to those nutrients is limited.

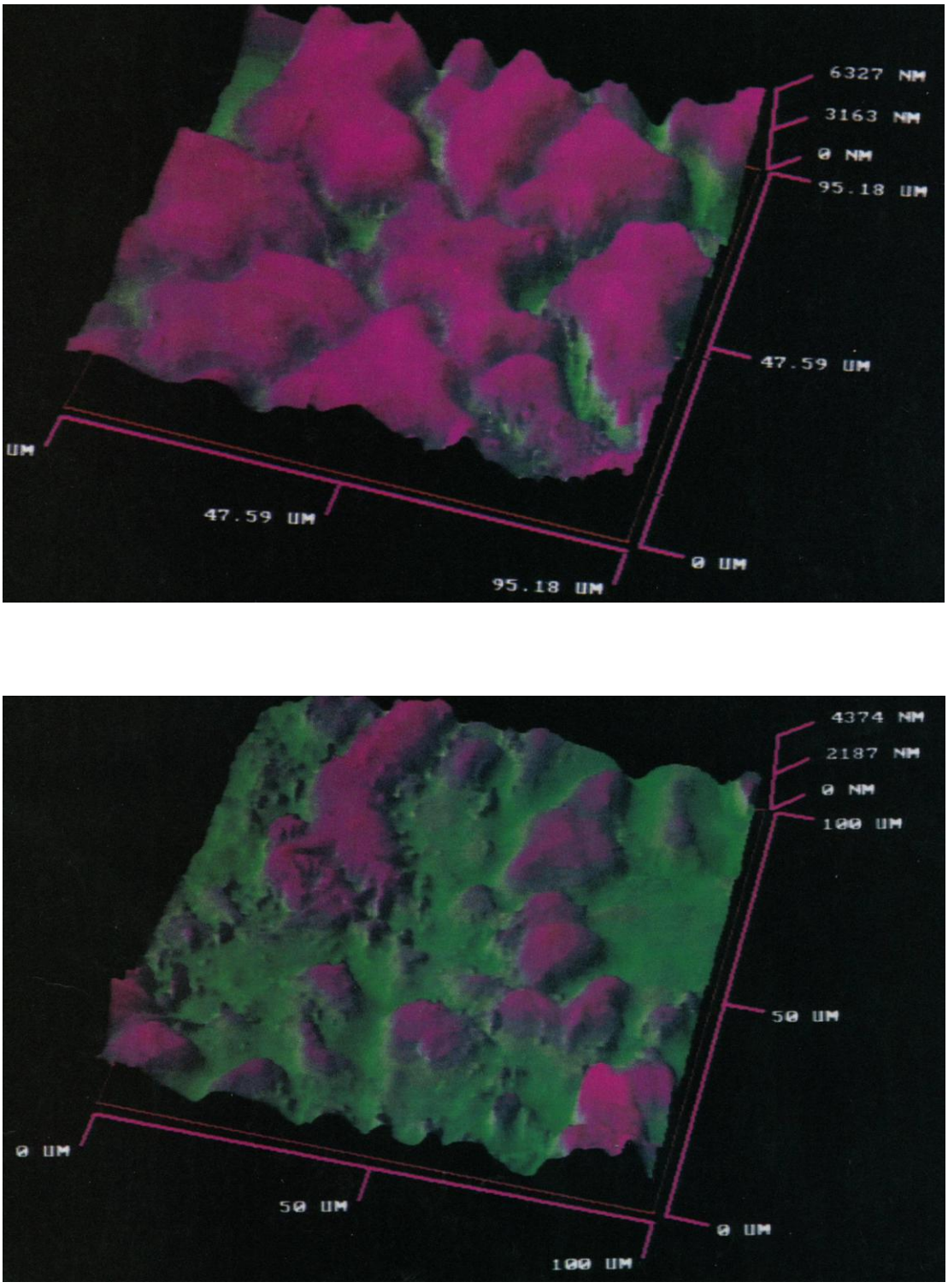
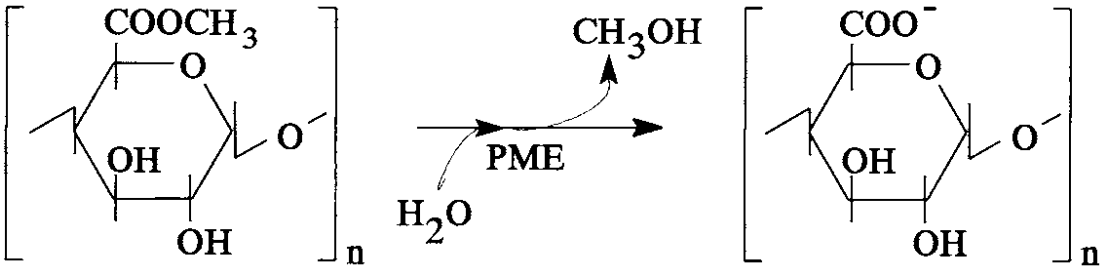


Figure 1.1: Mapping of adaxial (upper) leaf surface landscape by scanning of atomic force microscopy (AFP). Upper one is from young leaf and lower one is from old leaf; x and y axes are in micrometers and z axis is in nanometers (Mechaber et al., 1996).

This heterogeneous landscape is also exposed to intensely fluctuating environmental conditions, including harmful ultraviolet rays, high and low temperatures, variations in nutrient and water availability, and winds (Hirano & Upper, 2000; Lindow & Brandl, 2003). However, this habitat serves as home to many beneficial and pathogenic microbes. Usually phytopathogens avoid the stresses and reside interior part of leafs and epiphytes survive on the exterior of leafs. Historically, pathogenic organisms have attracted more interest because of their potentially devastating effect on economically important crops. But a balanced ecosystem is also important for plant health, therefore all the phyllosphere inhabitants are significant. They also have the ability to answer a broad range of biological questions about the adaptation of phyllosphere organisms. The adaptation of bacterial species indicates biotechnological potential, which has driven scientists to explore adaptive mechanisms, including identifying new bacterial species which possess unique enzymes that can be exploited in agriculture and industry (Dias et al., 2009). Hence, the nature of the phyllosphere makes it an interesting environment in which to study microbial diversity. The phyllosphere emits methanol, a waste product of pectin metabolism, and *Methylobacterium* spp. utilize methanol as sole source of carbon and energy. *Methylobacterium* species are non-pathogenic, and in nature, they are ubiquitous, found in variety of habitats (Green, 2006). Their capacity for adapting to changing environmental conditions and growing at high rates on methanol, and a variety of C_1-C_n compounds (facultative) reflects their metabolic potentiality. Aerobic plant-associated methylobacteria are phytosymbionts and their ability to synthesize cytokinins, auxins and vitamins expands their significance and biotechnological potential. Moreover, a recent study has increased interest in the phyllosphere, *Pseudomonas syringae*, a plant pathogen typically found in non-agricultural environments, and *Escherichia coli* O157:H7, a human entero-pathogen, were found on leaf surfaces (Brandl, 2006; Morris et al., 2007).

1.2.1 What is the source of methanol in leaves?

A reliable source of methanol in leaves comes from pectin demethylation in the cell walls (Fall & Benson, 1996). Pectin is the major polysaccharide of plant cell walls and is mainly composed of homogalacturonan (HGA-70%), xylogalacturonan (XGA), apiogalacturonan, rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) (Harholt et al., 2010; O'Neill et al., 1990). Pectin synthesis occurs in the Golgi apparatus in the early stage of cell development. Initially synthesized as a less modified form in the cis- and medial- Golgi, and gradually modified via methylesterification in the medial- and trans- Golgi. Eventually the highly esterified molecule is transported to the primary cell wall via the plasma membrane (Mohnen et al., 2008). Methylesterification in HGA is catalyzed by pectin methyltrasferase. This enzyme transfers the methyl group from S-adenosyl-L-methionine (SAM) to the carboxyl group of α-1,4-linked galactusyluronic acid residues in HGA (Mohnen et al., 2008). Cells grow by elongation of the primary cell wall, and during cell wall extension esterified HGA is demethylated through the action of the enzyme pectin methylesterase (PME) to produce ionized galacturonic acid residues and methanol (Galbally & Kirstine, 2002) (as illustrated in Figure 1.2). The structure of pectin polymers is variable among plant species, and even within the tissues of the same plant (Galbally & Kirstine, 2002). The rate of emission of methanol varies significantly during leaf development (Nemecek-Marshall et al., 1995), with higher emissions from growing leaves, which declines with increasing leaf age (Bosch et al., 2005; Obendorf et al., 1990). This indicates that pectin demethylation is significantly higher during leaf expansion, a period of rapid cell wall synthesis, and then declining demethylation and thus methanol production in older leaves. Pectin methylesterases are also involved in the regulation of tip growth of growing pollen tubes (male gametophytes of angiosperm) (Fall & Benson, 1996), and growth and maturation of seeds (Walter & Schurr, 2005). So growth processes are a likely source of leaf methanol (Hüve et al., 2007). Leaf growth varies greatly during the day and has a pronounced day/night cycle; the exact timing of these variations differs among plant species (Macdonald & Fall, 1993; Nemecek-Marshall et al., 1995).



Galacturonan methyl ester **Galacturonan demethylated**

Figure 1.2: Formation of methanol from pectin and reaction catalyzed by pectin methylesterase (PME) (Galbally & Kirstine, 2002).

1.2.2 How does methanol exit plant surfaces?

Experiments have revealed that the rate of leaf methanol emission depends on stomatal distribution and conductance and increased with increasing light intensity on stomatal conductance, and is emitted mainly through stomata **(**(Nemecek-Marshall et al., 1995) (Figure 1.3). The study also found that methanol was emitted from the abaxial surface of hypostomatous leaves (stomata mainly in the abaxial surface), but from both surfaces of amphistomatous leaves (stomata in both surfaces) (Nemecek-Marshall et al., 1995) (Figure 1.4).

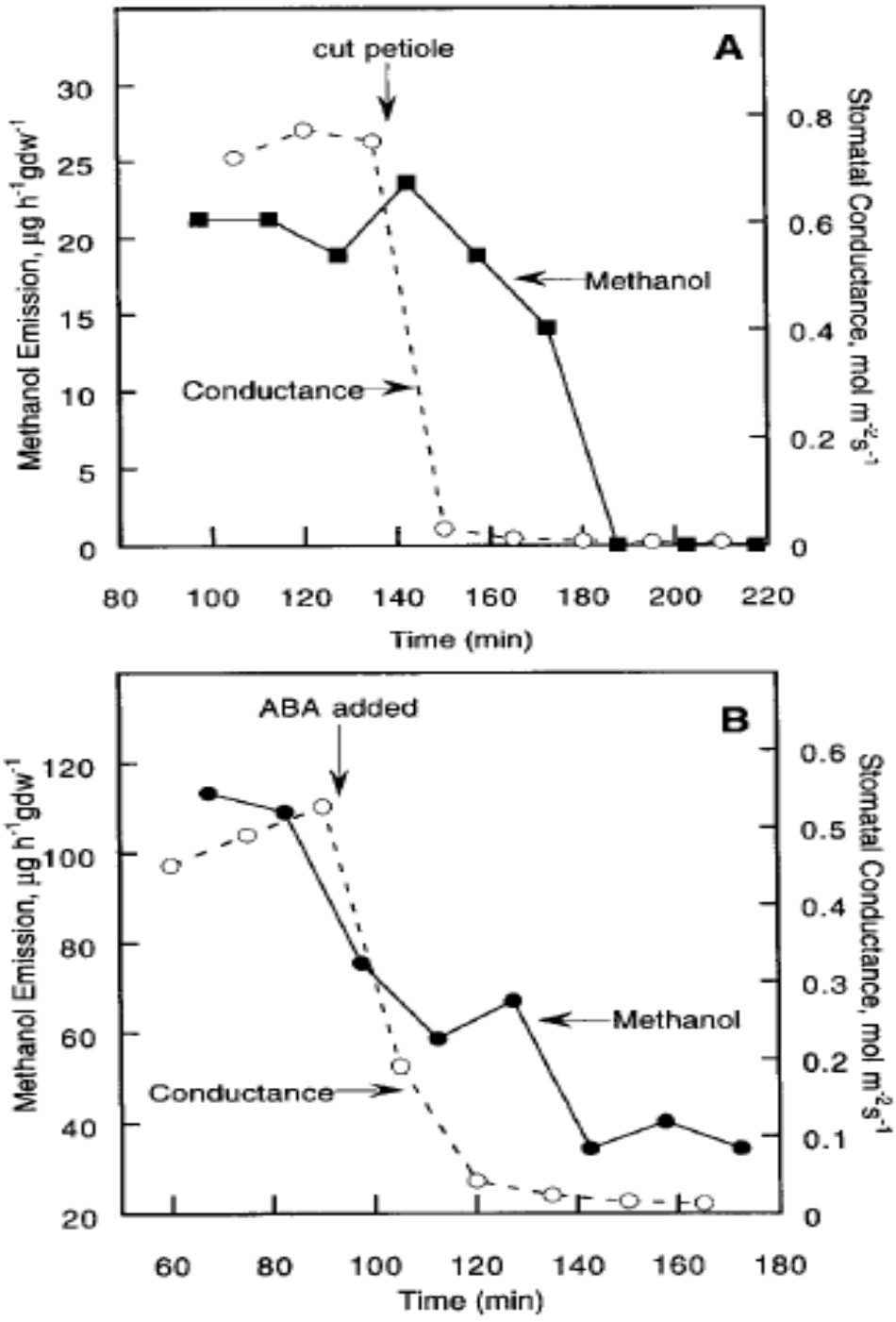


Figure 1.3: Relationship between methanol emission and stomatal conductance (Nemecek-Marshall et al., 1995). The two figures have showed the effect of excision of leaf petiole (A) and ABA treatment (B) on methanol emission and stomatal conductance. Both experiments have showed that methanol emission rates decreased with decreasing stomatal conductance.

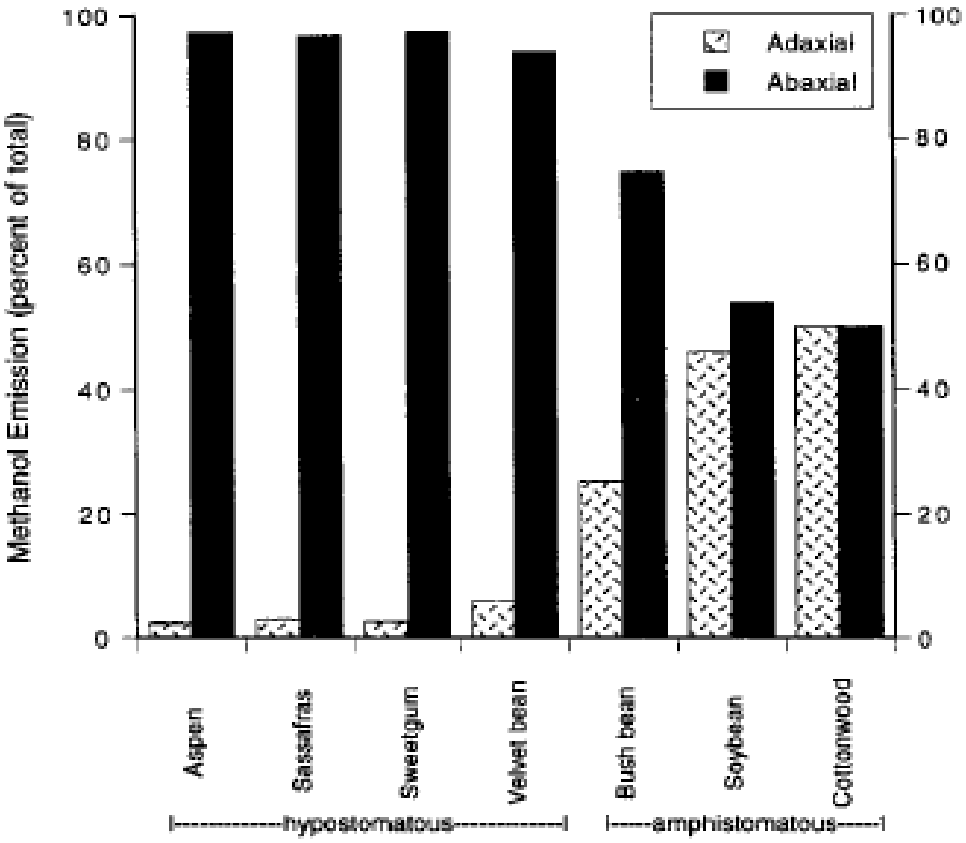


Figure 1.4: Methanol emissions from both leaf surfaces of hypostomatous and amphistomatous plants (Nemecek-Marshall et al., 1995).

1.3 Phyllosphere Microbiology

Despite the hostility of the phyllosphere environment, it is normally colonized by a variety of epiphytes, including bacteria, algae, fungi, yeasts, protozoa, and nematodes. Among them, bacteria are the most abundant inhabitants (Lindow & Brandl, 2003), and there are estimated on average to be 10^6-10^7 cells per square centimeter of leaf (Andrews & Harris, 2000; Beattie & Lindow, 1995; Hirano & Upper, 2000). The abundance of epiphytic bacteria differs among and within plants of the same species. In addition, the plant species influences the microbial carrying capacity of a leaf. For example, broad leaf plants (such as cucumber and beans) have a greater carry capacity of cultivable bacteria, than grasses or waxy broad leaf plants (Kinkel et al., 2000; O'Brien & Lindow, 1989). This variation is largely affected by

fluctuation of the physical and nutritional conditions of the phyllosphere. The phyllosphere physicochemical environment also selects for some bacterial populations, rarely found in the rhizosphere. For example, pigmented bacteria generally dominate leaf surfaces (Fokkema & Schippers, 1986; Stout, 1960a; Stout, 1960b), and common root colonizers such as *Rhizobium* (O'Brien & Lindow, 1989) and *Azospirillum* (Jurkevitch & Shapira, 2000) fail to become established on leaves. Of the environmental constraints, ultraviolet radiation (UVR) is particularly damaging to living organisms because photons of these wavelengths are of sufficiently high energy to cause direct damage to DNA. A study including UVR sensitivity analysis and survival strategies of a bacterial community from field grown peanut (*Arachis hypogeae* L.) phyllosphere, found significant numbers of pigmented organisms (Sundin & Jacobs, 1999). The minimal inhibitory dose (MIDc) of UVR of 56.1% of pigmented isolates was greater than that of *Pseudomonas syringae* 8B48, a UV tolerant bacterium. They concluded that pigmentation is a survival strategy on leaf surfaces under UV radiation. They also studied a non-pigmented strain of the genus C*urbobacterium*, which is highly UVR tolerant, and found colonization in the abaxial (lower) leaf surface, and concluded that for non-pigmented strains colonization of the abaxial leaf surface is an important phyllosphere strategy.

Many studies have investigated strategies for establishment of epiphytic bacteria in the phyllosphere, as it is generally a nutrient-poor environment (Leveau & Lindow, 2001), and found multiple physical and chemical factors limit their growth and survival in the phyllosphere. Many phyllosphere bacteria have traits or phenotypes which confer an ability to modify their microenvironment to increase nutrient availability for colonization and survival. For example, 50% of *Pseudomonas* strains produce compounds with surfactant properties (Bunster et al., 1989; Hutchison & Johnstone, 1993; Neu et al., 1990), like tolaasin and syringomycin (Hutchison et al., 1995), to increase the wettability which allows for solubilization and diffusion of substrates, making them more readily available. Extracellular polysaccharide (EPS) production is another survival strategy, by which epiphytic bacteria make a shield to protect themselves from desiccation stress (Costerton et al., 1995), and from reactive oxygen (Kiraly et al., 1997). Synthesis of alginate by *P. syringae* is stimulated by

desiccation stress (Keith & Bender, 1999; Singh et al., 1992) and H_2O_2 (Keith & Bender, 1999), and therefore contributes to the epiphytic fitness on dry leaves (Yu et al., 1999). High cell density induces cross-talk via quorum-sensing signals (Bassler, 1999; Pierson et al., 1998) which trigger neighboring microbes to express traits for their own benefit, increasing fitness on plants.

A community proteogenomics approach gave insights into the success of leaf surface microbial colonists (Delmotte et al., 2009). The predominant community members for all the plant hosts were *Methylobacterium* and *Spingomonas*, and porins and TonB receptor proteins (which allow active transport of substrates) were consistently seen from different plant hosts. Stress resistance was also common, the study found proteins such as superoxide dismutase, catalase, DNA protection proteins, chaperones, osmoprotectant trehalose formation proteins, reflecting adaptation to stressful conditions by various bacteria. There is also evidence that general stress response is an essential mechanism for plant colonization by *Methylobacterium* (Gourion et al., 2008; Gourion et al., 2006), and that it consists of a two-component response regulator, PhyR, that triggers, upon activation, the regulation of stress-related protein functions via a sigma factor of the EcfG family (Francez-Charlot et al., 2009). Protein flagellin for motility and fasciclin/beta-I_g-H_3 protein for cell-cell adhesion were also found, which are potentially important for bacteria living in the phyllosphere.

In conclusion, the phylloplane and its inhabitants undergo substantial changes of environment during different growing seasons (Ellis et al., 1999; Legard et al., 1994; Lilley et al., 1996; Thompson et al., 1995), and expressing traits for fitness. As such the potential for extensive mixing of genes in this community seems large. Suggesting that the leaf surface is a hot spot for horizontal dissemination of genetic information, and therefore, an interesting environment for studying diversity (Lindow & Leveau, 2002).

1.3.1 The Association between *Methylobacterium* and Plants

The genus *Methylobacterium* is an abundant colonizer of the phyllosphere (Knief et al., 2010a). They have been detected by cultivation-dependent methods (Corpe & Rheem, 1989; Elbeltagy et al., 2000; Mano et al., 2007; Omer et al., 2004b; Van Aken et al., 2004b), and by cultivation-independent methods (Araujo et al., 2002; Idris et al., 2004; Jackson et al., 2006; Pirttilä et al., 2000; Rasche et al., 2006). However, the degree of association with plants is varied. Published papers suggest that *Methylobacterium* are root-nodulating symbionts (Jaftha et al., 2002; Sy et al., 2001), endophytic (Elbeltagy et al., 2000; Lacava et al., 2004; Pirttilä et al., 2000; Van Aken et al., 2004b) and epiphytic (Corpe & Rheem, 1989; Hirano & Upper, 1991; Omer et al., 2004b) on plant surfaces. It is speculated that plant-*Methylobacterium* association is ancient (Kutschera & Niklas, 2005) and permanent (Fedorov et al., 2011), and that plant-associated *Methylobacterium* is a co-evolved phytosymbiont (Kutschera, 2007), due to their symbiotic interaction. Although this has only been seen with haploid cells of bryophytes, which is moisture dependent (Kutschera, 2007). However, this association mainly relies on methanol, which is a waste product of pectin metabolism (Fall & Benson, 1996) as discussed earlier. *Methylobacterium* spp. has also efficiently competed with other microorganisms for successful establishment in the phyllosphere. Two main traits, methylotrophy and general stress response, are found to be important in *Methylobacterium* for successful colonization on leaf surfaces (Gourion et al., 2008; Gourion et al., 2006; Sy et al., 2005). Quantitative studies, including whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis (WC-MS), conclude that more than 80% of viable bacteria isolated from leaf surfaces are members of the genus *Methylobacterium* (Corpe & Rheem, 1989; Fall & Benson, 1996; Tani et al., 2012c). Around 45 species of the genus *Methylobacterium* have been isolated from different environments (Table 1.1) to date. Half of the species are associated with plants and plant associated environments (highlighted in Table 1.1). And half of the new *Methylobacterium* spp. described in the last few years have been isolated from the phyllosphere namely *M. bullatum* (Hoppe et al., 2011), *M. funariae* (Schauer &

Kutschera, 2011), *M. gnaphalii* (Tani et al., 2012a), *M. marchanitae* (Schauer et al., 2011), *M. cerastii* (Wellner et al., 2012), *M. gossipiicola* (Madhaiyan et al., 2012), *M. longum* (Knief et al., 2012b), and *M. oxalidis* (Tani et al., 2012b).

1.3.2 The Role of Methylotrophy in Plant Colonization

Methylotrophy is the ability to use C1 compounds, reduced organic compounds containing no carbon-carbon bonds, as substrates. Several studies have established that C1 metabolism plays the key role in the ability of *Methylobacterium* to colonize plants (Jourand et al., 2005; Sy et al., 2005). Enzymes involved in methylotrophy of this organism have been identified and characterized in a metaproteomic study (Vorholt, 2002).This study indentified 4600 proteins and found a methanol based methylotrophy linked protein repertoire in the phyllosphere microbiota. First, methanol is oxidized by methanol dehydrogenase (MDH) in the periplasm of the cell to produce formaldehyde (CH_2O), which is then transferred into cytoplasm. In the cytoplasm, part of the formaldehyde is oxidized to carbon dioxide (CO_2) for energy generation, and part is assimilated via the serine cycle (as illustrated in Figure 1.5). *Methylobacterium nodulans* is the causal organism of nodulation of the *Crotalaria* genus of leguminous plants (Jourand et al., 2004), and possesses an *mxa* gene cluster for coding MDH. The expression and role of methylotrophy between *M. nodulans* strain ORS 2060^T and its host plant *Crotolaria podocarpa* were studied (Jourand et al., 2005). Results showed that an *M. nodulans* mutant, with the *mxaF* gene deleted (one of two key genes along with *mxaI* coding for MDH) resulted in up to 60% decrease in total root nodule number in *C. podocarpa*, up to 42% decreased nitrogen fixation capacity, and up to 46% reduction in plant total dry biomass, compared with the wild type strain. The symbiotic wild phenotype was restored with a non-methylotrophic mutant with a functional *mxaF*. When a *lacZ – mxaF* fusion was used, expression of methylotrophy genes in the root nodule was shown (Jourand et al., 2005).

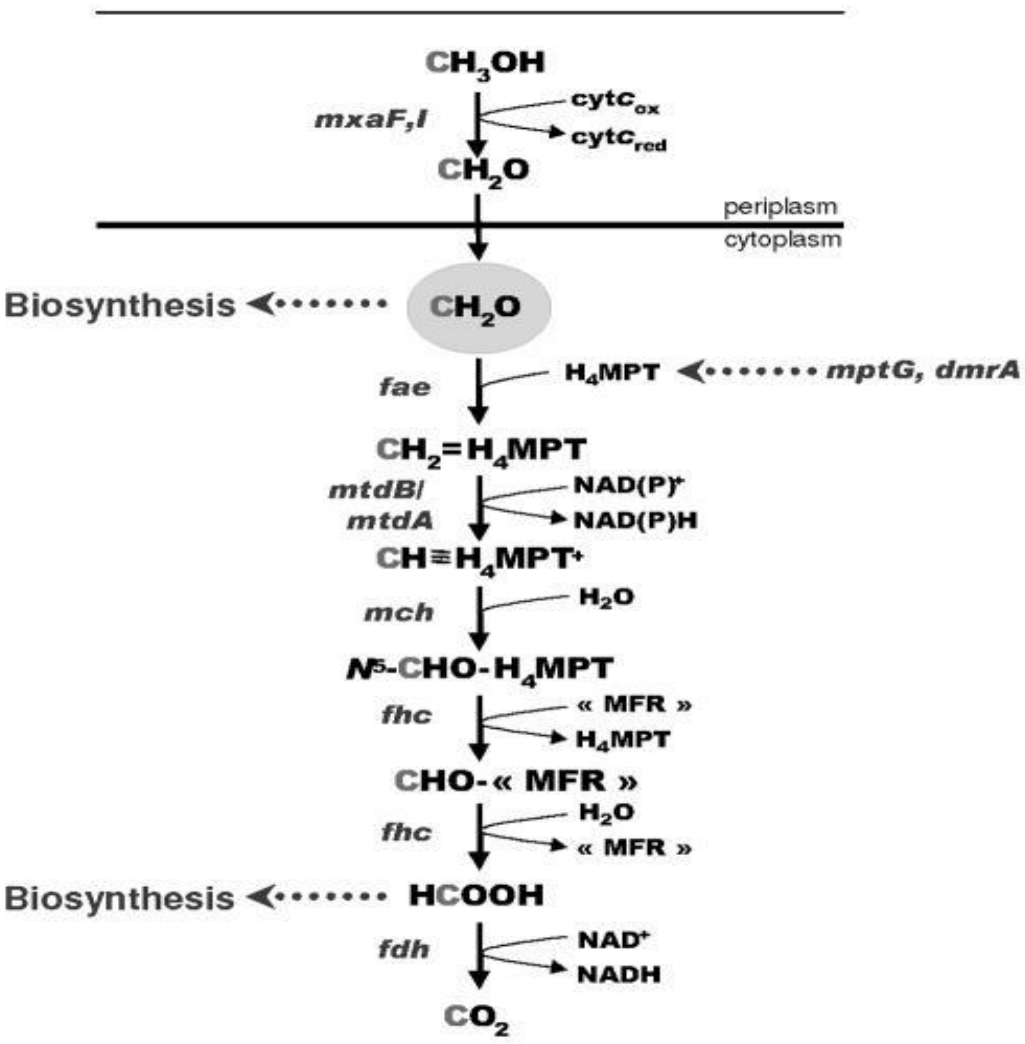


Figure 1.5: Carbon conversion in *Methylobacterium* during methylotrophic growth (Sy et al., 2005).

A similar study with *M.extorquens* AM1 and the model legume *Medicago truncatula* (Sy et al., 2005) found that the ability to use methanol as a carbon and energy source provides a selective advantage during colonization, with impaired fitness found with an *mxaF* mutant. They also found that some impaired mutants successfully colonize at the same level as the wild type and concluded that methanol is not the only carbon source supporting their association with plants. A proteogenomics study found an alternate gene for MDH, XoxF, which exhibits 50% sequence identity to MxaF (Delmotte et al., 2009). The biochemical properties of XoxF-MDH are ability to

oxidize formaldehyde and ethanol, different from methanol (Schmidt et al., 2010). It should also be noted that the majority of *M.extorquens* cell were found on the surface, on the lower abaxial side and also in intercellular spaces in the leaves (Sy et al., 2005). The phyllosphere habitat is known to be a constantly changing environment, and the same rate of adaptation of metabolic rearrangement happened with their inhabitants (Skovran et al., 2010). On the other hand, the quantity of methanol released from plants varies during the day and depends on the state of the stomata, methylobacteria have to continuously adapt their metabolism. XoxF is responsible for more rapid methanol oxidation and also decreases the concentration of formaldehyde (Schmidt et al., 2010), which is toxic product of methanol oxidation.

1.3.3 Synthesis of Bioactive Substances by Epiphytic Methylobacteria

There is speculation that aerobic methylobacteria are phytosymbionts (Trotsenko et al., 2001). There is evidence that *Methylobacterium* species can benefit from methanol that is released by plants, and may also grow on other plant-derived compounds (Abanda-Nkpwatt et al., 2006; Gourion et al., 2006; Sy et al., 2005). It is also apparent that these bacteria produce phytohormones such as cytokinins and auxins (Koenig et al., 2002; Omer et al., 2004a; Trotsenko et al., 2001), and stimulate seed germination and growth of certain plants (Abanda-Nkpwatt et al., 2006; Kutschera, 2007; Lee et al., 2006; Meena et al., 2012; Ryu et al., 2006). Cytokinins are adenine derivatives, and the key enzymes of cytokinin biosynthesis are isopentenyl transferases. In *Methylobacterium* spp., cytokinins are produced indirectly from the hydrolysis of tRNA molecules (Chen, 1997; Koenig et al., 2002; Morris, 1986) to produce isopentenylated adenine (zeatin). The product of the *miaA* gene is responsible for isopentenylation of a specific adenine in some tRNA. This was confirmed when an *M. extorquens* mutant (with deleted *miaA* encoding isopentenyl-tRNA synthetase) completely lost its ability to synthesize cytokinins (Koenig et al., 2002). But in a germination experiment in soybean seeds, the *miaA* mutant stimulated germination as well as the wild type bacteria.

Methylobacterium spp. have the ability to synthesize auxins (Indole-3-acetic acid, IAA) (Doronina et al., 2002a; Ivanova et al., 2001). The key enzymes of IAA biosynthesis are indole-3-pyruvate decarboxylase, tryptophan decarboxylase, tryptophan-2-monooxygenase, and tryptophan side-chain oxidase (Trotsenko et al., 2001). The availability of the complete genome sequence of *M. extorquens* AM1 (Fedorov et al., 2010) revealed an open reading frame (ORF) of the gene encoding a thiamine pyrophosphate (TPP) dependent decarboxylase of α-ketoacids, showing similarity to indolepyruvate decarboxylases (IpdC). The genus *Methylobacterium* use several pathways of IAA synthesis, which was confirmed by the absence of an IpdC-coding gene in the genome of *M. nodulans*, indicating great diversity of IAA biosynthetic pathways (Fedorov et al., 2010). An impaired IAA biosynthesis mutant of *M. extorquens* AM1, Δ*ipdC*, showed enhanced activity of the enzymes of C1 and central metabolism when auxin was added to the growth media. This indicates that *M. extorquens* AM1 can synthesize IAA, which serves as an autoregulator of bacterial metabolism during epiphytic growth (Fedorov et al., 2009).

The 1-aminocyclopropane-1-carboxylate (ACC) deaminase (*acds*) genes were found in many plant associated bacteria (Glick et al., 2007), and bacteria with this gene stimulate plant growth by decreasing the concentration of ethylene released by plants. Several studies support this phenomenon including: inoculation of *Brassica campestris* with *M. fujisawaense* (Madhaiyan et al., 2006) and *M. oryzae* (Madhaiyan et al., 2007b), and rice and tomato with *M. radiotolerans* (Chinnadurai et al., 2009). These strains are all known to contain ACC deaminase. PPFM also synthesize vitamin B_{12} (6-800 ng/l), and this accumulates intracellularly, especially when growing on methanol (Trotsenko et al., 2001). *M. mesophilicum* and *M. extorquens* G10 synthesize the largest amount of vitamin B_{12} (Ivanova et al., 2006), and stimulate plant growth. The stronger effect of PPFMs on germination and plant growth were found in native plants over invasive and suggested PPFMs could be useful in improving coastal sage scrub/ grassland restorations (Irvine et al., 2011).

1.3.4 Biotechnological Potential of Methylobacteria

The genomes of six strains of *Methylobacterium* were selected according to their key adaptations to different plant associated niches and environmental constraints for sequencing (Marx et al., 2012), which will facilitate the understanding of the key features behind these adaptations. Presence of the *nifH* gene in the genome of *M. nodulans* ORS 2060 was confirmed by genomic analysis, which is responsible for atmospheric nitrogen fixation (Jaftha et al., 2002; Sy et al., 2001). Detailed knowledge is available about their taxonomy, physiology, biochemistry, and the variety of enzymatic pathways for the specific metabolism (Chistoserdova, 2011). For example: large-scale cultivation of forage protein, RMP (ribulose-5-mono-phosphate) pathway are considered because of higher values of specific growth rate, economic coefficient, highest protein and lysine content and digestibility than RBP (ribulose-1,5-bis-phosphate) and serine pathways of metabolism (Chetina & Trotsenko, 1984; Doronina & Trotsenko, 1986). M. *extorquens* and other methylotrophic bacteria utilize the serine pathway to synthesis PHB and PHBV (Korotkova et al., 1997; Korotkova et al., 1999; Trotsenko, 1983; Trotsenko, 1985; Trotsenko & Belova, 2000). Other products such as EPSs (exopolysaccarides), ectoine, and S-layers production are also promising.

The other benefit of methylotrophs is their ability to degrade a broad range of highly toxic compounds such as: mono- and dichloromethane (Doronina et al., 1996a; Doronina et al., 1996b; Firsova et al., 2004; Trotsenko & Doronina, 2003; Zyakun et al., 2003). In addition, aerobic methylobacteria tolerate heavy metals such as nickel, cadmium, copper, zinc, chromium, mercury, lead and arsenic (De Marco et al., 2004; Dourado et al., 2012b; Idris et al., 2006), reduces nickel and cadmium toxicity to promote plant growth (Madhaiyan et al., 2007c) and colonizes and reduces phytotoxicity of torrefied grass fibre (Trifonova et al., 2008). The important factors for industrial ecology are the end products of biodegradation. Aerobic methylotrophs assure the complete utilization of toxic compounds without undesirable adverse effects to the environment (Trotsenko et al., 2005).

1.3.5 Phyllosphere Methylotrophic Bacterial Diversity

Phyllosphere methylotrophic communities are often difficult to fully characterize, mainly because the phyllosphere itself is a highly diverse environment. Plant associated methylotrophic bacterial diversity has been studied by several researchers worldwide, on different plant species using a variety molecular technique. For example, PCR-based DNA fingerprinting techniques like RAPD, ARDRA, BOX-PCR, ERIC-PCR, RISA on tropical plants (e.g. cotton, maize, sunflower, soybean, menthe) (Balachandar et al., 2008; Raja et al., 2008a), ARISA on *Arabidopsis thaliana* and *Medicago truncatula* (Knief et al., 2008; Knief et al., 2010a; Knief et al., 2010b), DGGE on *Trifolium repens* and *Cerastium holosteoides* (Wellner et al. 2011), and using 16S rRNA gene sequencing (Hiraishi et al., 1995).

The distribution of *Methylobacterium* spp. isolates from six different plant species, coffee, sugarcane, citrus, sweet pepper, eucalyptus and sweet broom weed, was analyzed by comparative phylogenetic analysis of the sequences of 16S rRNA (structural) and *mxaF* (functional) genes. Congruency was found between phylogeny and specific host inhabitance, which was higher for *mxaF* than for the 16S rRNA gene, overall, colonization of *Methylobacterium* was found to be host-species specific (Dourado et al., 2012a). A study of a oil-contaminated mangrove ecosystem by sequence analysis of 16S rRNA genes, found that oil spills selected for specific *Methylobacterium* spp. isolates, and suggested those isolates have the potential to bioremediate mangrove environments by immobilizing metals (Dourado et al., 2012b).

The isolation of *Methylobacterium* species from specific plant species (Table 1.1) highlights their niche-specificity diversity. Moreover, specific plant species carried significantly higher abundance and composition of *Methylobacterium* species (Wellner et al., 2011). Specific plant induced biofilm formation by *Methylobacterium* species has also been identified (Rossetto et al., 2011). Calculation of diversity indices, including the Shannon index of diversity (Shannon & Weaver, 1949), Pielou

index of evenness (Pielou, 1969), and Margalef index of richness (Margalef, 1958), based on ARDRA profiles and carbon utilization patterns of *Methylobacterium* isolates, showed that *Methylobacterium* populations vary between plant species (Balachandar et al., 2008; Raja et al., 2008b). Cultivation-independent analysis, ARISA tool performed that similar plant species harbor very similar *Methylobacterium* communities, which differ from other plant species grown at the same site indicating host species-specific *Methylobacterium* community composition (Knief et al., 2008). But plant sampling site has greater impact on colonization pattern on leaves and competition experiments showed that the efficiency of colonization depends on strain phylogeny rather than the geography and plant species (Knief et al., 2010a; Knief et al., 2010b).

From the above discussion it can be concluded that the ability of *Methylobacterium* spp. to dominate the phyllobacterial population is typical of many plant species and suggests that they are the most diverse and ecologically significant group of culturable phyllobacteria.

1.4 Molecular Approaches

Molecular approaches are driven by culture based and non culture based methods, which mainly employ the Polymerase chain reaction (PCR) to study biodiversity. PCR was developed by Kary Mullis in 1983 (Bartlett & Stirling, 2003) revolutionized the molecular era, and in 1993 awarded noble prize for his invention. *Methylobacterium* is cultivable genus and have established culture method and selective media (Corpe, 1985; Holland et al., 2000).

In the case of *Methylobacterium*-plant association, culture independent techniques have been employed by many scientists. One of the studies is Automated Ribosomal Intergenic Spacer Analysis (ARISA) and has been performed by Knief et al., in 2008; to get better insight in this ecosystem and recommend this method a useful tool to study *Methylobacterium* communities in different ecosystem. DNA was extracted

from environmental samples and pure culture as well. They have designed *Methylobacterium* genus specific primer which target 16S rRNA gene and used this primer and combination with reverse primer that binds to the $tRNA^{Ala}$ gene, which is located upstream of the 23S rRNA gene in the 16S-23S intergenic spacer (IGS). Different length of PCR products were obtained due to the length heterogeneity of the IGS of different *Methylobacterium* species. *Methylobacterium*-specific ARISA provides rapid comparisons of *Methylobacterium* communities (Knief et al., 2008; Knief et al., 2010a; Knief et al., 2010b). ARISA definitely is a high resolution fingerprinting method (von Mering et al., 2007). This method use 16S-23S rRNA inter-genic spacer, allow better differentiation of isolates of a particular bacterial species or genes (Acinas et al., 2004). The IGS has frequently been used to study of phylogeny, molecular evolution or population genetics and to differentiate between very closely related bacteria (Carr et al., 2003; Hirano & Upper, 2000; Yang et al., 2001). The length variability of the IGS has been used as a basis for more complex community profiling methods with environmental samples (Carr et al., 2003; Hirano & Upper, 2000; von Mering et al., 2007). Sequence analysis of the IGS revealed that every strain have rrn operons, in which two tRNA genes, $tRNA^{Ile}$ and $tRNA^{Ala}$ (Delmotte et al., 2009). Similar results observed with other taxa as well (Yang et al., 2001). For which, most species of the same genus and most strains of the same species showed differences in IGS sequence length. Same results also observed in different *Methylobacterium* strains and two species *M. chloromethanicum* CM4 and *M. dichloromethanicum* DM4 were proposed to reclassify as *M. extorquens* strain (Kato et al., 2005).

No other techniques implied in the molecular biology are fully advantageous. Every technique has some merits and demerits points. Researchers always find the techniques which are less laborious, less time consuming and mainly less cost-effective. In the case of ARISA, besides the advantages, it has some disadvantages like other techniques. It does not perfectly reflect the diversity of the community. The presence of different rrn operons can lead to overestimation of diversity. Secondly, a peak of a particular size usually can represent different strains. So, similar to other finger printing techniques, constructions of clone libraries are essential for linking

bacterial strains to peaks in ARISA patterns. In some strains, multiple different IGS copies were present and ARISA cannot differentiate the several highly similar sequences precisely (Delmotte et al., 2009).

1.4.1 16S rRNA is the molecular marker to study biodiversity

In all cells, ribosomal RNAs are a conserved and ancient molecule. These molecules are phylogenetically distant and are not influenced by environmental changes (Rosselló-Mora & Amann, 2001). The sequence of 16S rRNA gene is remarkably similar between organisms, which means that sequences from distantly related organisms can be aligned precisely, making differences easy to measure. For this reason, the 16S rRNA gene has been used extensively to determine evolutionary relationships (Barry et al., 1990; Rahman et al., 2004; Weisburg et al., 1991), and is less likely to be influenced by lateral gene transfer (Cole et al., 2003; Jain et al., 1999; Olsen et al., 1986; Woese et al., 1990).

In 1970, Carl Woese proposed the three domain system of classification – Archaea, Bacteria and Eucarya based on 16S rRNA gene sequences, and classified 11 bacterial divisions based on sequences of 16S rRNA from cultured microorganisms (Woese, 1987). The species concept of the prokaryotes, very important in microbiology, is based on 16S rRNA gene analysis, and is proposed that an organism (species) whose 16S rRNA gene sequence differs more than 3% from another organism (species) (less than 97% similar to any other sequence in the database) is considered a new species (Madigan et al., 2006). This gene is about 1,550 bp long and is composed of an alternating pattern of nine hypervariable and conserved regions (Neefs et al., 1990; VandePeer et al., 1996) (Figure 1.6 &1.7). Primers are designed to bind to conserved regions, and amplify variable regions. Conserved regions have been used to design universal primers for amplification of microorganisms, and variable regions can be used to distinguish species from one another.

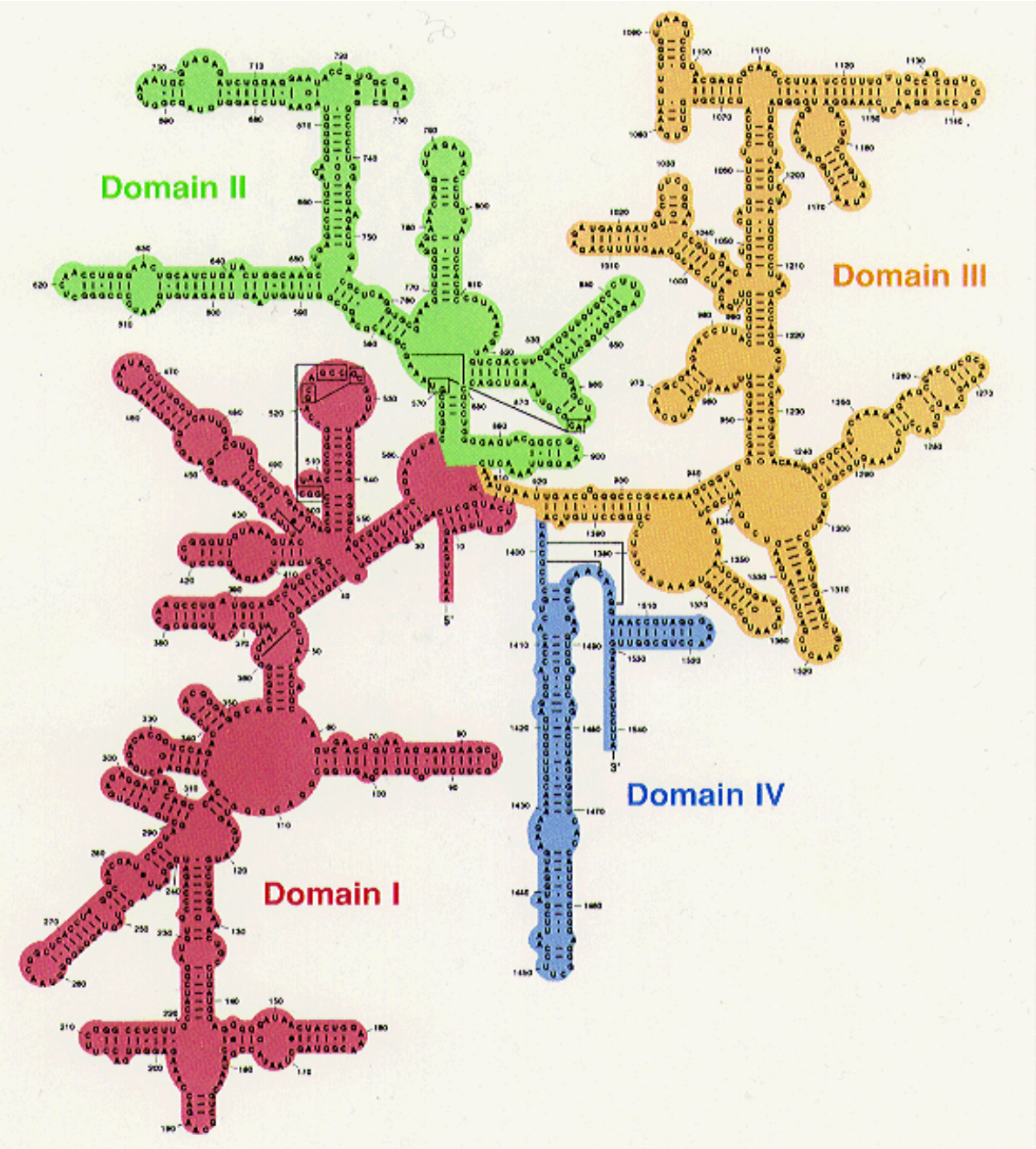


Figure 1.6: 16S rRNA molecule secondary structure (Garrett & Grisham, 2005). Red corresponds to the 5□ domain (nucleotides 27-556), green to the central domain (nucleotides 564-912), yellow (923-1391) and blue (1392-1541) correspond to the 3□ domain**.**

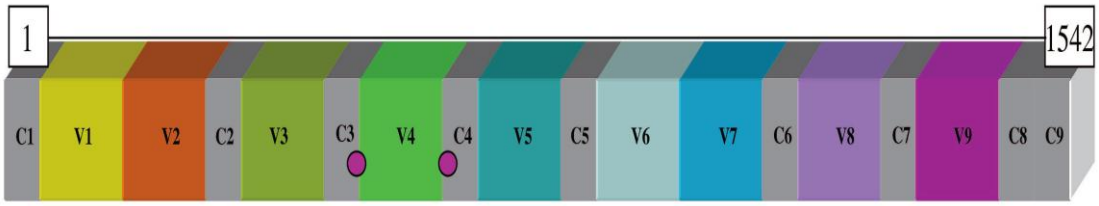


Figure 1.7: Conserved and hypervariable regions in the 16S rRNA gene (Petrosino et al., 2009). C1-C9 are conserved regions (shown in grey) and V1-V9 are variable regions (shown in variable colors).

Different bacterial species and even strains of a species can have dissimilar 16S rRNA gene sequences (Amann et al., 1990), and this variability can be used to assess diversity within a bacterial community. A huge number of 16S rRNA gene sequences are available via the internet through the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) and the Ribosomal Database Project (RDP) (www.cme.msu.edu/RDP/html/index.html), which also provides search algorithms to compare new sequences to these databases.

Use of the 16S rRNA gene also has drawbacks; differences in primer binding affinity can cause bias in PCR which can result in over-representation of some microbial species. Microorganisms often contain more than one copy of the 16S rRNA gene, if this is not taken into account during analysis there will be an over-representation of species containing more than one 16S rRNA gene. Heterogeneity (up to 5%) in 16S rRNA genes from the same organism has been reported (Amann et al., 2000; Mylvaganam & Dennis, 1992), which can also lead to errors in diversity measurements.

1.4.2 Restriction Fragment Length Polymorphism (RFLP)

Restriction endonucleases are enzymes that first recognize short, specific sequences (four, six and eight base pair sequences) within DNA molecules and precisely cut the DNA at that site. Generally, four base-cutter enzymes give greatest resolution because they are more common in the DNA molecule, resulting in relatively small

fragments being produced, providing a better chance of identifying single base alterations in a sequence (Semagn et al., 2006). RFLP can reveal a different pattern between two DNA sequences of an organism. Generally two individuals of the same species have almost identical genomes, but the insertion, deletion, point mutation, inversion, duplication and translocation of genetic material between individuals can generate differences in DNA sequences. Hence, digestion of DNA with restriction enzymes can result in fragments whose number and size can vary among individuals, populations, and species (Semagn et al., 2006). RFLP was first used to identify DNA sequence polymorphisms for genetic mapping of a temperature sensitive mutation of adenovirus serotypes (Grodzicker et al., 1975). Application of restriction analysis for amplified rRNA for identification of microorganisms has been described independently in many researchers (Deng et al., 1992; Gurtler et al., 1991; Henrion et al., 1992; Jayarao et al., 1991; Vaneechoutte et al., 1992; Vilgalys & Hester, 1990), and established a rapid and accurate method for bacteria identification at the species level on the basis of their phylogenetic relationship (Carlotti & Funke, 1994; Choudhury et al., 1994; Moyer et al., 1995; Ralph et al., 1993; Urakawa et al., 1999).

1.4.3 Aims, approach and outline of this research

Geographically, New Zealand is an isolated country, does not have any land boundaries, and has a unique flora, most of which are endemic. The climate is largely temperate, but the weather can change unexpectedly - four seasons a day. There is relatively little air pollution compared to many other countries, and UV rays are very strong during the summer months, due to a hole in the ozone layer, and the average rainfall is high (640-1500 mm). New Zealand is often considered a biodiversity hotspot, meaning the region is extremely biologically diverse with a high proportion of species not found elsewhere of the Earth. Previous research on the abundance of bacterial populations on pasture leaves from Claudelands Showground (Hamilton, New Zealand) concluded that Gram-negative pigmented flavobacteria dominate the leaf surface (Stout, 1960a). Until now, in New Zealand, no research has been undertaken to study of the diversity of *Methylobacterium* species associated with

plants. As these organisms are abundant in the phyllosphere, it is hypothesized that New Zealand native plant species will harbor diverse *Methylobacterium* species. The main objective of this study is to investigate the variation of *Methylobacterium* species from the phyllosphere of native plants. To address this study cultivable *Methylobacterium* were isolated from leaves of native plants, genomic DNA was extracted from pure isolates and PCR undertaken to amplify 16S rRNA genes. Restriction enzymes were used RFLP analysis the isolates and representative isolates were to be sequenced (outline of research is presented in Figure 1.8). And results are presented in Chapter 2 of this thesis.

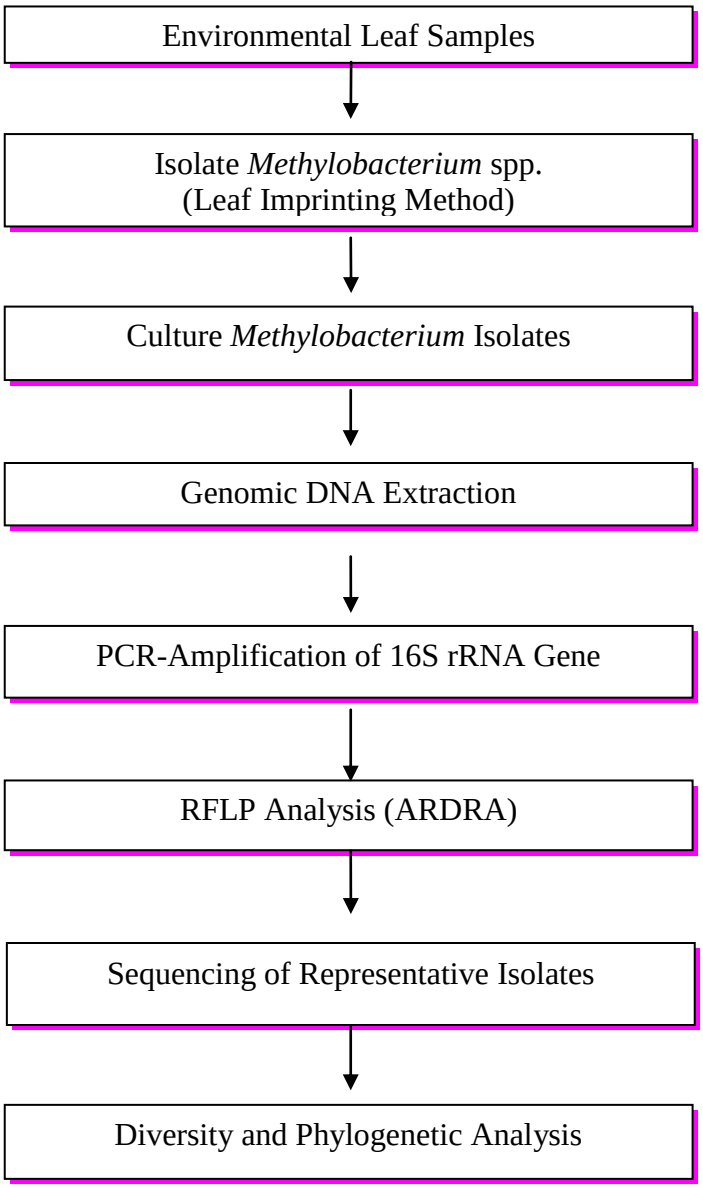


Figure 1.8: Outline of the research undertaken to study plant associated methylobacterial diversity.

Chapter 2: Diversity of *Methylobacterium* Species Associated with New Zealand Native Plants

2.1 Introduction

> *"Under the microscope, aerial plant leaves resemble eerie landscapes, with deep gorges, tall peaks and gaping pits that riddle the waxy surface. Add to this scenery a climate that features temperature highs of 50 °C [122 °F] or more, exposure to harmful ultraviolet rays, erratic periods of drought and limited access to nutrients, and one gets the picture that this is a hostile environment. Still, many bacteria, fungi, yeast and other microorganisms dwell in great abundance in this 'phyllosphere'…"*
>
> Leveau, J. (2009) "Microbiology: Life on leaves." *Nature* vol. 461, p. 741.

The term phyllosphere (above ground parts of plants) was first coined by Dr. Jakoba Ruinen (Ruinen, 1956). This is the Earth's biggest biological surface, estimated to be approximately two times bigger than the land surface (Woodward & Lomas, 2004). This surface is responsible for many essential processes including CO_2 fixation, O_2 release and primary biomass production (Delmotte et al., 2009). The physicochemical environment of the phyllosphere is highly diverse, due to fluctuating nutrient availability, water availability, temperature, wind pressure, exposure to pollutants, UV radiation, and the variable biology of the waxy protective layer of plant (plant cuticle). This diverse environment provides a habitat for many bacteria, it has been estimated that 10^{26} bacterial cell are living on every square centimeter (cm^2) of leaf globally (Lindow & Brandl, 2003). These inhabitants are called epiphytes and their importance is enormous. Global processes of carbon (C), oxygen (O_2) and nitrogen (N_2) cycling are greatly influenced by phyllosphere bacteria, as well as plant health; growth and productivity are also enhanced by phyllosphere inhabitants (Holland & Polacco, 1994; Lindow & Brandl, 2003; Lindow & Leveau, 2002; Trotsenko et al.,

2001). Conversely, the number and composition of phyllosphere bacteria is greatly influenced by plant species, sampling site, growing season, plant growth stage, location on the plant, leaf properties and surrounding plant species (Finkel et al., 2011; Hunter et al., 2010; Inceoglu et al., 2010; Kim et al., 2012; Kinkel et al., 2000; Knief et al., 2010b; Rasche et al., 2006; Rastogi et al., 2012; Redford et al., 2010; van Overbeek & van Elsas, 2008; Whipps et al., 2008; Yang et al., 2001). The alpha-proteobacterial genus *Methylobacterium* has been found to be a dominant group of bacteria in the phyllosphere (Delmotte et al., 2009). *Methylobacterium* spp. have been estimated at between 10^4-10^7 colony forming units (CFU) per gram weight of fresh plant material (Holland et al., 2002). *Methylobacterium* association with plants is varied from epiphytic to endophytic (Corpe & Rheem, 1989; Delmotte et al., 2009; Elbeltagy et al., 2000; Hirano & Upper, 1991; Knief et al., 2010b; Mcinroy & Kloepper, 1995; Pirttilä et al., 2000), and symbiotic (Jaftha et al., 2002; Sy et al., 2001). There are no reports of *Methylobacterium* as a causal agent of plant diseases. However a hypothesis that plant-associated methylobacteria are co-evolved phytosymbionts has been proposed (Kutschera, 2007).

The association of *Methylobacterium* spp. with plants relies on methanol (CH_3OH), a volatile organic compound (VOC) which is released by plants during growth through stomatal pores in the epidermis (Galbally & Kirstine, 2002; Hüve et al., 2007; Nemecek-Marshall et al., 1995). However some studies have shown that methanol is not the only reason for colonization of plants by *Methylobacterium* spp., other plant derived carbon compounds may support their colonization (Abanda-Nkpwatt et al., 2006; Delmotte et al., 2009; Gourion et al., 2006; Sy et al., 2005). Methanol is produced inside leaves as a byproduct of pectin metabolism during cell wall synthesis, and pectin methylesterases (PMEs) are important cell wall enzymes involved in modeling the pectin matrix. During cell elongation and division, PMEs catalyze the C-6 demethylation of homogalacturonan (HG) within plant cell walls. As a result, methanol is released and negatively charged carboxyl groups are created (Körner et al., 2009). The global methanol emission from plants is estimated to be 100-128 Tg per year (Galbally & Kirstine, 2002). The colonization of plants by methylotrophic bacteria, especially *Methylobacterium*, is of interest because they play

an important role in the atmospheric methanol cycle by utilizing methanol as their sole source of carbon and energy (Corpe & Rheem, 1989). They also produce plant growth promoting substances such as cytokinins, auxins and vitamin B_{12} (Doronina et al., 2002a; Ivanova et al., 2000; Ivanova et al., 2001; Ivanova et al., 2006; Omer et al., 2004a; Trotsenko et al., 2001). Several studies have reported their involvement in plant growth and development, including seed germination, root development and increasing yield of agricultural plants (Abanda-Nkpwatt et al., 2006; Lee et al., 2006; Meena et al., 2012; Ryu et al., 2006; Trotsenko et al., 2001; Verginer et al., 2010).

Members of the genus *Methylobacterium* are pink-pigmented facultative methylotrophs (PPFMs). The pink pigments are carotenoids mainly xanthophylls (Konovalova et al., 2007). They utilize one-carbon compounds, including methane (CH_4), methanol (CH_3OH), methylamine (CH_3NH_2), and formaldehyde (HCHO), and multi-carbon compounds containing no carbon-carbon bonds, as well as organic substrates with carbon-carbon bonds as sole source of carbon and energy (Green, 2006; Patt et al., 1976; Patt et al., 1974). They belong to the proteobacterial subgroup (class) Alpha-proteobacteria, order Rhizobiales, and family Methylobacteriaceae. They are strictly aerobic, Gram-negative and rod shaped. *Methylobacterium* are found worldwide on the leaves of many different plant species: including agricultural plants like cotton, clover, maize, rice, soybean, sunflower, and winter wheat (Balachandar et al., 2008; Delmotte et al., 2009; Knief et al., 2010b; Madhaiyan et al., 2007a; Madhaiyan et al., 2009; Omer et al., 2004b; Raja et al., 2008b); grasses, weeds, herbs, mosses and liverwort (Austin & Goodfellow, 1979; Hoppe et al., 2011; Knief et al., 2008; Schauer et al., 2011; Schauer & Kutschera, 2011; Tani et al., 2012a; Tani et al., 2012b); flowering plants (Idris et al., 2006; Knief et al., 2012b; Wellner et al., 2012) and trees (Doronina et al., 2004; Kang et al., 2007; Knief et al., 2010a; Van Aken et al., 2004a).

Research using cultivation independent and dependent methods has shown that species composition of *Methylobacterium* is varied from plant to plant. Results of both methods have shown that different plant species harbor different complexities of *Methylobacterium* species (Balachandar et al., 2008; Knief et al., 2008; Raja et al.,

2008b). *Methylobacterium* species composition was found to be similar on the same plant species at the same sampling site, but was different on other plant species at the same sampling site. However the sampling site was shown to be a stronger influence than plant species on the *Methylobacterium* community composition (Knief et al., 2010b). The stronger impact of the site on phyllosphere community composition was also supported by other findings (Dourado et al., 2012b; Finkel et al., 2011; Rastogi et al., 2012; Wellner et al., 2011).

Geographically, New Zealand is a large island lying in the Southwestern Pacific Ocean, and has been isolated from other landmasses for millions of years. The climate is mostly cool to temperate, with a strong maritime influence. The weather fluctuates very rapidly, even within a day, and can experiences very strong winds and harsh UV exposure (Warlond, 2009). These unique features and long isolation make New Zealand a biodiversity hotspot, most of the native plants (80%) are endemic – found nowhere else on Earth.

From the light of previous studies we hypothesize that native endemic plants of New Zealand will harbor methylobacteria. Therefore, the primary objective of this study is to investigate the diversity *Methylobacterium* species in the phyllosphere of native plants of New Zealand. This will be achieved via the isolation of methanol utilizing bacteria, and 16S rRNA gene amplification and sequencing, which has been used as a powerful molecular technique to discriminate species level diversity of methylotrophs (Tsuji et al., 1990). Amplified ribosomal DNA restriction analysis (ARDRA) will be used to select appropriate isolates for sequencing. ARDRA is a powerful technique to discriminate microbial diversity at the species level, and involves amplification of the 16S rRNA gene, followed by digestion with selected restriction enzymes (Heyndrickx et al., 1996). This will be significant as the first exploration of species composition of the genus *Methylobacterium* in the phyllosphere of native plants in New Zealand.

2.2 Materials and Methods

2.2.1 Sample collection and isolation of *Methylobacterium* species

Twenty four different native New Zealand plants were selected for isolation of *Methylobacterium* species (Table 2.1). Growing leaves of each plant species were collected in sterile snap lock bags at different times during 2011 to 2012. The majority of samples were collected from the campus of the University of Waikato, Hamilton, New Zealand, where they grow naturally. Leaves were excised and handled aseptically, and depending on leaf size, several leaves were used for bacteria isolation. Large leaves were cut into desired size from different portion of the leave surface and small leaves were used as a whole. The plants were chosen to represent a diversity of plant types: trees (*Agathis australis, Alectryon excelsus, Griselinia littoralis, Knightia excelsa, Litsea calicaris, Melicystis ramiflorus, Metrosideros excelsa, Pittosporum tenuifolium, Plagianthus regius,* and *Podocarpus totara*); shrubs (*Coprosma robusta, Hebe elliptica, Macropiper excelsum, Oleria albida,* and *Pomaderris kumeraho*); herb species (*Olearia traversii*); ferns (*Asplenium oblongifolium, Blechnum novea-zealandiae, Cyathea cunninghamii, Cyathea dealbata, Cyathea medullaris,* and *Nephrolepis cordifolia*) and flax (*Phormium cookianum* and *Phormium tenax*).

Leaf-imprinting techniques were used to isolate *Methylobacterium* species from the plant phyllosphere (Corpe, 1985; Holland et al., 2000). Immediately after collecting the leaves, they were laid directly on the surface of the 0.5% methanol supplemented ammonium mineral salt medium (AMS) (Corpe & Basile, 1982) and impressed carefully. After making an impression, the leaves were carefully lifted from the surface of the medium and discarded. Individual plates were sealed around the edge with parafilm and incubated at 30^{o}C for up to 2 weeks (Holland & Polacco, 1992; Holland & Polacco, 1994) as they are relatively slow growers (Corpe, 1985; Corpe & Rheem, 1989).

Ammonium mineral salts (AMS) agar medium contained: 0.7 g/l dipotassium orthophosphate (K_2HPO_4), 0.54 g/l ammonium chloride (NH_4Cl), 0.1 mg/l zinc sulfate ($ZnSO_4.7H_2O$), 0.03 mg/l manganese chloride ($MnCl_2.4H_2O$), 0.3 mg/l orthoboric acid (H_3BO_3), 0.2 mg/l cobalt chloride ($CoCl_2.6H_2O$), 0.01 mg/l copper chloride ($CuCl_2.2H_2O$), 0.02 mg/l nickel chloride ($NiCl_2.6H_2O$), 0.06 mg/l sodium molybdate ($Na_2MoO_4.2H_2O$), 15.0 g/l Difco^{TM} agar (1.5%), and 1.0 L distilled water (see Appendix A for further details). pH was adjusted to 6.8 and sterilized in an autoclave. After sterilization, and when the media had cooled, 5 ml (0.5%) of sterile methanol was added aseptically and mixed thoroughly before plates were poured. After incubation, colonies were chosen randomly from plates and streaked and re-streaked 5-6 times on agar plates of the same composition to obtain a pure culture. A total of 329 pure culture plates were obtained.

Table 2.1: Plant species used in this study

Scientific name	Common name	Family
Agathis australis	Kauri pine	*Araucariaceae*
Alectryon excelsus	Titoki	*Sapindaceae*
Asplenium oblongifolium	Shining spleenwort	*Aspleniaceae*
Blechnum novea-zealandiae	Kiokio	*Blechnaceae*
Coprosma robusta	Coprosma	*Rubiaceae*
Cyathea cunninghamii	Gully tree fern	*Cyatheaceae*
Cyathea dealbata	Silver tree fern/ponga	*Cyatheaceae*
Cyathea medullaris	Black tree fern/mamaku	*Cyatheaceae*
Griselinia littoralis	Griselinia	*Griseliniaceae*
Hebe elliptica	Hebe	*Plantaginaceae*
Knightia excelsa	Rewarewa	*Proteaceae*
Litsea calicaris	Mangeao	*Lauraceae*
Macropiper excelsum	Kawakawa	*Piperaceae*
Melicystis ramiflorus	Mahoe	*Violaceae*
Metrosideros excelsa	Pohutokawa	*Myrtaceae*
Nephrolepis cordifolia	Tuber sword fern	*Polypodiaceae*
Olearia traversii	Catham island akeake	*Asteraceae*
Oleria albida	Daisy bush	*Asteraceae*
Phormium cookianum	Mountain flax/Whararikí	*Hemerocallidaceae*
Phormium tenax	Common flax/Harakeke	*Hemerocallidaceae*
Pittosporum tenuifolium	Kohuhu/Kohukohu	*Pittosporaceae*
Plagianthus regius	Ribbon wood/Manatu	*Malvaceae*
Podocarpus totara	Golden totara	*Podocarpaceae*
Pomaderris kumeraho	Kumarahou	*Rhamnaceae*

2.2.2 Extraction of chromosomal DNA from pure cultures

Genomic DNA was extracted from pure cultures using either a boiling method or the Marmur method (Marmur, 1961). The boiling method was used to extract DNA from all isolates for ARDRA analysis, and the Marmur method was used to extract DNA from those isolates selected by ARDRA for sequencing.

The boiling method is simple and quick. Usually high temperatures break the cell walls and cell membranes (Goodwin & Lee, 1993; Hofler, 1994; Jose & Brahmadathan, 2006; Merk et al., 2006; Soumet et al., 1994; Strus, 1997). In this study, 1-2 colonies were picked with a sterile toothpick into a 1.5 ml eppendorf tube containing 1 ml of MQH_2O. The tube was vortexed vigorously until fully mixed, boiled for 10 min, centrifuged for 5 min at 1000 rpm, then kept in an ice. Five microlitres of supernatant was used as PCR template immediately after boiling and centrifuging.

Prior to extraction of DNA using the Marmur method isolates were grown in 50 ml AMS broth at 30^{o}C. The 50 ml cultures were transferred into 50 ml sterile falcon tube and centrifuged for 3 min at 3000 rpm. After centrifugation, the supernatant was removed and the cell pellet was resuspended in 400 µl SET (Tris-Sucrose-EDTA) buffer. Lysozyme solution was added (20 µl) and incubated at 37^{o}C for 1 hour. After incubation, 20 µl of 20% SDS (Sodium dodecyl/lauryl sulfate) and 10 µl of proteinase K solution were added to the lysates and incubated at 60^{o}C for 3 hour. After incubation, the digested lysates were purified by phenol chloroform extraction and concentrated by ethanol precipitation. The extracted DNA was stored at -20^{o}C. DNA concentration was quantified using a NanoDrop^{TM} 1000 Spectrophotometer and concentration was adjusted to 50-60 ng/µl with TE (Tris-EDTA) buffer and stored at -20^{o}C. The details of the protocol and reagents involved in this procedure are presented in Appendix B.

2.2.3 PCR amplification of 16S rRNA genes

Polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene from extracted DNA using the universal eubacterial forward primer 27F (EubB) (5'- AGA GTT TGA TCM TGG CTC AG -3') and reverse primer 1492R (5'- TAC GGY TAC CTT GTT ACG ACT T -3') (Lane, 1991). The nucleotide ambiguity codes M and Y are represents amino (C & A) and pyrimidine (C & T) respectively.

For each isolate, 5 µl of template DNA (supernatant product of the boiling method) was added to 45 µl PCR master mixes in a 200 µl thin walled PCR reaction tube. Master mix of each reaction contained 24.75 µl of milli-Q water, 5 µl of 10x PCR buffer (Invitrogen Ltd, New Zealand), 5 µl of $MgCl_2$ (50 mM) (Invitrogen), 5 µl of 2 mM dNTPs (Invitrogen), 2 µl of 10 µM of each primer (27F and 1492R) (Integrated DNA Technologies, Inc), 1 µl of bovine serum albumin (BSA) (Promega Corporation, USA), and 0.25 µl of Taq DNA polymerase (Invitrogen). The PCR amplification was run on a Bio-Rad DNA Engine® (PTC-200) Peltier Thermal Cycler (Bio-Rad Laboratories Inc, Hercules, CA) in a total reaction volume of 50 µl. The thermal cycling conditions consisted of 35 cycles (30 sec at 94^{o}C, 30 sec annealing at 60^{o}C, and 1 min 30 sec at 72^{o}C) with an initial denaturation of 2 min at 94^{o}C and a final elongation step of 10 min at 72^{o}C. An *E. coli* positive and 2 negative control was run alongside of every PCR.

2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualize PCR products. Detailed procedures are in Appendix C. Five microlitres of PCR products were combined with 2 µl of 10X loading dye (Invitrogen) and loaded into the wells at the top of the gel. To estimate the size of the PCR products, an aliquot (3 µl) of 250 bp DNA ladder (Invitrogen) was also loaded onto each gel. Gels were run for 40 min at 80 V. After running, the gels were removed from the gel box, stained with ethidium bromide solution (15 min)

and destained with water for 15 min. Visualizing and imaging of gels was carried out by UV transillumination.

2.2.5 Amplified ribosomal DNA restriction analysis (ARDRA)

The comparison of RFLP fingerprints of amplified 16S rRNA genes is an established method of determining taxonomic relatedness between isolates (Broda et al., 2000; Heyndrickx et al., 1996; Pukall et al., 1998; Swaminathan & Barrett, 1995). Each isolate was screened by ARDRA to select representative isolates for sequencing. 16S rRNA PCR amplicon of each isolate was digested with the four base cutter endonuclease, Afa I (Rsa I) (Invitrogen) in a total reaction volume of 20 µl. Each digestion contained in 10 µl of PCR product, 2 µl of 10X buffer T (BSA free), 2 µl of 0.1% BSA, 0.5 µl of restriction enzyme and 5.5 µl of sterile distilled water. Digestion was performed for 3 hours at 37^{o}C, and inactivation of the enzyme was at 65^{o}C for 20 min according to the manufacturer's protocol. After digestion, products (20 µl) were analyzed by agarose gel electrophoresis using 2% agarose in 1X Tris acetate EDTA (TAE) buffer, and visualized with ethidium bromide by UV transillumination. A 1 kb+ DNA marker (Invitrogen) was run on every gel to identify the size of the restriction fragments. All isolates were compared visually for matching fingerprints and grouped with each group representing a different restriction types. Representative isolates from each restriction group were selected randomly for sequencing.

2.2.6 Sequencing of 16S rRNA genes

PCR products of isolates selected for sequencing were purified using ExoSAP. This technique involves two enzymes: Exonuclease I, which removes leftover primers, and Shrimp Alkaline Phosphatase, which removes unincorporated dNTPs (Bell, 2008). According to the manufacturer's protocol, 1.5 µl of ExoSAP mix was added to 10 µl of PCR amplicon, and incubated for 30 min at 37^{o}C, followed by 15 min at 85^{o}C for complete inactivation of both enzymes. Sequencing reactions were performed at the

Waikato DNA Sequencing Facility (WDSF) using both primers (27F and 1492R). DNA sequences were resolved using an ABI 3130XL (Applied Biosystems) fitted with 50 cm capillary arrays. All sequences are presented in Appendix D.

2.2.7 DATA analysis

Each 16S rRNA gene sequence was searched for closest identities with BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990). Alignment of the 16S rRNA gene sequences and phylogenetic analysis was performed using the ARB software program (Ludwig et al., 2004), and PHYLIP (Felsenstein, 1989). Distances were obtained using the distance options according to the Kimura's two-parameter distance model (Kimura, 1980). Phylogenetic trees were constructed with the neighbor-joining method (Saitou & Nei, 1987).

2.3 Results

2.3.1 Isolation of *Methylobacterium* species

In order to investigate *Methylobacterium* species diversity from New Zealand native plants, a total of 24 plant species from different plant types (tree, shrub, herb, fern and flax) were selected. Most samples (75%, 18 out of 24) were collected from naturally growing plats on the campus of The University of Waikato, Hamilton, New Zealand. The other six samples (25%) were collected from the native nursery, The Plant Depot, Hamilton, Waikato, New Zealand. Different numbers of leaves were used to isolate bacteria because the size and shape of the leaves from different plants were not the same. In the case of *Phormium cookianum* and *P. tenax*, different portions of the leaf were used for isolations. After 10-14 days of incubation of leaf imprinted plates at 30^{o}C, colonies were selected for streaking on fresh plates and over an extended time period these were restreaked 4-5 times, resulting in the isolation of 329 pure cultures of methanol grown strains. Among them, 266 strains (83.4%) were pale pink to vivid pink pigmented, 38 isolates (11.6%) were cream in color and 25 isolates (7.6%) were

dark orange to red in color. Methylotrophs were isolated from leaves of every plant species, but the abundance of methylotrophs varied from species to species (Figure 2.1). The highest abundance of isolates were from *Micropiper excelsa* (68 isolates, 20.7%) followed by *Plagianthus regius* (57 isolates, 17.3%), *Phormium tenax* (50 isolates, 15.2%), *Phormium cookianum* (35 isolates, 10.6%), and *Asplenium oblongifolium* (18 isolates, 5.5%) and the lowest abundance was from *Blechnum novea-zealandiae* (0.3%), *Knightia excelsa* (0.6%), *Litsea calicaris* (0.6%), and *Podocarpus totara* (0.6%). All 329 bacterial isolates were investigated further in this diversity study because all were grown on methanol supplemented AMS medium, even though the majority of *Methylobacterium* spp. is known to be pink-pigmented.

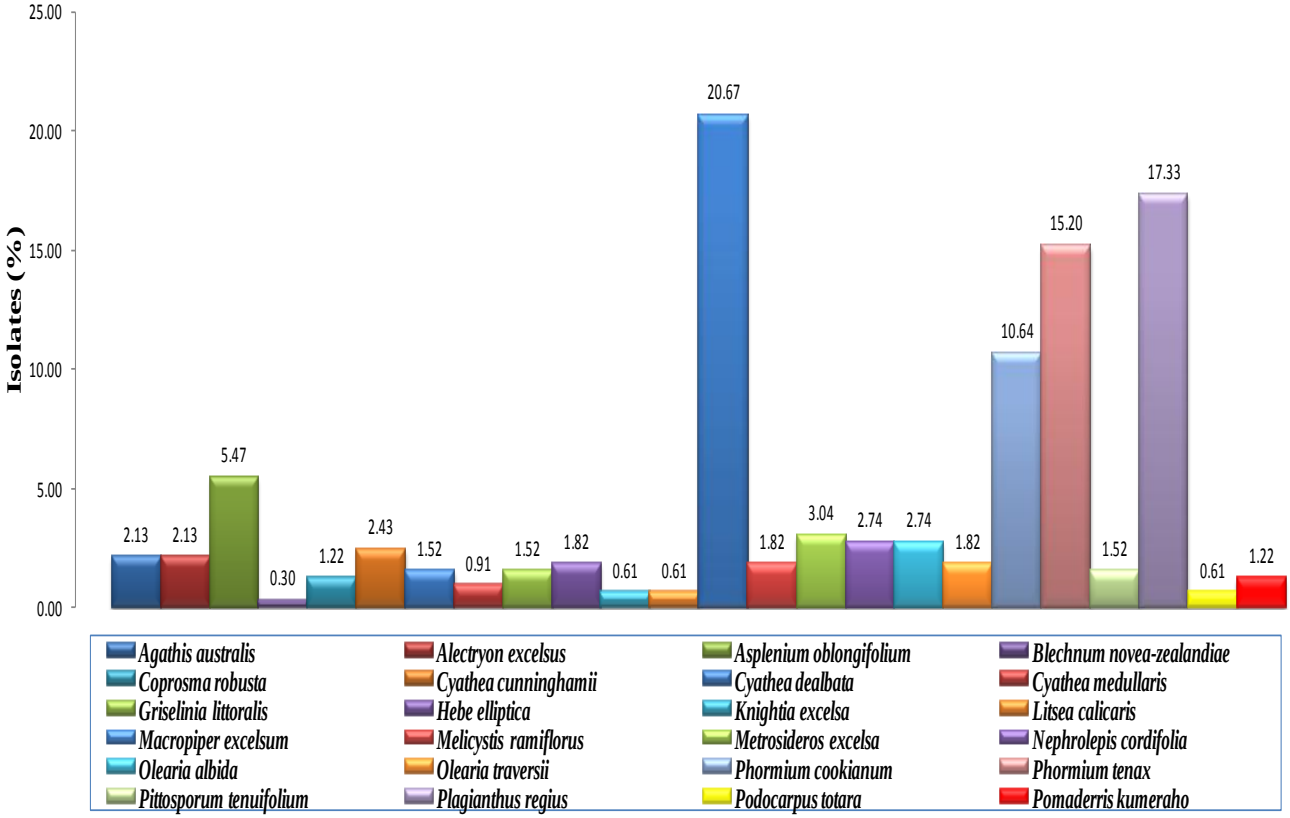


Figure 2.1: Plant species and abundances of methanol utilizing isolates.

2.3.2 PCR amplification and ARDRA analysis

The 16S rRNA gene was amplified from all 329 isolates using the universal bacterial primers (27F and 1492R). In the amplification step, 72 isolates (21.9%) were not amplified. Eight isolates from three plant species namely *Litsea calicaris*, *Podocarpus totara*, and *Pomaderris kumeraho* (2, 2 and 4 respectively) did not show any amplification at all. Therefore, they were not used in further studies. A total of 249 amplicons (75.7 %) from 21 plant species were used for ARDRA analysis.

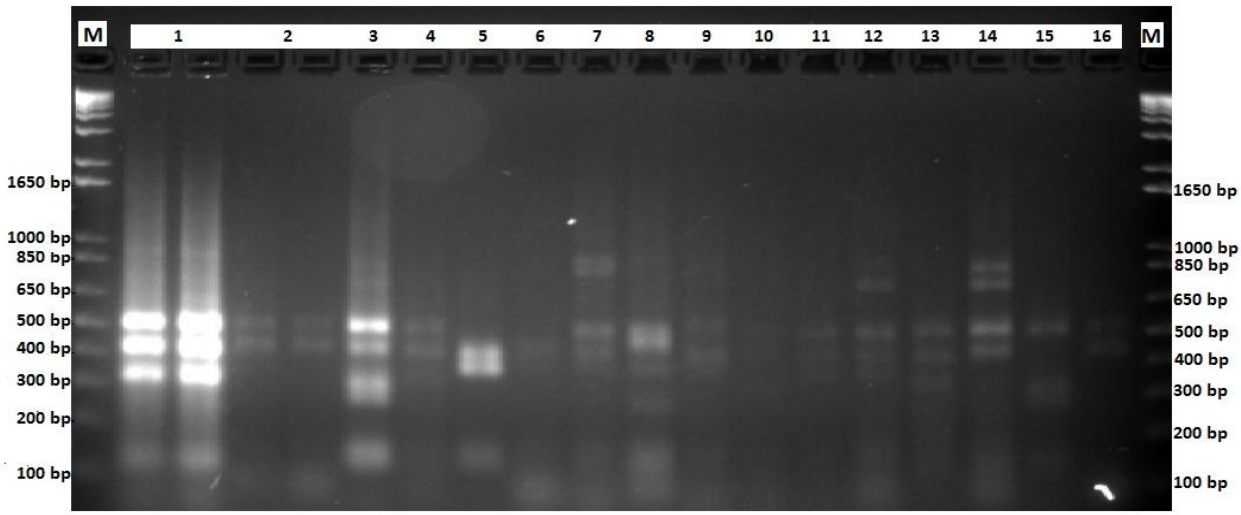


Figure 2.2: The sixteen different ARDRA patterns of the isolate. M represents 1kb+ DNA marker. The lane number corresponds to the ARDRA pattern listed in Table 2.3.

ARDRA analysis using a four base cutter restriction enzyme (Afa I) resulted in a number of different restriction patterns, reflecting the diversity of methylotrophs isolated from the different plant species (Figure 2.2 and Table 2.2). Isolates with identical restriction patterns were grouped together in one operational taxonomic unit (OTU). For all 249 isolates, a total of 16 different OTUs were identified (Table 2.3). The majority of isolates grouped in four OTUs, OTU 2 with 108 isolates (43.4%),

OTU 1 (67 isolates, 26.9%), OTU 3 (27 isolates, 10.8%), and OTU 16 (16 isolates, 6.4%). OTUs 5, 9 and 10, each had 5 isolates (2.0%) followed by OTU 13 (4 isolates, 1.6%), OTU 8 (3 isolates, 1.2%), OTU 4 and 15 (2 isolates of each, 0.8%), and the remaining OTUs had only one isolate (0.4%). From the ARDRA analysis a total of 34 isolates were selected randomly as representatives from each OTU for sequencing.

Table 2.2: Sizes of the major restriction fragments of the sixteen OTUs

Operational taxonomic unit	Fragment size (bp)
1	525, 425, 325
2	500, 400
3	500, 400, 325, 275
4	425, 400, 375, 250
5	430, 375
6	450, 350
7	725, 700, 500, 300
8	500, 450, 350, 250
9	550, 425, 375
10	500, 400, 350, 300
11	500, 425, 350
12	700, 500, 400, 350
13	500, 400, 300
14	800, 700, 500, 425
15	500, 325, 275
16	550, 450

2.3.3 Analysis of 16S rRNA gene sequences

The 16S rRNA genes of 34 representative isolates were sequenced. BLAST analysis was undertaken via blastn through the National Center for Biotechnology Information

(NCBI) (http://www.ncbi.nlm.nih.gov). BLAST analysis revealed that most of the isolates (79.1%, 197 of 249) were members of the genus *Methylobacterium*, which is typically the dominant Alpha-proteobacterial methanol utilizer genus found in association with plant leaves. Among the isolates belonging to the genus *Methylobacterium*, the majority (54.8%, 108 of 197) belonged to OTU 2 and had 98-99% similarity to *M. komagatae*, *M. mesophilicum*, *M. brachiatum*, and *M. phyllosphaerae*; followed by OTU 1 with 34.0% of isolates (67 out of 197) with 99-100% similarity to *M. marchantiae* and *M. adhaesivum*; OTU 16 with 8.1% isolates (16 out of 197) with 99% similarity to *M. oryzae*, *M. radiotolerans*, *M. tardum*, and *M. fujisawaense*; OTU 13 with 2.0% isolates (4 out of 197) with 99% similarity to *M. zatmanii* and OTU 4 with 1.0% isolates (2 out of 197) with 97% similarity to *M. marchantiae*.

The remaining isolates were related to other genera of bacteria, this included 29 isolates (11.6%, OTUs 3 and 15) which were members of the genus *Hyphomicrobium*, which also belongs to the class Alpha-Proteobacteria. These isolates were either more closely related to *H. methylovorum* (OTU 3) or *H. facile* (OTU 15). The isolates in OTU 10 were closely related to *Methylopila musalis* (83%), isolates in OTU 14 to *Rhizobium endophyticum* (95%), isolates in OTU 5 were closely related to the Beta-proteobacterial species *Methylophilus rhizosphaerae* (98%), the isolate in OTU 6 was most closely related to the Beta-proteobacterial genus *Ramlibacter* sp. (99%), the isolate in OTU 7 was related to the Beta-proteobacterial species *Achromobacter xylosoxidans* (94%), and the isolate in OTU 12 was related to Gamma-proteobacterial genus *Xanthomonas* sp. (84%).

Only three isolates from OTU 8 (1.2%) were closely related to actinobacterial species *Janibacter melonis* (94% identity). The isolates from OTU 9 were closely related to the Bacteroidetes species *Niastella populi* (99%). Only one isolate from OTU 11 belonged to the class Firmicutes and showed 99% similarity to *Paenibacillus lautus*.

Table 2.3: BLAST analysis of sequences derived from PCR amplification of 16S rRNA from the sixteen OTUs

Restriction type (OTU)	Isolates (%)	Isolate code	Isolation source	Closest species	Accession number	Identity (%)	Number of matched bp
I (1)	26.9	kk002 kk040 rw104	*Macropiper excelsum* *Macropiper excelsum* *Plagianthus regius*	*M. marchantiae* *M. adhaesivum*	FJ157976 GU992364	100	619/619
		kh130	*Pittosporum tenuifolium*	*M. marchantiae*	AB698714	99	677/680
		cf283	*Phormium tenax*	*M. adhaesivum*	AB698724	100	461/461
II (2)	43.4	kk034	*Macropiper excelsum*	*M. komagatae*	AB703238	98	550/564
		kk036 rw080 pk208	*Macropiper excelsum* *Plagianthus regius* *Metrosideros excelsa*	*M. mesophilicum* *M. brachiatum*	AM910537 AB698745	99	822/832
		ss136	*Asplenium oblongifolium*	*M. phyllosphaerae*	NR 044105	99	641/649
III (3)	10.8	kk004 kk063 ss288 db262 mf303	*Macropiper excelsum* *Macropiper excelsum* *Asplenium oblongifolium* *Olearia albida* *Phormium cookianum*	*Hyphomicrobium methylovoram*	AB680579	99	711/719
IV (4)	0.8	rw087	*Plagianthus regius*	*M. marchantiae*	AB698714	97	830/857
V (5)	2.0	rw113	*Plagianthus regius*	*Methylophilus rhizosphaerae*	AB698738	98	763/773
VI (6)	0.4	rw083	*Plagianthus regius*	*Ramlibacter* sp.	AM411935	99	476/481
VII (7)	0.4	cf159	*Phormium tenax*	*Achromobacter xylosoxidans*	HQ678684	94	298/317
VIII (8)	1.2	cf150	*Phormium tenax*	*Janibacter melonis*	JN084150	94	472/502

Restriction type (OTU)	Isolates (%)	Isolate code	Isolation source	Closest species	Accession number	Identity (%)	Number of matched bp
IX (9)	2.0	ciaa197 ti234 cf305	*Olearia traversii* *Alectryon excelsus* *Phormium tenax*	*Niastella populi*	AB682649	99	666/675
X (10)	2.0	ciaa203	*Olearia traversii*	*Methylopila musalis*	JQ173144	83	278/334
XI (11)	0.4	cf153	*Phormium tenax*	*Paenibacillus lautus*	HM133943	99	704/714
XII (12)	0.4	ss132	*Asplenium oblongifolium*	*Xanthomonas* sp.	DQ339599	84	651/778
XIII (13)	1.6	rw086	*Plagianthus regius*	*M. zatmanii*	AB698689	99	652/660
XIV (14)	0.4	kk035	*Macropiper excelsum*	*Rhizobium endophyticum*	EU867317	95	731/769
XV (15)	0.8	kk005 kk022	*Macropiper excelsum* *Macropiper excelsum*	*Hyphomicrobium* sp. (VV3) *Hyphomicrobium sp.* (AT4)	AY436815 FN667866	99	710/712 650/652
XVI (16)	6.4	kk037 kk048 pk243 cf284	*Macropiper excelsum* *Macropiper excelsum* *Metrosideros excelsa* *Phormium tenax*	*M. oryzae* *M. radiotolerans* *M. tardum* *M. fujisawaense*	GU294332 AB698731 AB698727 AB558142	99	683/692

2.3.4 Diversity and distribution of *Methylobacterium* species in the phyllosphere of native NZ plants

A total of 197 isolates (79.1%) were identified as members of the genus *Methylobacterium* by BLAST analysis (Table 2.3) and were isolated from 19 of 21 plant species. Most of the remaining isolates (11.6% of total) were closely related to *Hyphomicrobium* spp. Maximum abundances of these isolates were from with *Macropiper excelsum* (3.8%), and *Phormium cookianum* (2.8%), but were also isolated from eleven other plant species in this study, including two plant species from which only *Hyphomicrobium* spp. were isolated (*Cyathea dealbata* and *Cyathea medullaris*). *Methylophilus rhizosphaerae, Methylopila musalis,* and *Niastella populi* like isolates were at the same abundances (2.0% of each species), and were isolated from three, five and four plant species respectively with only one species, *Phormium cookianum,* in common. All other bacteria isolates were in low numbers and therefore only isolated from a small number of plant species (Table 2.3 and Figure 2.3).

The majority of isolates from all plants were members of the genus *Methylobacterium* (79.1%). Results showed that all of the plant species harbor a methylobacterial community, of different complexity, except for *Cyathea dealbata* and *Cyathea medullaris* from which no *Methylobacterium* spp. were isolated (Figure 2.3, 2.4 & 2.5). Only *Hyphomicrobium methylovoram* isolates were found to be associated with these tree ferns (Figure 2.3). Of the 197 methylobacterial isolates, 54.8% (OTU 2) were closely related to *M. komagatae, M. mesophilicum, M. brachiatum* and *M. phyllosphaerae* (98-99%). They were broadly distributed (18 plant species) and isolated frequently from most of the plants in this study. Maximum abundances were found in association with *Phormium tenax* (13.7%), *Plagianthus regius* (12.7%), *Macropiper excelsum* and *Phormium cookianum* (6.6% in each), *Metrosideros excelsa* (2.5%), and *Griselinia littoralis* (2.0%). While the abundance in the remaining species was below 2%: *Agathis australis, Asplenium oblongifolium, Olearia albida, Alectryon excelsus, Coprosma robusta* and *Hebe elliptica, Blechnum*

novea-zealandiae, *Cyathea cunninghamii*, *Knightia excelsa*, *Melicystis ramiflorus*, *Nephrolepis cordifolia*, and *Pittosporum tenuifolium* (Table 2.3).

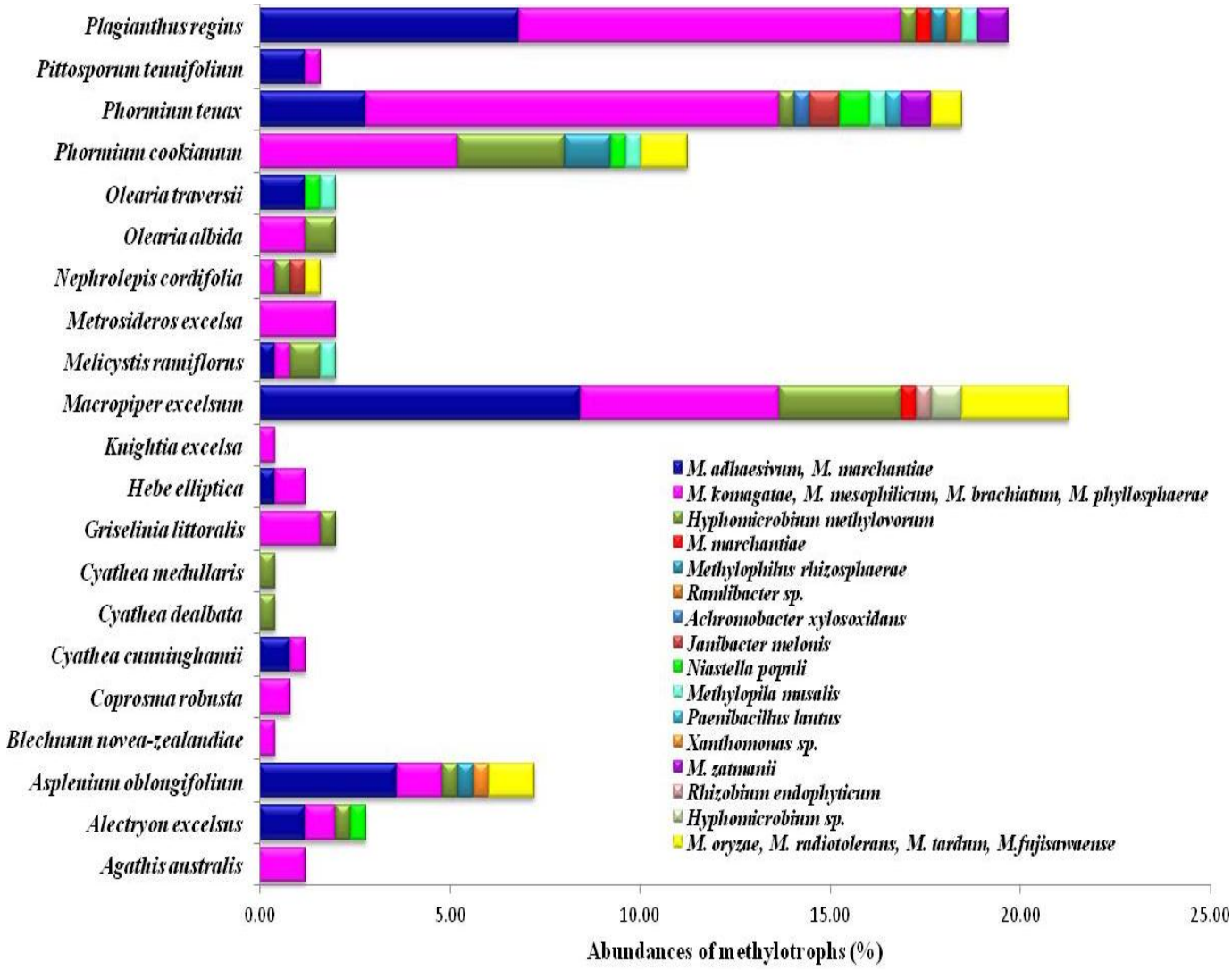


Figure 2.3: Abundance of methylotrophs isolated from leaves of native NZ plants.

The second largest group of *Methylobacterium* isolates (34.0% of all methylobacteria isolates, OTU 1) was closely related to two methylobacterial species namely *M. marchantiae* and *M. adhaesivum* (99-100%), and was isolated from 10 different plant

species. These isolates are also widely distributed in different plant species, but also with varying abundance. Most isolates were from *Macropiper excelsum* (10.7%), *Plagianthus regius* (8.6%), *Asplenium oblongifolium* (4.6%), and *Phormium tenax* (3.6%). Abundance of isolates from the other plant species was below 2%: *Alectryon excelsus*, *Olearia traversii*, *Pittosporum tenuifolium*, *Cyathea cunninghamii*, *Hebe elliptica*, and *Melicystis ramiflorus* (Figure 2.4 & 2.5).

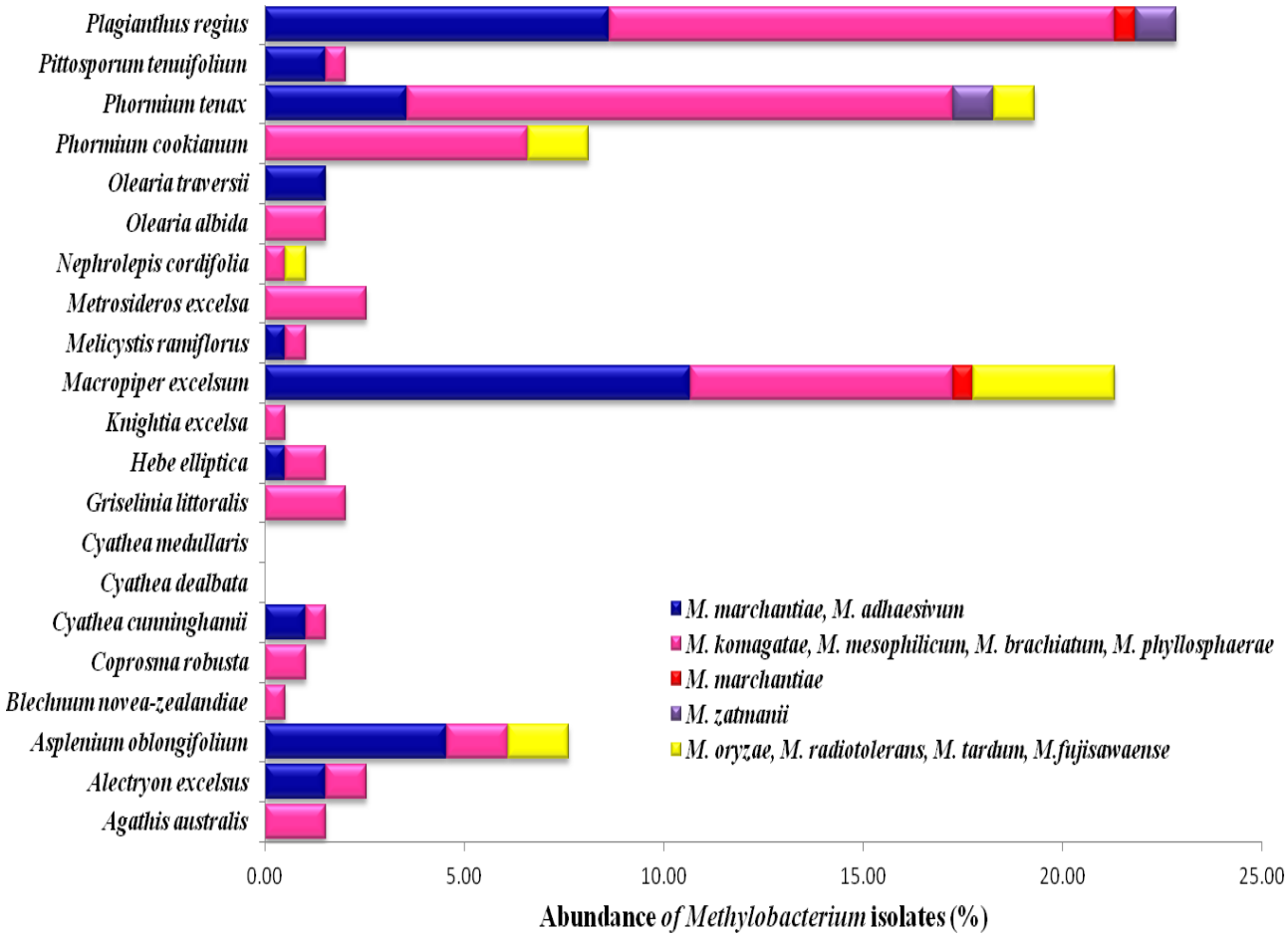


Figure 2.4: Diversity of Methylobacterium spp. in the phyllosphere of native NZ plants.

The third group of *Methylobacterium* isolates (8.1%, OTU 16) was closely related to *M. oryzae*, *M. radiotolerans*, *M. tardum* and *M. fujisawaense* (99%), but were only

isolated from five plant species namely *Asplenium oblongifolium*, *Macropiper excelsum*, *Nephrolepis cordifolia*, *Phormium cookianum*, and *Phormium tenax*. Among them, *Macropiper excelsum* had most isolates (3.6%). The fourth group (OTU 13) was closely related to *Methylobacterium zatmanii* and were only isolated from two species of plants in this study *Phormium tenax* and *Plagianthus regius*. The fifth group (OTU 4) was also related to *Methylobacterium marchantiae* (97%), and were only isolated from *Macropiper excelsum* and *Plagianthus regius*. The other group of isolates (OTU 1) related to *M. marchantiae* had a greater similarity with *M. marchantiae* (99%) (Table 2.3).

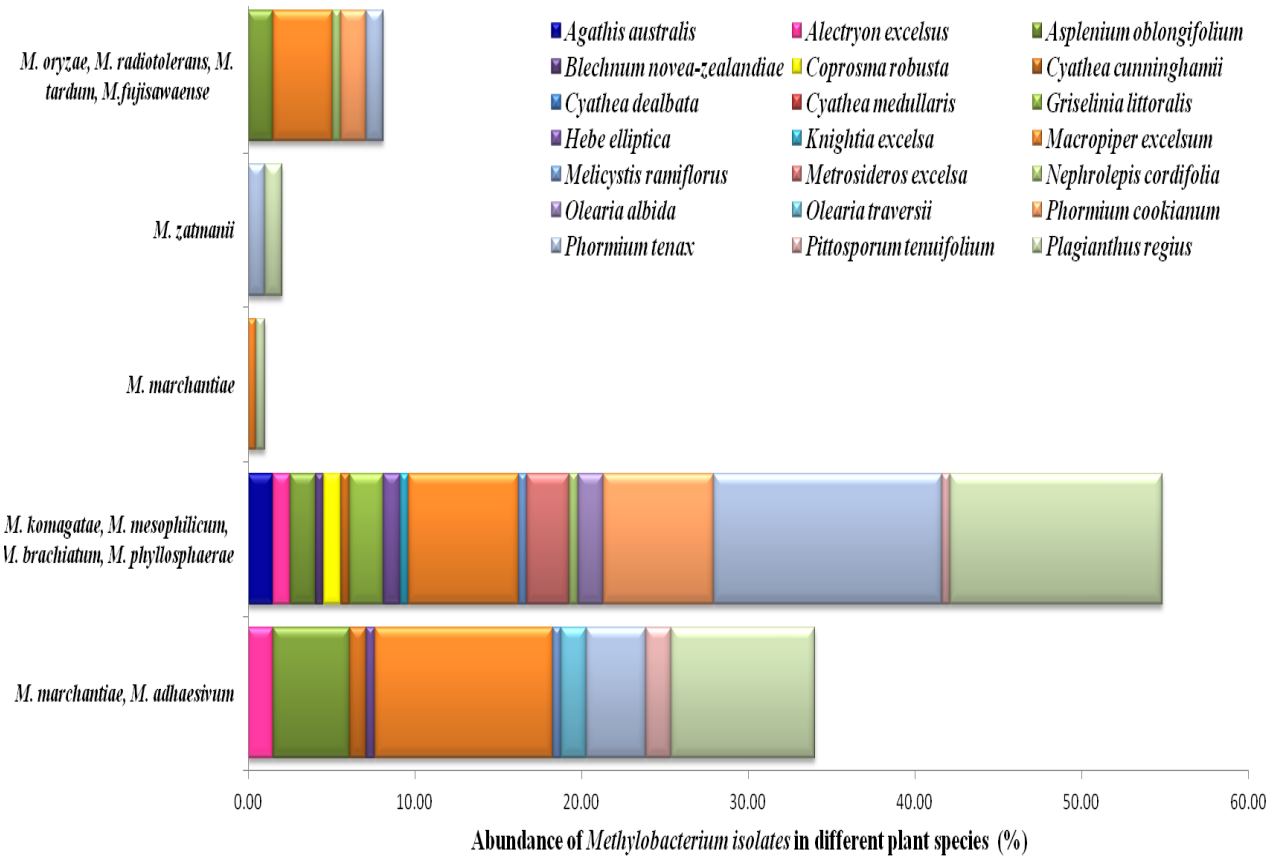


Figure 2.5: Distribution of *Methylobacterium* species in native NZ plants.

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Phylogenetic analysis of the 16S rRNA gene sequences of the representative *Methylobacterium* isolates (Figure 2.6) supported the five groups of the isolates seen from ARDRA analysis. The phylogenetic analysis revealed that representative isolates (kk037, kk048, pk243 and cf284) of OTU 16 are grouped together. They are closely related and in the same cluster (Figure 2.4 & Table 2.3). Representative isolate of OTU 13 (rw086) make different cluster in the tree. The major *Methylobacterium* group, OTU 2 are representing by isolates kk034, kk036, rw080, pk208 and ss136. Phylogenetic analysis revealed that isolate kk034 was closely related to *M. komagatae*, isolate kk036 was most closely related to *M. brachiatum and M. mesophilicum*, isolate ss136 was closely related to *M. phyllosphaerae*. Isolates rw104, kh130 and rw087 were found closely related to *M. marchantiae* in the phylogenetic tree which is similar with BLAST results.

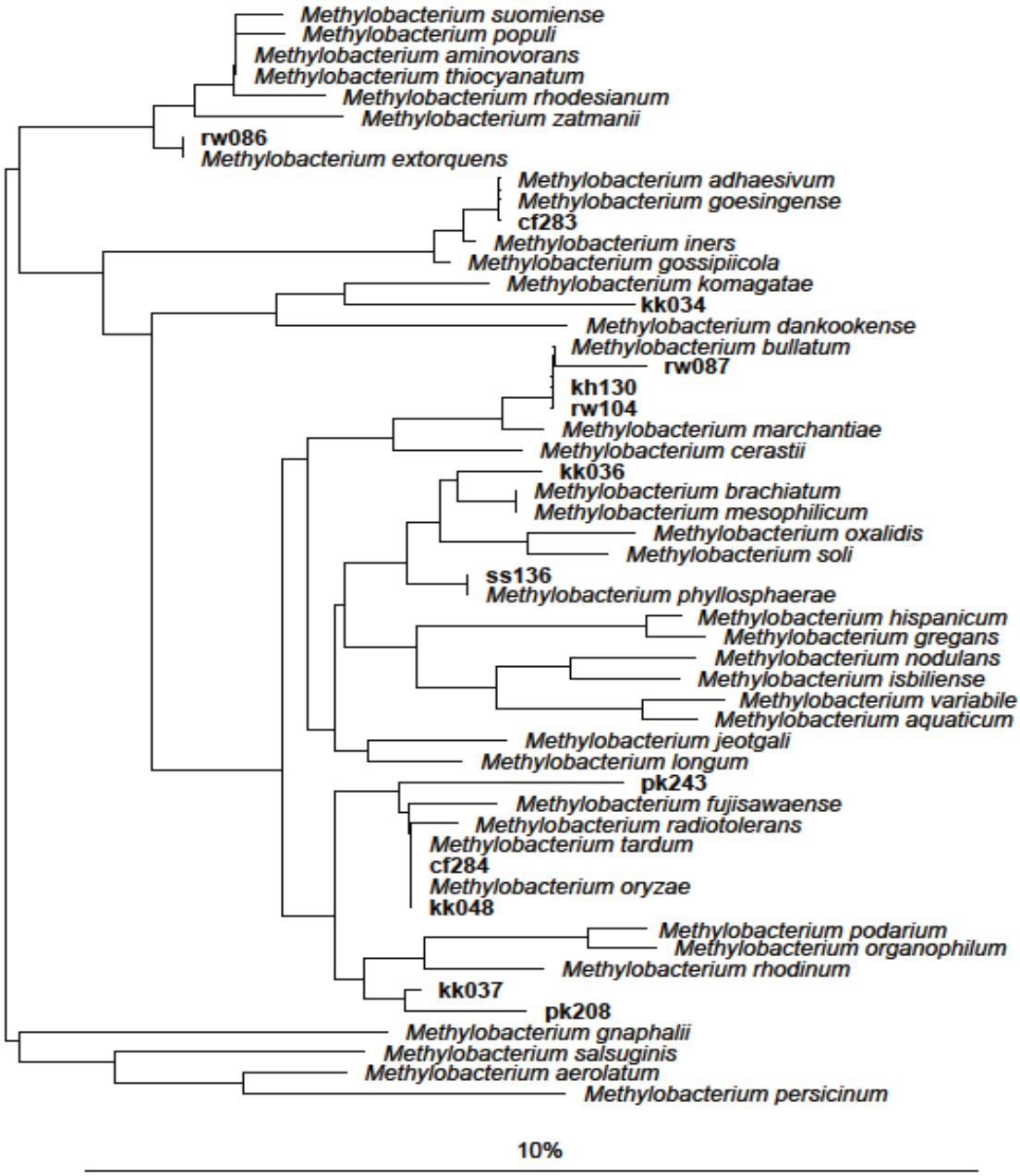

Figure 2.6: Phylogenetic analysis of 16S rRNA gene sequences of *Methylobacterium* isolates.

2.4 Discussion

To investigate *Methylobacterium* species diversity from leaves of native plants in New Zealand, leaf imprinting on methanol supplemented AMS agar was used to isolate methylotrophic bacteria. Methanol is a key substrate for growth of *Methylobacterium* and in plants methanol is the waste product of pectin metabolism, produced during plant cell division. A total of 329 isolates were obtained from 24 different native New Zealand plants. Methylotrophs were isolated from leaves of every plant, but the species isolated and the number of isolates varied from plant to plant. This interspecies variability is supported by results from other studies of the phyllosphere community (Lambais et al., 2006; Redford et al., 2010; Whipps et al., 2008; Yang et al., 2001).

To select isolates for sequencing of the 16S rRNA gene a finger printing technique was applied (ARDRA). In this study, a four base cutter endonuclease Afa I (Rsa I) was used and sixteen different restriction patterns were obtained. Sequencing of the 16S rRNA gene of representative isolates of each restriction group revealed that most of the isolates (79.1%) are *Methylobacterium* isolates. The *Methylobacterium* isolates in this first study of New Zealand native plants were most closely related to: *M. adhaesivum*, *M. brachiatum*, *M. fujisawaense*, *M. komagatae*, *M. marchantiae*, *M. mesophilicum*, *M. oryzae*, *M. phyllosphaerae*, *M. radiotolerans*, *M. tardum*, and *M. zatmanii*. In this study, *Methylobacterium* were found to be a common colonizer of the investigated plants. This is consistent with studies undertaken by several researchers worldwide using both cultivation dependent and independent methods (Delmotte et al., 2009; Hirano & Upper, 1991; Holland & Polacco, 1994; Knief et al., 2012a; Wellner et al., 2011). The abundance and distribution of *Methylobacterium* species isolates varied between plant species although they were collected in the same location. This finding is supported the findings of other cultivation independent (Knief et al., 2008) and cultivation dependent studies (Balachandar et al., 2008; Raja et al., 2008b). Sixteen different *Methylobacterium* spp. distributed worldwide (Table 1.1) with *M. extorquens* being found to be ubiquitous colonizer of the phyllosphere of

many plants (Delmotte et al., 2009), however in this study leaves of New Zealand native plants only yielded isolates related to 11 *Methylobacterium* species. A reason for this may be the isolation media used which was highly selective, with only methanol used as a carbon source. Generally, *Methylobacterium* species are differentiated according to their carbon substrate utilization pattern (Green, 2006). For example, *M. aminovorans* was proposed (Urakami et al., 1993) due to utilize tetramethylammonium hydroxide (TMAH) and N,N-dimethylformamide (DMF). *M. thiocyanatum* was proposed (Wood et al., 1999) because of utilization of cyanate and thiocyanate as sole source of nitrogen and energy. *M. dichloromethanicum* was proposed (Doronina et al., 2000) due to utilize dichloromethane and *M. chloromethanicum* was proposed due to utilize chloromethane (methyl chloride) as sole carbon and energy source (McDonald et al., 2001). Another reason could be the degree of association between plants and *Methylobacterium*, with results from other research showing that *Methylobacterium* association with plants varies from epiphytic (Omer et al., 2004b) to endophytic (Lacava et al., 2004) and symbiotic (Jourand et al., 2004). In this study only epiphytes were isolated from the surfaces of leaves. There is some indication that *Methylobacterium* colonization of plants first starts as an epiphytic association, and then establishes an endophytic association (Andreote et al., 2006). The final reason could be the geographic location, which has been shown to be an important determinant in shaping *Methylobacterium* colonization in the phyllosphere of *Arabidopsis thaliana* plants under natural conditions (Knief et al., 2010b).

All the *Methylobacterium* species identified in this study are common leaf epiphytes. For example, *M. adhaesivum* was isolated from the phyllosphere of *Gallanthus nivalis* and *Fragaria moschata* (Verginer et al., 2010); *Narcissus tazetta* var. *chinensis*, *Gnaphalium spicatum*, *Vulpia* myuros, *Oxalis articulate*, *Galium spurium* var. *echinospermon*, *Bryum argenteum*, *Conyza sumatrensis*, *Physcomitrium sphaericum*, *Ptychomitrium gardneri*, and *Brachythecium plumosum* (Tani et al., 2012c). *M. marchantiae* was isolated from the phyllosphere of *Marchantia polymorpha* (Schauer et al., 2011); *Gnaphalium spicatum*, *Lactuca sativa*, *Papaver*

dubium, and *Viburnum odoratissimum* var. *awabuki* (Tani et al., 2012c). *M. komagatae* was found on the phyllosphere of *Haplocladium microphyllum*, *Narcissus tazetta* var. *chinensis*, and *Trifolium repens* (Tani et al., 2012c); *Dipterocarpus* sp. (Kieu et al., 2009). *M. mesophilicum* was found on the phyllosphere of *Gallanthus nivalis*, *Hedera helix* (Verginer et al., 2010); *Helianthus annuus* (Knief et al., 2008). *M. brachiatum* was found on the phyllosphere of *Lactuca sativa*, *Brachythecium plumosum*, *Geranium carolinianum*, and *Trachycarpus fortunei* (Tani et al., 2012c). *M. phyllosphaerae* was found on the phyllosphere of *Oryza sativa* (Madhaiyan et al., 2009). *M. zatmanii* was found on the phyllosphere of *Trigonotis peduncularis*, *Lactuca indica*, *Solidago sempervirens* (Tani et al., 2012c). *M. oryzae* was found in association with the phyllosphere of *A. thaliana* (Knief et al., 2008); *Oryza sativa* (Kieu et al., 2009; Madhaiyan et al., 2007a); *Viola odorata* (Esposito-Polesi, 2012). *M. radiotolerans* was found associated with the phyllosphere of *Bryum argenteum*, *Vulpia myuros* (Tani et al., 2012c); *A. thaliana* (Knief et al., 2008); *Dipterocarpus* sp. (Kieu et al., 2009); *Centella asiatica* (Rafat et al., 2010). *M. tardum* was found associated with the phyllosphere of *Poa annua* (Tani et al., 2012c), and *M. fujisawaense* was found association with *Funaria hygrometrica* (Tani et al., 2012c) and *Racomitrium japonicum* (Tani et al., 2012d).

The largest numbers of isolates in this study were found from leaves of *Plagianthus regius* followed by *Macropiper excelsum*, *Phormium tenax*, *Phormium cookianum*, and *Asplenium oblongifolium*. This may reflect the leaf texture and structure of the investigated plants. Leaves of these plants (*M. excelsum*, *P. tenax*, *P. cookianum*, and *A. oblongifolium*) are thin, not glossy and hard, easily lie on the media, and making imprint is easier than for some of the other plants in this study. The other plants (*A. australis*, *A. excelsus*, *C. robusta*, *G. littoralis*, *H. elliptica*, *K. excelsa*, *M. excelsa*, *O. albida*, *O. traversii*, *and P. tenuifolium*) have leaves which are thick, glossy, hard, shiny, spiky, or velvety and therefore did not lie easily on the media. Leaf texture and structure have been shown to be important factors for obtaining epiphytes from the surface of the leaves using the imprinting technique (Holland et al., 2000). The low number of *Methylobacterium* isolates from *B. novea-zealandiae*, *C. cunninghamii* and

N. cordifolia may have been due to the size of plants sampled in this study. But in general, in this study, there were few *Methylobacterium* species isolated from fern species, and there were none isolated from *C. dealbata* (black fern) and *C. medullaris* (silver fern). However, plant species has been shown to be the main driver for community structure of *Methylobacterium* species in several studies (Kinkel et al., 2000; Knief et al., 2008; Knief et al., 2010b; Omer et al., 2004b; Redford et al., 2010; Wellner et al., 2011).

The most frequently isolated *Methylobacterium* colonizers are *M. brachiatum*, *M. komagatae*, *M. mesophilicum*, and *M. phyllosphere*. They were isolated from 18 different plants species (out of 21 in this study). For seven plants species these were the only *Methylobacterium* sp. to be isolated, possibly indicating some specialization to these plant species (*A. australis*, *B. novea-zealandiae*, *C. robusta*, *G. littoralis*, *K. excelsa*, *M. excelsa*, *O. albida*). This may be due to the special feature of some NZ plants which are divaricating (adaptation to a dry, windy and frosty climate). Similarly, only *M. adhaesivum* and *M. marchantiae* were isolated from *O. traversii*. From this study, it is difficult to explain the reasons for the association of individual *Methylobacterium* sp. with specific plants. Plant species and the generalist behavior of some strains of *Methylobacterium* have been found to have a combined role in colonization (Dourado et al., 2012a).

In this study, no single species of plant was not found to harbor of all the 11 species of *Methylobacterium* identified. In general, maximum association was found with *Macropiper excelsum*, *Phormium tenax*, and *Plagianthus regius* followed by *Asplenium oblongifolium*, *Phormium cookianum*, *Alectryon excelsus*, *and Pittosporum tenuifolium*. Seven plant species *Agathis australis*, *Blechnum novea-zealandiae*, *Coprosma robusta*, *Griselinia littoralis*, *Knightia excelsa*, *Metrosideros excelsa*, and *Olearia albida* were found having only four *Methylobacterium* species such as *M. brachiatum*, *M. komagatae*, *M. mesophilicum*, and *M. phyllosphaerae*. Only one plant, *Olearia traversii* was found having only two *M.* species such as *M. marchantiae* and *M. adhaesivum*. No other *M*. species were found within this plant at

all. *Phormium tenax* and *Plagianthus regius* have showed association with *M. zatmanii*. No other plants have showed association with *M. zatmanii* at all. This species was found rarely in New Zealand plants. Only five plants namely *Asplenium oblongifolium, Macropiper excelsum, Nephrolepis cordifolia, Phormium cookianum,* and *Phormium tenax* have showed association with another four *M.* species such as *M. fujisawaense, M. oryzae, M. radiotolerans,* and *M. tardum*. Among all plants, leaves of *Macropiper excelsum* and *Plagianthus regius* have showed ideal habitat for colonization of *M. adhaesivum* and *M. marchantiae* followed by *Asplenium oblongifolium* and *Phormium tenax*. Another flax species *Phormium cookianum* have not showed any association with *M. adhaesivum* and *M. marchantiae*. Among the fern species, *Asplenium oblongifolium* have shown maximum association with *M.* followed by *Cyathea cunninghamii, Nephrolepis cordifolia*, and *Blechnum novea-zealandiae*. *Cyathea dealbata* and *Cyathea medullaris* have not showed any association with *M.* species at all. Among the shrub species, *Macropiper excelsum* have showed maximum association with the species of *M.*; *Coprosma robusta* and *Olearia albida* have showed association with only OTU 2 which represents the group of four species namely *M. brachiatum, M. komagatae, M. mesophilicum,* and *M. phyllosphaerae*; *Hebe elliptica* have showed little association with OTU 1 and OTU 2 only. Among the tree species, only *Plagianthus regius* have shown maximum association with *Methylobacterium* species except the OTU 4 group of *M.* species (Figure 2.4 & 2.5).

Besides *Methylobacterium*, other Alpha-proteobacteria (*Hyphomicrobium methylovoram, Hyphomicrobium* sp., *Methylopila musalis, Rhizobium endophyticum*); Beta-proteobacteria (*Achromobacter xylosoxidans, Methylophilus rhizosphaerae, Ramlibacter sp.*); Gamma-proteobacteria (*Xanthomonas* sp.); Actinomycetes (*Janibacter melonis*); Bacteroidetes (*Niastella populi*); and Firmicutes (*Paenibacillus lautus*) were also isolated on methanol media in this study. Similar species have also been seen in other culture independent studies (Delmotte et al., 2009; Jackson et al., 2006; Lambais et al., 2006; Rastogi et al., 2012; Redford et al., 2010; Yang et al., 2001). Several studies have demonstrated the association of some

of these bacterial species with plants. *Hyphomicrobium* sp. are restricted facultative methylotrophs (Harder & Attwood, 1978), and association with the phyllosphere of plants has been shown previously (Nadalig et al., 2010). They have also been detected as pollutant resistant methylotrophic bacteria for the use in bioremediation (De Marco et al., 2010; De Marco et al., 2004; Isabelle et al., 2011). An isolate (ciaa203) had 83% similarity with *Methylopila musalis* in this study, and has been found from *Musa paradisiaca* L. fruit (Doronina et al., 2011). *Rhizobium endophyticum* was found with seeds and roots of *Phaseolus vulgaris* (Lopez-Lopez et al., 2010), and plant tissue of *Jatropha curcas* L. (Madhaiyan & Ji, 2012). But there are no reports of the ability of *R. endophyticum* to utilize methanol. In this study one isolate (kk035) had 95% similarity with *R. endophyticum,* and may be a novel species, however more investigation is required. *A. xylosoxidans* was found in the rhizosphere of *Prosopis juliflora* (Cibichakravarthy et al., 2012). *Methylophilus* is a methanol utilizer (Kolb, 2009) and gas been found in association with leaves of *Cyperus microiria* (Tani et al., 2012c). *Ramlibacter* sp. have been found on tobacco leaf (Lv et al., 2011), and *Malus domestica* roots (Bulgari et al., 2012). In this an isolate (ss132) had 84% identity with *Xanthomonas* sp., previously *Xanthomonas* sp. have been identified in association with plant leaves and stems (Corpe & Rheem, 1989; Sheng et al., 2011), and have been isolated as an orchard epiphyte from an apple tree (Jeng et al., 2001). *Janibacter melonis* has been found from cucumber leaf (Sun et al., 2012), and plant tissue of *Jatropha curcas* L. (Madhaiyan & Ji, 2012). *Niastella populi* has been isolated from soil of *Populus euphratica* forest (Zhang et al., 2010). *Paenibacillus lautus* has been found from *Delesseria sanguine* (Goecke, 2011), and in the rhizosphere of *Dichanthium* spp. (Sharma et al., 2010).

Achromobacter, *Janibacter*, *Niastella*, *Ramlibacter*, *Rhizobium*, *Paenibacillus*, and *Xanthomonas* isolates were identified in this study with the ability to grow on methanol. To date, no clear evidence has been found for methanol utilization by bacteria from these genera. Further studies are therefore required to understand more about the utilization of carbon substrates by these bacteria. Percent similarity of some isolates (cf159, cf150, ciaa203, ss132, and kk035) with *Achromobacter*, *Janibacter*,

Methylopila, *Rhizobium*, *Xanthomonas*, and *Paenibacillus* respectively, may be indicating new lineages within the respective genus or within the *Methylobacterium* genus. However this study does present evidence for the association of these bacterial species with plants.

Although leaf texture and structure are not the identical for all of investigated plants, *Methylobacterium* spp. was observed in all samples, indicating general and fittest colonizers of the phyllosphere. But the divaricating feature of New Zealand endemic plants also has shown some specificity of the isolated 11 *Methylobacterium* spp., where more investigation is required. Low number of isolates yielding plants are recognized by hardy, thick and glossy appearance of leafs may be leaf imprinting technique is not suitable for retrieving bacteria, but other isolation technique using stomacher should be ideal choice for isolation bacteria from tougher and specialized leafs (Opelt & Berg, 2004; Wellner et al., 2011).

The findings from several studies revealed that environmental parameters govern by the geographic location and site specific factors have huge impact on shaping up plants species composition which ultimately determines bacterial community composition. Therefore biogeographical aspects can play a dominant role to diversity of plant associated bacteria (Finkel et al., 2011; Wellner et al., 2011). Since phyllosphere represents an ecological niche, harbors diverse well adapted epiphytic bacteria; and New Zealand geographically isolated island and recognized as a biodiversity hotspot, specialized for unique flora, most of them are endemic. So the extents to which New Zealand native plants are associated with *Methylobacterium* species community in this geographical region are therefore an important area to study.

2.5 Summary

In order to study *Methylobacterium* spp. diversity, leaf samples from a total of 24 native plant species were collected from the campus of The University of Waikato and the native nursery, Hamilton, New Zealand and assayed by using leaf imprinting technique on methanol supplemented AMS agar media resulting in the isolation of 329 pure cultures of methanol grown strains. Every plant species have shown methylotrophs association and maximum association was found with the *Micropiper excelsa* leaf.

A total of 249 amplicon were used for ARDRA analysis as their 16S rRNA gene was amplified and rest of the isolates including 3 plants (*Litsea calicaris*, *Podocarpus totara*, and *Pomaderris kumeraho*) were not undertaken for the experiments for ARDRA and subsequent analysis. Restriction endonuclease Afa I was used for grouping isolates according to identical restriction patterns resulting in 16 different OTUs and 34 representatives from 16 OTUs have been selected for sequencing. Therefore, in the combination of ARDRA, 16S rRNA gene sequencing and phylogenetic analysis were used successfully to identify 197 *Methylobacterium* isolates (79.1%) associated with New Zealand native plants used in this study, however species composition varied between species.

In this study, 11 methylobacterial species were found in association with native plants of New Zealand. They are *M. adhaesivum, M. brachiatum*, *M. fujisawaense*, *M. komagatae, M. marchantiae*, *M. mesophilicum*, *M. oryzae*, *M. phyllosphaerae*, *M. radiotolerans*, *M. tardum*, and *M. zatmanii.* Among them OTU 2 group, representing *M. brachiatum*, *M. komagatae*, *M. mesophilicum*, and *M. phyllosphaerae* were isolated most frequently from the studied plants. The second largest group was OTU 1 (and OTU 4) representing *M. adhaesivum* and *M. marchantiae,* the third group was OTU 16 representing *M. fujisawaense*, *M. oryzae*, *M. radiotolerans*, and *M. tardum*, and the final group was OTU 13 representing only *M. zatmanii* strains.

The remaining isolates (52) belonged to the other members of α, β, γ-Proteobacteria, Actinomycetes, Bacteroidetes and Firmicutes namely: *Hyphomicrobium*, *Methylopila*, *Rhizobium*, *Achromobacter*, *Methylophilus*, *Ramlibacter*, *Xanthomonas*; *Janibacter melonis*, *Niastella populi*, and *Paenibacillus lautus*. Among them *Achromobacter*, *Janibacter*, *Methylopila*, *Rhizobium*, *Paenibacillus*, and *Xanthomonas* were identified as possible novel methanol utilizers, this will require more in-depth study to confirm, but their association with plants has also found in other studies.

Chapter Three: General Conclusion

The aim of this study was to investigate the association of *Methylobacterium* community with the phyllosphere of native plants of New Zealand. The phyllosphere represents a heterogeneous landscape, is the biggest biological surface on Earth, is an intensely fluctuating environment, and emits methanol as the waste product of pectin metabolism during plant growth. *Methylobacterium* spp. utilize methanol as a carbon substrate for their growth and energy. Growing plants are the main methanol producer to the atmospheric methanol budget. The alpha-proteobacterial genus *Methylobacterium* was found to be the dominant group in the phyllosphere community, and produces phytohormones which directly increase plant growth and productivity. Methodologies were successfully used to isolate and identify *Methylobacterium* species community from native plants of New Zealand.

This was the first study of *Methylobacterium* species diversity in New Zealand native plants, 21 different plants species (from trees, shrubs, herbs, ferns and flax) were used in this study. A leaf imprinting technique was used to isolate the *Methylobacterium* species, and methanol was used as the only carbon substrate in the AMS media. Some leaf samples used in this study were found to be glossy, thick, hard, and irregular in shape, and were difficult to imprint on the media, which may be the reason for retrieving a low number of isolates from these species of plants. A future study should explore an alternative technique to isolate bacteria from all plant species. One possibility would be to use a more physical technique to homogenize the samples (i.e. a stomacher), transferring the microbes into a liquid which could then be plated onto media. This type of isolation strategy has been successfully used for moss (Opelt et al., 2007), which typically has a highly specialized morphologically difficult to sample.

The combination of ARDRA, 16S rRNA gene sequencing and phylogenetic analysis were used successfully to identify *Methylobacterium* isolates associated with New Zealand native plants. To analyze the isolates, restriction endonucleases were used to group the isolates and 16 different OTUs were found. ARDRA successfully identified representative isolates which were then subsequently sequenced and analyzed. Eleven different methylobacterial species were found in association with native plants of New Zealand. Six species: *M. adhaesivum*, *M. brachiatum*, *M. komagatae*, *M. marchantiae*, *M. mesophilicum*, and *M. phyllosphaerae* were found more frequently suggesting their potential adaptation to the NZ environment and NZ native plants. This dominance of specific species may also reflect their metabolic potential. *Methylobacterium* species are divided into two physiological groups according to their phenotypic characteristics (Green & Bousfield, 1982), one group contains metabolically less reactive species such as *M. extorquens*, *M. rhodesianum*, and *M. zatmanii* and the other group contains more reactive species such as *M. fujisawaense*, *M. komagatae*, and *M. marchantiae*. Phylogenetic analysis also groups them accordingly. Except for carbon and energy source utilization, other phenotypic and chemotaxonomic characteristics are similar among *Methylobacterium* species (Hiraishi et al., 1995), which hinders species identification. More investigation will be required to identify novel species.

Biogeographical aspects also influence biodiversity of plant associated bacteria (Ramette & Tiedje, 2007). This study provides a foundation for future work on the native plants of New Zealand. Plant leaf physiology, which is affected by geographic parameters (particularly climate), may have a strong influence on *Methylobacterium* species composition. To address a possible biogeographic influence on diversity the same plant from different locations within New Zealand would need to be studied, and bacteria would need to be sampled from all plant species using a more efficient technique. An analysis of the phyllosphere of the many different NZ native plants for methanol emission also needs to be undertaken as methanol is the main substrate for *Methylobacterium*, and different leaves have different potentials for methanol production and emission.

Overall this study provides a small scale overview of *Methylobacterium* species association with native plants of New Zealand, and identified that NZ native plants harbor a diverse group of *Methylobacterium* species. Association was varied from plant to plant, with maximum association found with *Macropiper excelsum*, *Phormium tenax* and *Plagianthus regius*. Indicating that a potential plant specific factor, and the metabolic potential of some *Methylobacterium* species, have a combined role to play in the *Methylobacterium/*phyllosphere association.

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Appendices

Appendix A: ANMS media

ANMS Salts solution (x10 stock)

KNO_3.....5 g

NH_4Cl.....5 g

$MgSO_4.6H_2O$.....10.79 g

$CaCl_2.7H_2O$.....2.65 g

Dissolved in above order in 700 ml and diluted to 1 litre. Stored at room temperature.

Iron solution (x10,000 stock)

Ferric-EDTA.....3.8 g

Final volume 100 ml. Wrapped in foil and kept in the fridge.

Molybdate solution (x1000 stock)

Na_2MoO_4.....0.13 g

Final volume 500 ml. Stored in the fridge.

Trace elements solution (x1000 stock)

$CuSO_4.5H_2O$.....0.1 g

$FeSO_4.7H_2O$.....0.25 g

$ZnSO_4.7H_2O$.....0.2 g

H_3BO_3.....0.0075 g

$CoCl_2.6H_2O$.....0.025 g

Na_2.EDTA.....0.125 g

$MnCl_2.4H_2O$.....0.01 g

$NiCl_2.6H_2O$.....0.005 g

The above was dissolved in the specified order in distilled water and made up to 500 ml. Stored in the dark at 4^oC.

Phosphate buffer (x100 stock)

$Na_2HPO_4.12H_2O$.....71.6 g

KH_2PO_4.....26 g

The above was dissolved in the specified order in 800ml of distilled water. The pH was adjusted to 6.8 and diluted to 1 litre. Stored at room temperature.

Media preparation

1xANMS contains 100ml ANMS salts, 1 ml molybdate solution, 1 ml trace elements, 0.1 ml Fe-EDTA solution per litre. Autoclaved with or without agar. 10 ml of sterile phosphate buffer per litre is added after autoclaving. Agar plates were prepared by addition of 1.5% Difco^{TM} agar, granulated (w/v) (15 g/L) prior to sterilization. 0.5% sterile methanol was added to the media prior to making plates.

Appendix B: Marmur DNA extraction method

DNA extraction

1. Centrifuge 50 ml enrichment culture at 3 min at 3000 rpm
2. Remove supernatant and resuspend the pellet in 400 µl SET buffer
3. Add 20 µl lysozyme solution (5 mg/ml in TE + 10 mM NaCl)
4. Incubate 37^0C for 1 hour
5. Add 20 µl 20% SDS (to give 1% final volume)
6. Add 10 µl proteinase K solution (20 mg/ml in TE)
7. Incubate at 60^0C for 3-4 hours for maximum protein digestion
8. Purified by phenol chloroform extraction and concentrated by ethanol precipitation

Removes protein (by phenol chloroform)

1. Add equal volume of phenol: chloroform: isoamyl alcohol (450 µl) to the DNA containing reaction mixture
2. Vortex gently
3. Spin 10 min at 13000 rpm 4^0C to separate the aqueous phase which contains DNA from the organic phase
4. Transfer supernatant (aqueous phase) with care into a fresh tube (no interlayer or organic phase)

Removes phenol (by chloroform)

1. Add equal volume of chloroform: isoamyl alcohol (400 µl)
2. Vortex
3. Spin 10 min at 13000 rpm at 4^0C
4. Transfer supernatant to a fresh microfuge tube with care (no interlayer or organic phase)

Precipitates DNA (by 100% ethanol)

1. Add 0.1 volume (one tenth) 3 M sodium acetate, pH 5.5
2. Add 2 volumes (double) of cold 100% ethanol (-20^0C)
3. Vortex
4. Incubate at -20^0C overnight for precipitating
5. Spin 20 minutes at 12000 rpm 4^0C to recover the precipitated DNA
6. Carefully remove as much supernatant as possible with 1 ml micropipette (do not lose DNA pellet)

Washes out salt (by 70% ethanol)

1. Carefully add 1 ml cold (-20^{0}C) 70% ethanol (do not vortex)
2. Spin 10 min at 12000 rpm 4^{0}C (this is a wash)
3. Carefully pipette out supernatant (do not lose DNA pellet). There will be clear pellet on the bottom. It may be difficult to see.
4. Air dry pellet 10 min at room temperature (do not over dry) by placing the tube upside down on a rack. Just until all residual ethanol has evaporated.
5. Resuspend pellet (dissolve) in desired volume of 10 mM Tris pH 7.5 or TE buffer
6. Stored at -20^{0}C until further need.

Reagents

0.5 M EDTA (500 ml)

EDTA.....84.05 g

H_2O.....~250 ml

Add EDTA to the water and 5 M NaOH was added slowly with stirring and adjusted pH 8.0. 500 ml volume was adjusted with water and sterilize by autoclaving.

5 M NaOH (100 ml)

NaOH.....20 g

dH_2O.....100 ml

This solution was stored in a plastic bottle in room temperature.

1M Tris.HCl Buffer (50 ml)

Tris base.....6.057 g

1 M HCl.....2.85 ml

Adjust pH 8.0

6.057 g Tris base was added into one-third volume of the final amount and then adjusted pH 8.0 with 1 M HCl. Bring to the volume with water and sterilize by autoclaving.

SET Buffer (TSE): Tris-Sucrose-EDTA (10 ml)

dH_2O.....8.5 ml

20% Sucrose.....2 g

50 mM EDTA.....1 ml from 0.5 M EDTA

50 mM Tris.HCL.....500 µl from 1M Tris.HCl

Shake gently to mix. Sterilize by autoclaving.

TE Buffer (10:1) (100 ml)

1 ml of 1 M Tris.HCl (10 mM)

200 µl of 0.5 M EDTA (1 mM) P^H 8.0

98.8 ml of dH_2O

Sterilize by autoclaving.

5 M NaCl (100 ml)

29.22 g NaCl dissolved in 80 ml dH_2O over low heat and volume to 100 ml was adjusted with dH_2O. Sterilize by autoclaving.

20% SDS (Sodium dodecyl sulfate) (100 ml)

SDS, electrophoresis grade.....20 g

dH_2O.....90 ml

Solution was heated at 68^oC to dissolve. pH was adjusted to 7.2 using concentrated HCl and made the volume to 100 ml with dH_2O.

3 M Sodium acetate (50 ml)

Sodium acetate.$3H_2O$.....20.412 g

dH_2O.....40 ml

Adjust pH to 5.2 with glacial acetic acid

Make up volume to 50 ml with dH_2O and sterilize by autoclaving.

Lysozyme solution (1 ml)

Lysozyme powder.....0.005 g

TE (10µl 5 M NaCl in 5 ml TE).....1 ml

The solution was store at -20^oC.

Proteinase K solution (1 ml)

0.02 g proteinase K dissolves in 1 ml TE.

The solution can be used immediately and stored at -20^{o}C.

Appendix C: Agarose gel electrophoresis

30 ml Agarose gel

30 ml 1xTAE buffer was measured by measuring cylinder and put into a conical flask. 1% agarose, electrophoresis grade (0.3 g) was measured and put into the same flask. The flask containing the buffer and agarose was boiled in a microwave until all the agarose has dissolved. The clear dissolved gel was poured into the gel block in one continuous flow making sure no bubbles form in it and allow them at least 30 minutes to harden. Once hardened, the container was filled with 1xTAE buffer until all of the wells are submerged. After loading the PCR product, positive control, negative control and marker into the well, the electricity was turned on and set up the volt and time. After running the gel, gels were stained with 0.2 mg/L ethidium bromide for 10-30 min and destain with water for 20-30 min. Gels were visualized on the Alpha Imager™ and captured the image and saved.

50x TAE buffer (Tris-Acetate-EDTA) (1 L)

Tris base.....242 g

Acetic acid.....57.1 ml

0.5 M EDTA.....100 ml

Distilled water was added to make volume 1 L and adjusted pH to ~8.5 using KOH.

1x TAE (1 L)

50x TAE.....20 ml

ddH_2O.....980 ml

Mix well.

Appendix D: Sequences

OTU 1: kk002

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kk040

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CA

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OTU 2: kk034

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rw080

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pk208

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ss136

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OTU 3: kk004

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kk063

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ss288

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OTU 4: rw087

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OTU 5: rw113

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OTU 6: rw083

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OTU 7: cf159

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OTU 8: cf150

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OTU 9: ciaa197

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ti234

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OTU 10: ciaa203

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OTU 11: cf153

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OTU 12: ss132

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OTU 13: rw086

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OTU 14: kk035

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OTU 16: kk037

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