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Diversity of rhizobial *Methylobacterium* species associated with indigenous legumes in South Africa

by

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DECLARATION

I, **Sanele Moyana** declare that the thesis/dissertation, which I hereby submit for the degree of *Magister Scientiae* (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: ..... DATE: .....

This dissertation is dedicated to my late Father President Z. Moyana, who throughout his lifetime etched in the walls of my heart the importance of education, and to my Mother Thembakazi V. Moyana. Thank you for your unwavering support.

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SUMMARY

The genus *Methylobacterium* includes a variety of pink pigmented and cream white facultatively methylotrophic bacteria that are characterized by their ability to mainly utilize methanol as a carbon source. *Methylobacterium* includes only one known nitrogen fixing species (*Methylobacterium nodulans*), which was initially isolated from root nodules of the legume *Crotalaria podocarpa,* in Senegal. Additional *Methylobacterium* strains able to fix atmospheric nitrogen with members of *Crotalaria* and *Listia* legumes native to Southern Africa have since been isolated. The aim of this study thus was to investigate the taxonomic position and delineate the diversity of *Methylobacterium* isolates associated with *Crotalaria* and *Listia* species native to South Africa. This was achieved by employing housekeeping gene phylogenies and various phenotypic tests. Of the original 92 isolates investigated, 29 belonged to the genus *Methylobacterium*. Aligned sequences from the isolates, together with reference and outgroup sequences obtained from the National Center for Biotechnology Information (NCBI) database, were used for constructing phylogenetic trees. To confirm the phylogenetic results, phenotypic characterization tests were conducted. The phylogenetic analyses of the housekeeping genes of the *Methylobacterium* isolates grouped them into two clusters (A and B). Group A isolates were closely related to *M. nodulans*, while Group B formed a different cluster, grouping with a well-known *Methylobacterium* strain 4-46. From the results, it was clear that only isolates obtained from *Crotalaria* clustered in Group A with *M. nodulans*, whereas all *Listia* isolates and two *Crotalaria* isolates clustered in Group B. Results from this study showed that single phylogenies of 16S rRNA, *recA* and *rpoB* best delineated *Methylobacterium* isolates. Carbon utilization tests did not provide results that could be used for the separation of the *Methylobacterium* isolates according to the two assigned groups.

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PREFACE

Various members of the plant family Leguminosae are characterized by the ability to associate with certain nitrogen fixing gram negative soil bacteria. These diazotrophic bacteria (referred to as rhizobia) belong to a diverse group of Alpha-proteobacteria and Beta-proteobacteria (Chen *et al*., 2003). Most known nitrogen-fixing rhizobia belong to different genera within the Alpha-proteobacteria. In 1976, the genus *Methylobacterium* was proposed to house the strictly aerobic, gram negative, pink pigmented and facultatively methylotrophic bacteria which formed part of the Alpha-proteobacteria. Interactions amongst plants and *Methylobacterium* strains are varied as some associate with epiphytes, others with endophytes, while others interact with legumes as nitrogen fixing microsymbionts (Omer *et al*., 2004). Bacteria from this genus have been shown to be broadly distributed throughout most environments. The first species to be described belonging to this genus was *Methylobacterium organophilum*. To date, this genus is known to only contain a single symbiotic species, namely *M. nodulans*, which was isolated from root nodules of the legume *Crotalaria podocarpa* (Sy *et al*., 2001).

The work presented in this dissertation is focused on the rhizobial species of *Methylobacterium* that are associated with the indigenous legumes *Crotalaria* and *Listia*. *Crotalaria* species typically forms two categories of associations, first with the slow growing rhizobia said to be related to *Bradyrhizobium*, and secondly, fast growing rhizobial strains which were later identified and classified as *M. nodulans*. During further studies, a distinctive group of *Methylobacterium* strains was isolated from a diverse group of *Listia* species in Southern Africa, which adds on to the focus of this study. However, not much work has been conducted to further investigate the taxonomic standing of *Methylobacterium* strains isolated from *Listia*.

Section **one** and **two** of this dissertation covers the background information and review of the current literature, which focuses on the taxonomy of the Alpha- and Beta- rhizobia. Section **two** also covers the legume-rhizobium symbiosis interaction and genes involved in nodulation and nitrogen fixation. These sections also cover promiscuity and specificity of rhizobia and/or legumes. Finally, the second section concludes with a review of *Methylobacterium* species in terms of their habitats, associations with plants and further reviews the methods used to describe *Methylobacterium* species.

In Section **three** of this dissertation, methods that helped achieve the aim and objectives of the study are discussed in detail. Details regarding the origin of all the cultures used in this study are provided. In addition, details regarding the isolation and culturing of bacteria, as well as the protocols followed to describe and further characterize these bacteria are also laid out and explained in detail.

In Section **four**, the results of the study, aimed at investigating the taxonomic position of *Methylobacterium* isolates associated with *Crotalaria* and *Listia*, are described in detail. The phylogenetic analysis of the four hose-keeping genes (i.e. 16S rRNA, *atpD*, *recA* and *rpoB*) used to identify and delineate *Methylobacterium* isolates separated *Methylobacterium* isolates into two groups, A and B. The phenotypic tests used to study and investigate the taxonomic position of *Methylobacterium* isolates showed the influence on their growth by temperature, pH and NaCl, irrespective of the host or growth media, while carbon utilization tests did not provide reliable results regarding *Methylobacterium* host influence.

Section **five** forms the discussion of the findings of this study. Overall, single gene phylogenies of 16S rRNA, *recA* and *rpoB* best delineated *Methylobacterium* isolates mainly relating them with the known and available genome reference strains of *Methylobacterium nodulans* and *Methylobacterium* sp. 4-46. The section also discusses how the isolates separated according to their different host legumes i.e., *Crotalaria* and *Listia*, although the grouping of two of the *Crotalaria* isolates (SA531a and SA531c) with *Listia* isolates was not expected. The section further discusses the possible reasons for this outcome.

1. INTRODUCTION

Bacteria capable of fixing atmospheric nitrogen when symbiotically associated with plants, more specifically legumes, are referred to as rhizobia. The interactions between the rhizobia and their plant hosts have been studied in detail. Under nitrogen limiting soil conditions, host legume plants release signal flavonoids into the rhizosphere. Rhizobia present in the rhizosphere sense these compounds and will activate the expression of essential nodulation genes to form specific lipochitooligosaccharides known as Nod factors (Oldroyd, 2013). The Nod factors get recognized by the plant and this interaction triggers root hair curling to trap the rhizobia. These are the initial steps commonly required for the formation of specialized nodule structures where the symbiotic association occurs (Madsen *et al.*, 2010; Oldroyd, 2013; Maroti and Kondorosi, 2014). Within nodules, atmospheric nitrogen (N_2) is reduced by the bacteria into ammonium (NH_4^+), which is made accessible to the host plant. At the same time, the host plant offers protection and access to fixed carbon as energy source to the bacteria (Mus *et al.*, 2016; Remigi *et al.*, 2016).

Taxonomically, rhizobia are classified into various genera in either the Alpha- or Beta-proteobacteria. Those from Beta-proteobacteria belong to genera such as *Cupriavides* and *Paraburkholderia* (*Burkholderia sensu lato*). Most nitrogen-fixing rhizobia belong to different genera within the Alpha-proteobacteria such as *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Allorhizobium* (De Lajudie *et al.*, 1998). Further, within the Alpha-proteobacteria, an additional rhizobial branch, involving bacteria in the genus *Methylobacterium*, was first introduced by Sy *et al.* (2001). According to Ardley *et al.* (2009), interactions among legumes and *Methylobacterium* species are varied. For example, some associate as epiphytes (on plants) like *Methylobacterium phyllostachyos* (Omer *et al.*, 2004), others as endophytes (inside plants) (e.g., *Methylobacterium pseudosasicola*) (Van Aken *et al.*, 2004), and yet others interact with plants as nitrogen fixing symbionts (e.g. *Methylobacterium nodulans*) (Sy *et al.*, 2001; Jaftha *et al.*, 2002; Yates *et al.*, 2007).

Methylobacterium strains are mainly categorized by their capability to grow at the expense of reduced carbon compounds containing one or more carbon atom but containing no carbon-carbon bonds (Lidstrom, 2006). Such substrates include methanol, methane, methylated amines, methylated sulfur and galogenated methane, as well as different substrates that use

multi-carbon compounds like acetate, ethanol and ethylamine (Lidstrom, 2006). *M. nodulans*, first isolated from the legume *Crotalaria podocarpa* in Senegal, was found to possess the *mxaF* gene that codes for methanol dehydrogenase (MDH) (Sy *et al.*, 2001). Disrupting the *mxaF* gene via insertional mutagenesis negatively affected plant growth. The methylotrophic-minus mutants were linked to a drastic reduction in nodule number and plant biomass (Jourand *et al.*, 2005). As a result, the capability to utilize methanol was thought to be advantageous to *M. nodulans* for nodulation (Ardley *et al.*,2009).

The discovery of symbiotic nitrogen fixing *Methylobacterium* species was triggered by the observation of specific symbiotic associations between rhizobia and different *Crotolaria* species in Senegal (Samba *et al.*, 1999). *Crotalaria* species formed two types of associations, where the first was associated with fast growing rhizobia while the second was linked to slow growing rhizobia (Samba *et al*., 1999). At the time, the strains nodulated by the slow growing rhizobia were related to *Bradyrhizobium japonicum*, while the fast growing rhizobial strains were not related to any known species (Samba *et al.*, 1999). The fast growing rhizobial strains were isolated from *Crotalaria glaucoides, C. perrottetii,* and *C. podocarpa*, and were eventually identified and classified as *Methylobacterium nodulans* (Jourand *et al.*, 2005).

As molecular biology technologies have advanced, and interest in nitrogen fixation characteristics of *Methylobacterium* species grown, phylogenetic approaches based on the 16S rRNA and *mxaF* genes have been applied to further identify and classify *Methylobacterium* isolates from different legume hosts (Jaftha *et al.*, 2002; Jourand *et al*., 2004; Yates *et al.*, 2007; Ardley *et al*., 2009). The *nodA* and *nifH* genes have also been utilized, although genes which form part of the accessory genomes could be influenced by horizontal gene transfer (HGT) (Jourand *et al*., 2004).

During these additional studies, a distinct group of *Methylobacterium* strains was isolated from a diverse group of *Listia* species in South Africa, which included *L. angolensis, L. bainesii, L. listii,* and *L. solitudinis* (Jaftha *et al.*, 2002; Yates *et al.*, 2007). These isolates were closely linked to *Methylobacterium nodulans* (Yates *et al.*, 2007). Nevertheless, cross inoculation studies revealed that *M. nodulans* does not nodulate *Listia* species (Yates *et al.*, 2007). *Listia* species were nodulated by pigmented *Methylobacterium* strains (Ardley *et al.*, 2013) as opposed to *M. nodulans* which is specific to *Crotalaria* species. In addition, the

Methylobacterium strains that nodulated with *Listia* did not have the *mxaF* gene, which was present in *M. nodulans* and were therefore unable to use methanol as their single carbon source (Ardley *et al*., 2009). Taken together, there is limited knowledge of the diversity and distribution of *Methylobacterium* species associated with different legume species in South Africa. Therefore, the aim of this study was to utilize a set of housekeeping genes (i.e. 16S rRNA, *rpoB, recA* and *atpD*) and phenotypic characterization to study the diversity and distribution of several potentially new *Methylobacterium* species associated with *Crotalaria* and *Listia* species from different regions in South Africa.

1.1. Aim

To investigate the diversity of *Methylobacterium* rhizobial isolates associated with indigenous legumes in South Africa.

1.2. Objectives

- To investigate the taxonomic position of *Methylobacterium* isolates associated with *Crotalaria* and *Listia* hosts.
- To sequence housekeeping genes encoding 16S rRNA, ATP synthase beta subunit (*atpD*), DNA recombination and repair protein (*recA*) and RNA polymerase beta subunit (*rpoB*).
- To compare single gene phylogenies to identify consistent groups in order to delineate putative new species.
- Phenotypic characterization of the strains.

2. NITROGEN FIXATION

2.1. The legume-rhizobium symbiosis

The legume-rhizobium symbiosis involves a complex, but interesting, biochemical process that is carried out in an association between the plant host and the bacterial symbiont (Verma, and Long, 1983; Rolfe and Gresshoff, 1988; Long, 1989; Brewin, 1991; Fisher and Long, 1992; Long and Staskawicz, 1993) (Figure 2.1). In nitrogen limiting soil conditions, host legume plants release signal flavonoids (Figure 2.1) into the rhizosphere. The rhizosphere bacteria sense and activate the expression of essential nodulation genes to produce specific lipochitooligosaccharides known as Nod factors. The Nod factors play a crucial part in legume-rhizobium specificity (Spaink, 2000). Nod factors are recognized by the host plant, and this interaction initiates the first step in nodule formation by triggering root hair curling to trap the rhizobia and start the subsequent formation of the infection thread into the root hair (Verma and Long, 1983). Nod factors also trigger cortical cell division in the plant primordium to form a nodule. The bacteria move via the infection thread and then enter the nodule (Spaink, 2000). The rhizobia inside the infected nodule cells are surrounded by the plant plasma membrane which develops to form symbiosomes (unique structures that fix atmospheric nitrogen) (Emerich and Krishnan, 2014). Eventually, the symbiosomes, upon maturity, allow the rhizobia to switch to their bacteroid nitrogen-fixing form.

Within root nodules, inorganic forms of atmospheric nitrogen (ammonium and nitrates) are produced by bacteria, and form part of the earth's food webs through utilization by the legume plant (Emerich and Krishnan, 2014). The bacterial nitrogenase enzyme reduce atmospheric nitrogen into ammonia and eventually amino acids that in turn gets transported through the plant (Lindström *et al.*, 2002; Emerich and Krishnan, 2014; Gnat *et al.*, 2015) (Figure 2.2). In response, bacterial symbionts gain carbohydrates in the form of malate and succinate from the legumes (Emerich and Krishnan, 2014).

2.2. Diversity and specificity of legume-rhizobia interactions

Most of the biosphere's available nitrogen supporting plant nutrition is accounted for by the legume-rhizobium symbiosis (Oldroyd and Downie, 2008). Rhizobia are polyphyletic and represent a varied collection of bacteria from both Alpha-proteobacteria (e.g., genera *Rhizobium, Bradyrhizobium, Mesorhizobium, Ensifer* and *Methylobacterium*) and Beta-proteobacteria (e.g., genera *Paraburkholderia* and *Cupriavidus*) (Gyaneshwar *et al.* 2011; Gnat *et al*., 2015). Such diversity is partly explained by the fact that the nodulation (*nod*)

genes are often found on symbiotic plasmids (Rogel *et al.* 2001). Symbiotic plasmids are extremely portable genetic elements that can be exchanged through HGT between rhizobial species of different genera (Ding and Hynes, 2009).

Rhizobium-legume host specificity has been reported for host plants such as *Galega offcinalis* (tribe Galegeae) which nodulate with *R. galegae* (Andronov *et al.*, 2003). *Methylobacterium* species are also thought to have host plant specificity, i.e., between *Crotalaria* and *Listia/Lotononis* species (Ardley *et al.*, 2009). Modifications of Nod factors (i.e., length and acyl group concentration) are known to determine host specificity (Zhang *et al*., 2012). These unique partnerships amongst rhizobia and legumes were established over evolutionary time and have had a physiological adaptation effects that is linked to legumes becoming successful invasive species in various areas in the world such as those belonging to the sub-family Mimosoideae (Parker, 2001; Rodríguez-Echeverría *et al.*, 2014). In contrast, promiscuity either in host plants or rhizobial symbionts also exists. For example, members in the genera *Ensifer*, *Rhizobium* as well as *Bradyrhizobium* are nonselective as they nodulate a diverse range of legumes (van Rhijn *et al.*, 2001).

2.3. Rhizobia classification

2.3.1. The taxonomy of rhizobia

Initially, rhizobia were collectively grouped within the single genus *Rhizobium* in the family *Rhizobiaceae* and class Alpha-proteobacteria (Young *et al.*, 2003; Zakhia and de Lajudie, 2001). At first, all rhizobia were assumed to be capable of forming nodules in association with any legume host (Jordan, 1982; Sadowsky *et al*., Graham, 1991). Later studies, however, indicated that these bacterial strains could only interact with a specific range of legume host plants leading to differentiation of strains based on the specific legumes they nodulate (Young *et al.*, 2003; Willems, 2006; Zakhia and de Lajudie, 2006). Based on current classification methods, the known rhizobia belong to seven bacterial families, represented by 15 genera (Howieson and Dilworth, 2016; Lardi *et al.*, 2017) (Table 1).

2.3.2. Proteobacteria

The Proteobacteria are biologically important since they comprise of bacteria with medical (humans), veterinary (animals), industrial and agricultural potential (plants) pathogens as well as beneficial bacteria (Dreyfus *et al*., 1988; Balows *et al*., 1992; Holt *et al*., 1994; Friedland, 1998; Michod *et al*., 2008; Martínez-Hidalgo and Hirsch 2017; Smercina *et al*.,

2019). Proteobacterial cells are also viewed as the initial mitochondrial donor and in this way, contributed towards the origin of eukaryotic cells (Gray and Doolittle, 1982; Gray, 1992; Margulis, 1993; Gupta, 2000).

Proteobacteria represents the majority of gram-negative bacteria in prokaryotes (Madigan and Martinko, 2005; Gupta, 2000). This group contains complex phenotypic and physiological attributes like phototrophy, while others are heterotrophs and chemolithotrophs (Stackebrandt, 1992; Stackebrandt *et al*., 1988; Woese, 1987; Savarzin *et al*., 1991; Trüper, 1987). Although originally known as the purple bacteria and relatives, this name soon became inappropriate as the purple (photosynthesis) attribute was only found in a few members within the Proteobacteria (Gupta, 2000). As a result, the International Committee for Systematic Bacteriology proposed the name to be changed from 'Purple bacteria and relatives' to 'Proteobacteria' to cater for the diversity of traits within the group (Gupta, 2000; Stackebrandt *et al*., 1988). Proteobacteria (purple bacteria) was first circumscribed based on similarities of 16S rRNA/rDNA analyses (Woese, 1987; Fox *et al*., 1980; Woese *et al*., 1985). This criterion further grouped organisms within Proteobacteria into five classes which include Alpha-, Beta-, Gamma-, Delta- and Epsilon-proteobacteria (Garrity, 2001). Using 16S rRNA, other partial housekeeping gene sequences, full genomes and DNA-rRNA hybridization, new species continue to be added to the Proteobacteria (Gupta *et al*., 2000).

2.3.3. Alpha-proteobacteria

The members of this bacterial class are highly varied and although they have limited shared characteristics, they share a common ancestor. These bacteria are generally Gram-negative with some being parasitic with no peptidoglycan and therefore gram variable, (Brenner *et al*., 2005). The 16S rRNA phylogenies show clear separation of the Alpha-proteobacteria from the other Proteobacterial classes (Figure 2.3). Species from several genera within the Alpha-proteobacteria have been found to enter a symbiotic association with legumes (Gnat *et al*., 2015).

Alpha-proteobacteria have singular genome organisation and intracellular lifestyles which in certain instances lead to a reduction in their genome size, while others have enlarged genomes (Williams *et al*., 2006). Large genomes are, however, suggested to be essential for existence and competition in the rhizosphere (Young *et al*., 1996). Within the Alpha-

proteobacteria, all rhizobial isolates were initially classified into the genus *Rhizobium* which contained both fast and slow growing bacteria. Later, the fast growing and acid producing bacteria were separated from those that were slow growing and produced alkali (Jordan, 1982). The slow growing strains were moved to the novel genus *Bradyrhizobium* (Jordan, 1982; Young *et al.*, 2003). With time, taxonomic methods improved leading to more isolates being moved to other newly established genera (e.g. A*zorhizobium, Mesorhizobium, Rhizobium and Ensifer/Sinorhizobium)* (Gnat *et al*., 2015). Over the years, this has resulted in a controversy concerning the taxonomic status of some of the genera such as *Rhizobium* and *Agrobacterium* (Farrand *et al.*, 2003; Sahgal and Johri, 2003). The classification of rhizobia (alpha and beta) is guaranteed to remain dynamic and sometimes controversial, due to the relationship they have with non-symbionts, improvements in identification methods, and also because of the changing views on what bacterial species are and how they should be classified (Farrand *et al*., 2003).

2.3.4. Beta-proteobacteria

In 2001, members of Beta-proteobacteria were reported to be capable of nodulating legumes and fixing atmospheric nitrogen (Moulin *et al.*, 2001). Beta-proteobacteria members are found in the order Burkholderiales, where they were divided into four genera (i.e., *Burkholderia* sensu lato, *Cupriavidus*, *Herbaspirillum* and *Achromobacter*) (Euzeby, 1997; Barret and Parker, 2006; Gnat *et al*., 2015). Although potential pathogenic *Burkholderia* strains have been reported (e.g., *Burkholderia cepacian*), nodulation and nitrogen fixation ability are mainly associated with the non-pathogenic strains (Sprent *et al.*, 2017). The genus *Burkholderia* was later reclassified, creating five additional genera (i.e., *Burkholderia* sensu stricto, *Robbsia*, *Paraburkholderia*, *Caballeronia* and *Cupriavidus*) (Sawana *et al*., 2014).

The genus *Paraburkholderia*, which accommodated most of the rhizobial species, has since been validly published and now contains 17 of the legumes-nodulating species namely; *P. dilworthii, P. tuberum, P. nodosa, P. sabiae, P. rhynchosiae, P. dipogonis, P. piptadeniae, P. ribeironis, P. sprentiae, P. mimosarum P. caribensis, P. phymatum, P. caballeronis, P. aspalathi, P. phenoliruptrix, P. kirstenboschensis* and *P. diazotrophica*, (Beukes *et al.*, 2017). These nodulating *Paraburkholderia* strains have further been proposed to be split into two groups, i.e., those nodulating *Mimosa* and related species within Mimosoideae mainly found in South America (Gyaneshwar *et al.*, 2011; Bournaud *et al.*, 2013). The second group includes those that can nodulate varied papilionoid species of the Cape Floristic

region/Fynbos (De Meyer *et al.*, 2016; Lemaire *et al.*, 2015, 2016). It has been shown that *Mimosa* species are nodulated by Alpha-rhizobia as well, and that the two different rhizobial classes (Alpha and Beta-proteobacteria) can occupy diverse nodules on the same plant in *Mimosa* (Barret and Parker, 2006; Elliot *et al.*, 2009).

a. The genus *Rhizobium*

The genus *Rhizobium* was proposed for the fast-growing, nodulating, and polyphyletic bacteria (Mousavi *et al*., 2015). At present *Rhizobium* contains 103 species (http://www.bacterio.net) of which the widest host range species *R. leguminosarum* is the type species. Several species in this genus are nitrogen-fixing and form nodules. There are, however, those within the genus that do not have the capability to nodulate and fix atmospheric nitrogen e.g., *R. selenireducens* (Hunter *et al.*, 2007). Most species belonging to this genus were either isolated from nodules of the leguminous plants or roots of cereals as nitrogen fixing associates (Román-Ponce *et al*., 2016).

b. The genus *Mesorhizobium*

Jarvis *et al.* (1997) first described the genus *Mesorhizobium* to include the former *Rhizobium loti, Rhizobium haukii, Rhizobium cicero* and *Rhizobium tianshanense.* Presently, the genus *Mesorhizobium,* contains approximately 43 species of which *M. loti* is the type species (Euzeby, 1997; http://www.bacterio.net). Several isolates nodulating agroforestry legumes (e.g. *Acacia abyssinica, A. senegal, A. tortilis and Sesbania sesban*) were found to belong to the genus *Mesorhizobium* in Ethiopia (Degefu *et al*., 2011, 2013).

c. The genera *Ensifer and Sinorhizobium*

The genus *Sinorhizobium* was introduced to house the fast-growing soybean-nodulating species *S. xinjiangensis* and *S. fredii* (Chen *et al*., 2017; Chen *et al.*, 1988). *Ensifer* on the other hand, was first described to accommodate *Ensifer adhaerens*, a bacterial predator (Casida, 1982) apparently capable of nodulating legumes when it receives symbiotic plasmids from *Rhizobium tropici* (Rogel *et al.*, 2001). The genera *Ensifer* and *Sinorhizobium* have later been suggested to be synonymous and thus all *Sinorhizobium* species have been transferred to *Ensifer* (Willems *et al.*, 2003; Chen *et al*., 2017). Rhizobial strains isolated from the root nodules of *Astragalus* species were at first classified as belonging to the genera *Rhizobium*, *Mesorhizobium* or *Sinorhizobium* (Yan *et al*., 2016). Currently, 19 of these species have been published and reclassified into the genus *Ensifer,* including *E. alkalisoli* and *E. meliloti* (Yan *et al*., 2016; Young, 2003). Only three out of the 19 species (i.e., *E.*

fredii, *E. sojae*, and *E. glycinis*) can form nitrogen fixing root nodules with soybean (Chen *et al*., 1988; Yan *et al.*, 2016, and Chen *et al*., 2017).

d. The genus *Bradyrhizobium*

This genus was first described in 1982 by Jordan. It currently consists of 39 species (including one non-rhizobial species) (http://www.bacterio.net). This genus is known to include all slow-growing, Gram-negative aerobic bacteria (Jordan, 1982). The bacteria within this genus have a generation time between 10 and 12 hours. These rhizobia are rod shaped and use flagellum as a means of mobility, when grown on Yeast Mannitol Agar (YMA). *Bradyrhizobium* colonies are less than 1 mm in diameter (Vincent, 1970). Most *Bradyrhizobium* species establish effective symbiosis with a wide range of agriculturally important legume crops such as soybean, pigeon pea, cow pea and peanuts (Subramanian *et al*., 2006; Steenkamp *et al*., 2008; Pule-Meulenberg *et al*., 2010; Araújo *et al*., 2017).

e. The genus *Methylobacterium*

The first strain from the genus *Methylobacterium* to be described was isolated in 1913 by Bassalik. This genus consists of 55 validly published species, including a single nitrogen fixing symbiont (Jourand *et al.*, 2004). Species of *Methylobacterium* are identified by their ability to grow on methanol as a carbon source (Jourand, 2004). They are strictly aerobic, Gram-negative bacteria which catalyse methanol oxidation (Van Dien *et al*., 2003; Jourand *et al*, 2004). Many isolates have pink pigmentation owing to the existence of carotenoids (Van Dien *et al.*, 2003). These species grow at moderate temperatures (mesophilic), typically ranging from 25°C to 30°C.

The first non-pigmented nitrogen fixer *Methylobacterium* was initially isolated from *Crotalaria podocarpa* in Senegal where these isolates were asigned to a novel species, *Methylobacterium nodulans* (Sy *et al.*, 2001; Jourand *et al.*, 2004). Additional *Methylobacterium* strains that have a pink pigmentation have always been found in root nodules of *Listia* spp. (Jaftha *et al.*, 2002; Yates *et al.*, 2007). These *Listia* associated strains are specific to their host (Yates *et al.*, 2007) but their taxonomy has not yet fully been resolved. They are characterized by rod shaped and Gram-negative cells (Yates *et al.*, 2007). They can aerobically utilize single carbon compounds (C1) and other multi-carbon compounds as substrates, a process known as methylotrophy (Patt *et al.*, 1976).

2.4. Species of *Methylobacterium*

The interest to investigate and study the pink pigmented facultative methylotrophs (PPFMs) began between 1960 and 1970 due to the important impact they had on biotechnology through their involvement in the assimilation of single-carbon compounds. The first *Methylobacterium* strain to be found with methylotrophy ability was *M. organophilum*. However, after a thorough investigation concerning its physiology, morphology, and biochemical characteristics, it now falls under the *Methylobacterium* strains that cannot exploit methane as a single source of carbon (Green and Bousfield, 1981: Green and Bousfield, 1982). *M. organophilum* so far remains the only PPFM bacterium to have been reported at some point to grow on methane.

A later investigation at the National Collection of Industrial Bacteria and the American Type Culture Collection (ATCC) showed that neither of *Methylobacterium* strains were able to show growth on methane (Green and Bouslfield, 1983). Several failed attemps were made to demonstrate methane utilization for this strain. It was then proposed that its ability to integrate methane possibly was plasmid-borne and might have vanished due to the culture not being maintained in an organic medium under methane-containing atmosphere (Green, 1999). Consequently, the *Methylobacterium* description was amended to include bacteria that are not capable of utilizing methanol (Green and Bousfield, 1983**)**. It was also suggested that all PPFM bacteria be moved to the genus *Methylobacterium*. These also included *Pseudomonas rhodos*, *Pseudomonas rodiora*, and *Pseudomonas mesophilica* (Green and Bousfield, 1983; Green and Ardly, 2018). Nucleic acid hybridization and molecular techniques confirmed that the PPFM should form part of the genus *Methylobacterium* but that the *Pseudomonas* strains should be excluded (Wolfrum *et al.*, 1986**)**.

2.5. *Methylobacterium* habitat and association with plants

The PPFM are firmly facultative and aerobic and use a range of single carbon to multi-carbon substrates. The formerly named and described species of *Methylobacterium* live in diverse environments such as aquatic environments, plants (Green and Bousfield, 1982; Corpe and Rheem, 1989), ice lands (Moosvie *et al.*, 2005) and the Antarctica ice core (Antony *et al.*, 2012). The PPFMs are also found in hospitals (Furuhata and Koike, 1990; Kaneko and Hiraishi, 1991; Furuhata and Koike, 1993), bathrooms (Furuhata and Matsumoto, 1992) and on a human foot (Anesti *et al.*, 2006). Numerous species of *Methylobacterium* are infectious to humans, for example "*Persist bacteremia*" infected a child that had lymphoma (Fernandez

et al., 1977; Lai *et al.*, 2011). *Methylobacterium* species found in clinical surroundings have the capability to thrive under stressful conditions and antimicrobials (Yano *et al.*, 2013; Kovaleva *et al.*, 2014).

Some *Methylobacterium* spp. live in the plant environment (Ivanova *et al.*, 2001). For example, *Methylobacterium mesophilicum* was isolated from a citrus plant by Gai *et al.* (2009) and *Methylobacterium oryzaei* from rice phyllosphere (Yim *et al.*, 2010). The genus *Methylobacterium* is thought to be highly common phyllosphere colonizing bacteria (Knief *et al.*, 2010). The association with plants is, however, varied with some of the *Methylobacterium* being symbionts that nodulate roots of legumes (Sy *et al.*, 2001; Jaftha *et al.*, 2002), others are plant endophytes (Elbeltagy *et al.*, 2000; Pirttilä *et al.*, 2000; Lacava *et al.*, 2004; Van aken *et al.*, 2004), while yet others inhabit the surface of plants (Corpe and Rheem, 1989; Hirano and Upper, 1991; Omer *et al.*, 2004). It is suggested that these associations are long-term (Kutschera and Niklas, 2005; Fedorov *et al.*, 2011) and that the isolates are interdependent phytosymbionts (Kutschera, 2007).

2.6 The role of methylotrophy in plant colonization

Several studies have shown that the ability of *Methylobacterium* to colonize plants is aided by the key role played by the C1 metabolism (Jourand *et al.*, 2005; Sy *et al.*, 2005). Vorholt (2002) identified and characterized the enzymes which are key in methylotrophy. In the study, 46 000 proteins were identified in the phyllosphere which were linked to methylotrophy. Initially, formaldehyde (that is later transferred to the cytoplasm) is formed through the oxidation of methanol inside periplasm by the key enzyme methanol dehydrogenase (MDH). Part of the oxidation product is then broken down into CO_2 to produce energy and the remaining formaldehyde is then assimilated through the serine cycle (Figure 2.4).

The only known and described nodulating *Methylobacterium* to date is *M. nodulans* which possesses *mxaF* gene cluster for coding MDH. The role of methylotrophy and how it is expressed amongst *M. nodulans* strain OR2060^T and *Crotalaria podocarpa* was investigated by Jourand *et al.*, (2005). It was found that deletion of *mxaF* gene in *Methylobacterium nodulans* used to inoculate *Crotalaria podocarpa* resulted in a reduction in nodule formation, nitrogen fixation capacity and total biomass by approximately 60%, 42% and 46% respectively. The insertion of a functional *mxaF* genetic factor to the mutant of *M. nodulans* restored its symbiotic activity.

2.7. Methods used in describing *Methylobacterium* species

Bacterial species are usually described using already established or standard approaches like DNA-DNA Hybridization (DDH), 16S rRNA gene sequencing, phenotypic characteristics and genome-based criteria like average nucleotide identity (ANI). Until late 1960s, methods used to delineate species and genera were mainly based on observable phenotypic characteristics (Lehmann and Neumann, 1896). The most important development for clarifying species-strain relationship and the breaking down of the species and genera was initiated by introducing DDH methods (Kelly *et al.*, 2014). Today, the most advanced approaches are Multi-locus analysis (MLSA) and Multi-locus sequence typing (MLST) which permit a higher resolution regarding how the population of species is structured. This has led to a more conclusive decision about ecology, evolution, systematics, and epidemiology (Konstantinidis, 2006).

2.7.1. DNA-DNA Hybridization (DDH)

DNA-DNA Hybridization, sometimes referred to as the DNA-DNA reassociation method is grounded on an effort to create unbiased comparisons of whole genomes among diverse organisms to obtain their total identities at genomic level (Rossello-Mora, 2006). The DDH method is widely used in classification of organisms at lower ranks up to species level. The DDH approach was embraced as part of the 'modern spectrum' of techniques which made use of genetic measurements to circumscribe species instead of methods which employed biochemical and morphological characteristics (Goris *et al.*, 2007). The DNA-DNA Hybridization method employs the principle that denatured single DNA strands from organism A and B would duplex if their overall DNA nucleotide compositions were similar and that the two DNAs were isolated from genetically related organisms (Schildkraut *et al.*, 1961; Rossello-Mora, 2006).

There has generally been a universal agreement on the DDH cut-off limit of $\geq$ 70% relatedness for species as well as other conditions like variations in melting temperature within the range of 5 $^{\circ}$C or less for species (Schildkraut *et al.*, 1961; Stackenbrandt and Goebel, 1994; Rossello-Mora and Amann, 2001; Rosello-Mora, 2006; Stackenbrandt and Ebers, 2006). As a result, DDH has then been widely used to characterize prokaryote species (Stackebrandt, 2003). This approach, however, is faced with several drawbacks which have necessitated the development of other approaches. These drawbacks include: i) need for high

quantity and quality DNA compared to polymerase chain reaction (PCR) based techniques, ii) it is time consuming and labour intensive, iii) inter and intra laboratory variations while conducting DDH can yield different results and iv) due to the comparative nature of the the technique, no database can be built compared to the sequence based comparative techniques (Stackebrandt, 2003; Gevers *et al.*, 2005; Goris *et al*., 2007). Consequently, other promising methods to replace DDH like ANI and MLSA were proposed (Stackebrandt *et al.*, 2002; Konstantidis and Tiedje, 2005b).

2.7.2. Average nucleotide identity (ANI)

This method was proposed to determine how the prokaryotic strains are related on a genetic level by directly comparing complete genome sequences (Wayne *et al.*, 1987; Stackebtrandt *et al*., 2002; Goris *et al*., 2007). There were at least three conditions to be met: i) "that both advantages and disadvantages balance and justify the employment of whole genome," ii) "the approach should be supported by availability of strong bioinformatic tools and models to analyse and synthesize enormous information contained within the genomes," and iii) "that the ANI approach results should always be comparable and validated using DNA-DNA Hybridization data". For example, the 94% species delineation ANI value corresponded to 70% cut-off value for DDH (Konstantinidis and Tiedje, 2005a; Goris *et al.*, 2007; Rosello-Mora, 2009).

ANI is therefore considered being reliable for delineating archaeal and bacterial species as it is simple to use for the overall description of genetic relatedness between organisms. ANI uses diverse genes within a genome, representing a large dataset compared to the use of a few or a single gene (e.g. 16S rRNA), hence it provides an accurate measure of the level of relatedness between organisms (Konstantinidis and Tiedje, 2005a). The generated ANI results are largely not influenced by rates of evolution such as HGT because the slow evolving genes mitigate the effect of a few genes that evolve at faster rates (Konstantinidis and Tiedje, 2005a). Also, ANI values have been used to distinguish *Methylobacterium indicum* from the related *Methylobacterium platani* (Chaudhry *et al*., 2016).

2.7.3. MALDI-TOF/MS

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) technology, which profiles all proteins in a cell, has been used for identifying bacteria including *Methylobacterium* (Tani *et al.*, 2012). In MALDI-TOF-MS, the ribosomal proteins

are mainly represented by the mass spectral profiles since they are the most abundantly synthesized proteins during all organisms' growth conditions (Jarman *et al.*, 2000). This enables the MALDI-TOF-MS technique to accurately identify and characterize bacteria at different levels (i.e., genus, species, and subspecies) (Welker and Moore, 2011; Tani *et al.*, 2012). The MALDI-TOF-MS technique was used to classify approximately 200 *Methylobacterium* isolates from different environments (Tani *et al.*, 2012). The results were comparable to those from the 16S rRNA sequence analysis.

The MALDI-TOF-MS strategy cannot, however, capture the evolutionary relationships between species. This is because most of the generated spectral peaks from ribosomal translated proteins only show the diversity within species and not their evolution relationships (Tani *et al.*, 2012). Hence, phylogenetic analysis is recommended after utilization of MALDI-TOF-MS in selecting unique strains.

2.7.4. Phenotypic characterization methods

a. Carbon source utilization

Carbon source utilization data is a phenotypic strategy that is used to compare and differentiate taxa or species including members from *Methylobacterium* (Green and Ardley, 2018). The use of carbon substrates reveals physiological characteristics between strains (Antonie van Leewenhoek, 2012; Yang *et al*., 2012). Some of the carbon sources utilized include aspargine, aspartic acid, alanamide, D-fructose, D-galactonic acid, succinamic acid, saccharic acid, methanol, methyamineand pyruvate (Ardley *et al*., 2009; Yang *et al*., 2012).

There has been also development of tool kits like the GN2 Biolog Microplate containing up to 95 varied carbon sources that can be used to test for carbon utilization (Miller and Rhoden, 1991; Holmes *et al*., 1994). These Microplates employ the reduction of tetrazolium, responding to the respiration process of organisms. The carbon utilization trait is scored positive when a colour is developed as opposed to no colour development (Tang *et al*., 1989).

The GN2 Microplate method is easier to work with because it does not require additional chemicals to develop the colour. The GN2 Microplate can identify up to 500 species, being the largest kit containing database identification of species compared to other kits (Tang *et al.*, 1989). The Biolog method can be used by a wide range of users including animal, plant, clinical as well as environmental laboratories (Tang *et al*., 1989). For example, with

reference to 16S rRNA *Methylobacterium* clades, all the species under clade B utilized methylamine and methanol while the majority of the species under clade A (85%) could not (Green and Ardley 2018). Carbon utilization however, experience inter and intra laboratory variability of the results rendering them unreliable (Green *et al.*, 2018). Despite this disadvantage, carbon source utilization methods are still useful phenotypic means for differentiating between PPFM species.

b. Temperature, salt and pH tests

Organisms can be differentiated based on their growth responses under different temperatures, salt concentrations and pH in different growth media (Bochner, 2008). For example, species from genus *Methylobacterium* can grow at temperatures ranging from 18 °C to 42 °C, with optimum temperature range between 25 °C to 30 °C (Weon *et al.*, 2008; Kayash *et al*., 2014; Park *et al*., 2018). *Methylobacterium* species are known to thrive under extreme conditions, testing for growth at extreme temperatures and salt concentrations will be additional knowledge.

The ability of bacteria to grow in the presence of variable amounts of sodium chloride (NaCl) has been used to characterize several bacteria (Weon *et al*., 2008; Schauer and Kutschera, 2011). This is based on the analogy that bacteria must counteract stressful environments to thrive as NaCl is used to mimic such environments. This assumption is applicable for *Methylobacterium* as some are plant-growth promoting species, an activity that may take place in saline environments. Moreover, some *Methylobacterium* have been found to tolerate antimicrobials in clinical fields (Kayasth *et al*., 2014; Egamberdieva *et al*., 2015). These traits have led to bacteria including isolates from *Methylobacterium* being tested for salt tolerance using NaCl. Studies have reported the ability of *Methylobacterium* to grow at NaCl concentrations of up to 2% (Weon *et al*., 2008; Schauer and Kutschera, 2011). However, other *Methylobacterium* species like *M. currus* are only able to grow in 0-1.0% of NaCl, having an optimum salt concentration of 0% (Park *et al*., 2018).

Another interesting and important factor influencing microbial growth is the pH of the growth medium (Fierer and Jackson, 2006; Jones *et al*., 2009). Different organisms have variable pH ranges for optimum growth. For example, most rhizobia prefer a neutral pH (Somasegaran and Hoben, 1994). This parameter strongly influences species composition in different environments including the soil (Fierer and Jackson, 2006; Jones *et al*., 2009; Sofi

et al., 2013). Growth tests at various pH values are simple to conduct as the pH can be adjusted using an acid (HCl) or a base (NaOH) (Ratzke and Gore, 2018). Microbes that can survive at a low pH environment have generally been known to survive under nutrient limited conditions (Yang *et al*., 2012).

2.7.5. Multilocus Sequence Analysis (MLSA)

Multilocus sequence analysis is currently the most widely used method to study the taxonomy of prokaryotes. This method considers fragments of several protein-coding genes (Gevers *et al.*, 2005). The MLSA helps to resolve phylogenetic and taxonomic relations amongst strains (Gevers *et al.*, 2005; Rong and Huang, 2012). MLSA incorporates the DNA sequences of several housekeeping genes in order to genotypically distinguish between species even across the genus level. In this way, MLSA genotypically differentiates between species even across the genus level (Gevers *et al.*, 2005). For example, an MLSA study on *Ensifer* was conducted using a DNA chaperone (*dnaK*), glutamine synthetase type1 (*glnA*) that is central to nitrogen assimilation, citrate synthase (*gltA*) that is important in controlling the amount of ATP produced within cells and the 16S rRNA gene (Hernandez-Lucas *et al.*, 2001; Martens *et al.*, 2007; Martens *et al.*, 2008). This method was proposed as ideal for evolution-based studies to accurately investigate the limits between species (Venter *et al.*, 2016). Zeigler (2003) suggested that the efficiency of the MLSA approach might be identical to or even exceed that of DDH when measuring genome similarities. In addition, MLSA takes advantage of the extensive database of reference sequences, which are cheap to use for detailed phylogenetic results compared to other tools. In addition, the housekeeping genes used are protein coding genes which are conservative in nature. Commonly used housekeeping genes in MLSA approach studies include *atpD, recA, rpoB* and *gyrB* among others (Nishiguchi and Nair, 2003; Gevers *et al*., 2004; Thompson *et al.*, 2005; Thompson *et al.*, 2007; Tracz *et al*., 2007; Martens *et al.*, 2008).

Recently, the taxonomy of *Methylobacterium* was investigated using MLSA (Thompson *et al.*, 2005 and Meier-Kolthoff *et al.*, 2013). An MLSA, using standard housekeeping genes (*atpD, Dnak, gyrB, recA, rpoB, gInI*) in addition to 22 ribosomal protein (RP) genes and phenotypic tests were previously used to describe and reclassify *Methylobacterium* species (Green and Ardley, 2018). The results generally revealed three major clades (A, B, C) (Green and Ardley, 2018). Green and Ardley (2018), however, mainly considered reference strains earlier studied, with a few new *Methylobacterium* strains. The current study, therefore, aimed

to increase knowledge of *Methylobacterium* species, by using MLSA, ANI and phenotypic techniques to describe several potential new *Methylobacterium* species from different regions in South Africa which have not been studied previously.

3. MATERIALS AND METHODS

3.1. Bacterial isolates

A total of 92 bacterial isolates were included in this study (Table 3.1). These strains were all originally isolated in South Africa from either *Crotalaria* species (30 isolates) or *Listia* (initially known as *Lotononis*) native to South Africa (62 isolates). These strains were obtained from three different sources. The largest group of isolates came from the Agricultural Research Council, Plant Protection Institute (ARC-PPRI) at Roodeplaat, Pretoria. Twelve isolates were from previous studies conducted by the Rhizobial research group at the Department of Biochemistry, Genetics and Microbiology, University of Pretoria, and seven isolates were from University of Murdoch in Perth, Western Australia. All the obtained were originally directly isolated from root nodules collected from the field.

3.2. Maintenance of bacteria

All bacterial strains were routinely sub-cultured on Yeast Mannitol Agar (YMA) (Merk Chemicals Limited, Gauteng, South Africa) plates and incubated at 28 °C for between 7 days and 2 weeks (Vincent, 1970), as some were slow growing (Corpe, 1985; Corpe and Rheem, 1989). Yeast Mannitol Agar medium contained 0.5 g/L yeast extract, 10 g/L mannitol, 0.4 g/L potassium phosphate (K_2HPO_4), 0.2 g/L magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), 15 g/L bacteriological agar, 0.1 g/L sodium chloride (NaCl), and 1L distilled water (Somasegaran and Hoben, 1994).

3.3. Molecular identification of the bacterial strains

3.3.1. DNA extraction

Genomic DNA was extracted from pure cultures using the Quick-DNA^{TM} Miniprep kit (Zymo Research, USA) according to the manufacturer's instructions. The quality of the

isolated genomic DNA was confirmed on a 1.0 % (w/v) agarose gel (Lonza, USA) after electrophoresis at 80V for 20 minutes. The gel was viewed under the ultraviolet light using the Biorad gel documentation system. A 1 kb DNA ladder (N3232S) (Biolabs) was included. DNA concentration was also determined using a NanoDrop (NanoDrop™ 2000/2000c spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer.

3.3.2. Polymerase chain reaction (PCR) amplification conditions

High quality genomic DNA was used to amplify the 16S rRNA, *recA, rpoB* and *atpD* genes using 27F/1492R (Miller *et al*., 2013; You *et al*., 2008), F/R *recA* (Ardley *et al*., 2012) as well as 83F/1061R *rpoB* and 352F/871R *atpD* (Galibert *et al*., 2001) primer sets' respectively (Table 3.2). The PCR amplifications for each of the four different genes were conducted in a total reaction volume of 25 µl on a 96 well thermocycler (Applied Biosystems). Each PCR reaction contained 16.3 µl Nuclease-Free (NF) water (Anatech, South Africa), 2 µl of 25 mM $MgCl_2$, 2.5 µl of 10x buffer, 2 µl of 2.5 mM dNTPs (Lifetech scientific), 0.2 µl of 0.2 units of Super-Therm Taq DNA polymerase (Seperation Scientific, South Africa), and 0.5 µl of a 10 µM solution of each primer (both forward and reverse), and 1µl of the DNA template.

For the 16S rRNA gene the PCR conditions were: Initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 1 minute, annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes. For the *atpD* gene PCR, an initial denaturation at 95 °C for 5 minutes was followed by 30 cycles of denaturation at 94 °C for 2 minutes, primer annealing at 62 °C for 1 minute, extension at 72 °C for 1 minute, and final extension at 72 °C for 5 minutes. PCR conditions for the *rpoB* gene were: Initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 57.8 °C for 1 minute, extension step at 72 °C and final extension at 72 °C for 5 minutes. The *recA* PCR consisted of an initial denaturation at 95 °C for 5 minutes, followed by 32 cycles of denaturation at 95 °C for 45 seconds, primer annealing stage at 62 °C for 1 minute, extension at 72 °C for 30 seconds and a final extension at 72 °C for 5 minutes. Negative controls were included in all PCR assays. A 1 kb DNA ladder (N3232S Biolabs) was used to confirm the PCR products using gel electrophoresis. Electrophoresis was done using a 1.0 % (w/v) agarose gel (Lonza, USA) made with 1 X TAE buffer and run for 30 minutes at 80 volts. The expected band sizes were then visualized under UV light using the Biorad gel documentation system.

3.3.3. Amplicon purification and sequencing

All PCR products of the expected size for each gene (i.e., ~1500 base pairs (bp) for 16S rRNA, ~800 bp for *rpoB,* ~ 400 bp for *atpD* and ~ 446 bp for *recA*) were purified using the Exonuclease I (Exo I) and thermoactive alkaline phosphatase (FastAP) enzymes (Thermo Fisher Scientific, Waltham, MA) by adding 0.5 µl Exo I and 2 µl FastAP to the PCR products (Werle *et al*., 1994). The resulting reaction was placed on the heating block at 37 °C for exactly 15 minutes, and then at 85 °C for 15 minutes. Cleaned PCR products were visualized by gel electrophoresis on 1.0 % (w/v) agarose gel using 1 X TAE buffer at 80 volts for 20 minutes and viewed under UV light using the Biorad gel documentation system. The cleaned PCR products were then stored at -20 °C for further analysis.

The purified PCR amplicons were sequenced in both forward and reverse directions using the same primers that were used for amplifying each gene region (Table 3. 2). Sequencing was done using the ABI PRISM Big Dye Terminator version 3.1 Cycle Sequencing premix kit (Applied Biosystems). The sequencing reaction was carried out in a 12 µl reaction containing 10 X sequencing buffer (2µl), ddNTPs (1µl), 10 µM primer (1µl) and 4µl of the PCR product per reaction. Amplification was done at 96 °C for 5 seconds, followed by 25 cycles of 96 °C for 10 seconds, annealing at 55 °C for 5 minutes, and finally 60 °C for 4 minutes using the 96 well plate Vertigo thermal cycler (Applied Biosystems, USA). The sequenced DNA was purified using 100% ethanol, 3M sodium acetate (pH 4.8) and 70% ethanol and sequenced (at the Bioinformatics and Computational Biology Unit, University of Pretoria) using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA).

3.4. Sequence analysis and alignment, and Phylogenetic analysis

The reverse and forward raw sequence data generated were downloaded from the seqserve online system (Bioinformatics and Computational Biology Unit, University of Pretoria) and edited using Chromas version 2.6.5 software. For the 16S rRNA region, the closest type strains of species with valid names were obtained through comparing the bacterial sequences with the (NCBI) data bank and those type strains were further used as the reference strains when constructing the phylogenetic tree.

The sequences were then compared and identified using NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Only a subset of isolates (29) that were compared

and were identified as *Methylobacterium* were further analyzed. The edited sequences of the subset of isolates were uploaded to BioEdit software version 7.2.5 (Hall, 1999) for creating consensus sequences.

Both the bacterial culture isolates' sequences (from this study) and the selected type strains reference sequences (Table 3.3) were aligned using MAFFT (Multiple Alignment using Fast Fourier Transformation; https://mafft.cbrc.jp/alignment/server/) version 7.0, which takes the secondary structure of the RNA into account. For *atpD*, *recA* and *rpoB* house-keeping genes the reference sequences were also obtained from the NCBI database whereby the gene of interest was extracted from either the whole genome sequence of the most similar strain sequences, or as individual gene sequences available from the database. The sequences of the three genes for the references strains as well as the isolates from this study were manually aligned using BioEdit (Hall, 1999). Multiple sequence alignments for these protein encoding loci were generated using BioEdit based upon the inferred amino acid sequences. The same set of 29 bacterial strains and references were used for all the four genes.

For 16S rRNA, *atpD*, *recA* and *rpoB* genes, *Methylobacterium* species which formed part of Clade C1 (*Methylobacterium salsiginis* and *Methylobacterium platani*) and from clade B (*Methylobacterium populi*) (Green and Ardley, 2018) were used as outgroups. The aligned sequences for all four genes were trimmed using BioEdit and used for phylogenetic trees construction using Mega version 7.0 software as it has been cited many times, and it allows to select methods and algorithms best suited for your data (Kumar *et al*., 2016). In Mega, the analysis of individual genes was done; which included running analysis to find the best fit DNA/Protein model, then phylogenies were inferred using maximum likelihood (ML) analyses with 1000 bootstrap replicates. The evolutionary history was inferred by using maximum likelihood method based on the Tamura-Nei model for all four genes (Tamura and Nei, 1993; Kumar *et al*., 2016). The same best fit parameters used to infer the individual phylogenies were used to generate the MLSA concatenated phylogenetic tree.

3.5. Phenotypic characterization

3.5.1. Carbon utilization tests using the Biolog (GN2) method

Carbon utilization by *Methylobacterium* isolates was tested using the Biolog as described by Madhaiyan *et al* (2007). Based on the phylogenetic results, representative isolates from different groups of the 29 isolates that were identified as *Methylobacterium* were selected

for Biolog analysis. Accordingly, three representative isolates were selected from *Crotalaria* (i.e. Crot 99, Crot 100 and Crot 224), 3 isolates from WSM group (i.e., WSM 2598, WSM 3966 and WSM 3960), 2 XCT isolates (i.e., XCT 8 and XCT 17) and 2 isolates from SA53 group (i.e., SA53-1A and SA53-1C) (Table 3.1). The selected isolates were sub-cultured on R2A agar to obtain fresh culture. A colony of each bacterium was picked using a sterile plastic loop and suspended into a specified GN/GP inoculating fluid (IF). For each well, 150 µl of the suspension was pipetted into the well of the GN2 MicroPlate (Biolog catalog #1011) (BIOLOG Hayward, CA 94545 U.S.A). The MicroPlates were incubated at 28 °C. After 3 days the MicroPlates were scored and visually compared against the GN Database (Biolog catalog #1011), and the results recorded.

3.5.2. Temperature, salt concentration and pH tests

Temperature, salt concentration and pH tests were carried out on 29 out of 92 strains that turned out to be *Methylobacterium* using phylogenetics analysis. For all the phenotypic tests R2A and YMA agar or broth were used (Gallego *et al*., 2005 and Park, 2018). For the temperature test, 29 *Methylobacterium* isolates were cultured on the two different media (i.e., R2A and YMA) and incubated at six different temperatures (10 °C, 15 °C, 25 °C, 37 °C, 40, and 50 °C) for a maximum of 7 days. To test salt tolerance, growth at different sodium chloride (NaCl) concentrations (0, 1, 2, 3, 4 and 5%, w/v) were measured after 7 days. To test different pH concentrations, the isolates were inoculated in broth and incubated at 37 °C, shaking at 150 rpm for 5 days. The pH concentration tolerance was tested at pH values of 3, 5, 8, and 10. The pH of both R2A broth and YMB were adjusted using HCl and NaOH after sterilization (0.15MPa, 121 °C for 15 minutes) (Park *et al*., 2018).

4. RESULTS

4.1. Isolation of bacterial cultures

Among the 92 isolates, 17 (18%) of the isolates had pink to pale pink colonies on this medium while the remaining 75 (72%) had cream colonies. Of the 17 pink pigmented strains, 13 were isolated from the legume host *Listia*, while the remaining 4 strains were isolated from *Crotalaria*.

4.2. Identification of bacterial isolates using 16S rRNA phylogenetic analysis

Out of the 92 bacterial isolates used in this study, 85 (92%) isolates were successfully sequenced and analysed using 16S rRNA. From the sequenced strains, twenty-five corresponded to *Bradyrhizobium*, which is a sister genus to *Methylobacterium*, both of which are classified under Alpha-proteobacteria subclass (Table 4.1). The percentage of sequence similarity to *Bradyrhizobium* ranged from (94 to 98%). Twenty-nine isolates were affiliated with *Methylobacterium nodulans*, *Methylobacterium* WSM2598, and *Methylobacterium* sp 4-46 species, with percentage similarity ranging from 94-100%. Amongst the 29 isolates which compared to the genus *Methylobacterium*, 15 were from the legume genus *Listia*. These included 13 isolates from *Listia bainesii*, one isolate from *Listia listii* and the remaining one isolate from *Listia solitudinis*. The remaining 14 *Methylobacterium* affiliated isolates were from the legume genus *Crotalaria*. Of these, 7 isolates originated from *Crotalaria lotoides* and the remaining 7 isolates from *Crotalaria sphaerocarpa*. The remaining 31 isolates out of 85 compared either to *Agrobacterim, Microvirga Neorhizobium, Rhizobium, Mesorhizobium, Sinorhizobium*, *Sphingomonas*, or *Ochrobactrum* (Table 4.1). It was then decided that further analyses were going to be carried out using only the 29 *Methylobacterium* affiliated isolates.

The dataset used for phylogenetic analysis of the 16S rRNA sequences included sequences of all the 29 isolates identified as *Methylobacterium* strains and sequences of the type strains *Methylobacterium* species (*Methylobacterium nodulans* ORS 2060 and *Methylobacterium* sp. 4-46). These type strains fall under clade C2 members of the *Methylobacterium* genus according to the recent report by Green and Ardley (2018). The limited availability of sequences from previously characterized *Methylobacterium* strains and species using *recA*, *atpD* and *rpoB* genes in the GenBank database was the reason for the use of fewer taxa as reference strains for all the four genes. In addition, sequences belonging to (*Methylobacterium salsuginis* and *Methylobacterium platani*) falling under clade B and C1

(Green and Ardley, 2018) were also included as outgroups for all the phylogenetic analysis reported in this dissertation.

The results of the Maximum Likelihood (ML) phylogenetic analysis of the 16S rRNA gene sequences of *Methylobacterium* representative isolates are presented in Figure 4.1. From the results, the isolates separated and clustered into two distinct groups (Group A and B) (Figure 4.1). Group A comprised of *Crotalaria* isolates, which clustered together (62 bootstrap value) with the only known nodulating species of *Methylobacterium* genus, *M. nodulans* (Sy *et al*., 2001). Group B on the other hand included isolates from *Listia* spp. legume hosts (85 bootstrap value). As expected, these isolates grouped together with the known *Methylobacterium* sp. 4-46. Interestingly, two *Crotalaria* isolates (SA-53 1a and SA-53 1c) grouped with *Listia* isolates in cluster B (Figure 4.1). These findings were further investigated using additional housekeeping gene sequences (see below).

4.3. Identification of *Methylobacterium* isolates using *rpoB, recA* and *atpD* genes phylogenetic analyses

Three housekeeping genes (*rpoB, recA* and *atpD*) were used to analyse all the 29 isolates that were confirmed as *Methylobacterium* species using the 16S rRNA gene phylogeny (Figures 4.2, 4.3 and 4.4). The BLAST results showed percentage similarities that range between 94-99% against members of *Methylobacterium* genus. These results corroborated those revealed by 16S rRNA gene sequences analysis. The phylogenetic analyses results using *rpoB* (Figure 4.2) and *recA* (Figure 4.3) genes sequences of *Methylobacterium* representative isolates supported the two cluster groups (A and B) identified using 16S rRNA gene phylogenetic analysis. Similar to 16S rRNA gene, 12 *Crotalaria* isolates were closely related to *M. nodulans*. *Methylobacterium* isolates from *Listia*, as well as the two isolates isolates from *Crotalaria* (SA53 1a and SA53 1c) formed a different cluster, grouping with the *Methylobacterium* sp 4-46 reference strain. This pattern was observed for both *recA* and *rpoB* genes.

The *atpD* phylogeny is presented in Figure 4.4. The recovered results did not agree with those observed for the other genes. For example, the isolates separated according to the host legumes and consistently maintained the clustering pattern of the two *Crotalaria* isolates with the *Listia* isolates. However, the *M. nodulans* reference strain grouped with the *Listia* isolates, while *Methylobacterium* sp. 4-46 reference strain closely grouped with the

Crotalaria isolates (Figure 4.4). This contradiction necessitated ANI analysis between the reference strains.

To further investigate a concatenated tree of 16S rRNA, *recA*, *rpoB* and *atpD* genes was constructed. The concatenated gene analysis yielded results that were in agreement with the 16S rRNA, *recA*, and *rpoB* single phylogenies (Figure 4.5). *Listia* isolates closely grouped together with the *Methylobacterium* sp. 4-46, while all but two of the *Crotalaria* isolates clustered together with *M. nodulan*s. Two *Crotalaria* isolates (SA 51-1a, and SA 53-1c) clustered with *Listia* isolates rather than *Crotalaria* host isolates. However, with *M. salsuginis* as the outgroup, the supposedly second outgroup (*Methylobacterium platani*) moved up into the clusters and was closely grouping with the *Crotalaria* isolates. These results were to an extent not entirely congruent with the single gene trees, and the reason for this could be the inclusion of the *atpD* gene, as it was the only gene with differing phylogenetic results. An ML analysis was accordinly conducted with only 16S rRNA, *recA*, and *rpoB* (Figure 4.6), which showed the clear separation of the two clusters. This separation was in agreement with the single gene phylogenies of the gene sequences used for Figure 4.6. Two outgroups are evident, as *M. platani* now grouped closely with *M. salsuginis* which confirmed that the *atpD* gene was affecting the concatenated tree results.

4.4. ANI analyses

ANI analysis was conducted using Jspecies programme on three whole genome sequences of strains that were used as reference strains in this study. These included *M. nodulans*, *Methylobacterium* sp. 4-46 and *Methylobacterium* sp. WSM2598 (Table 4.2). These analyses were motivated by inconsistent results generated using *atpD* housekeeping gene with regard to reference strains *M. nodulans* and *Methylobacterium* sp. 4-46, in comparison with 16S rRNA, *recA* and *rpoB* housekeeping genes (Figures 4.1-4.4). In accordance to expectations, the ANI between *M. nodulans* and *Methylobacterium* sp. 4-46 was 83% (Table 4.2). The ANI value between *Methylobacterium* sp. 4-46 and *Methylobacterium* sp. WSM 2598 strains was 98.8% (Table 4.2). These results were consistent with the phylogenetic analyses results of 16S rRNA, *recA* and *rpoB*, but inconsistent with *atpD* phylogenetic results with regards to the two reference strains (Figures 4.1 - 4.4).

4.5. Phenotypic characterization of *Methylobacterium* isolates

4.5.1. Utilization of different carbon sources

Carbon source utilization tests were done using 96 well BIOLOG microplate (Table 4.9). Representative strains from the two distinct groups based on phylogenetic analyses were selected for Biolog tests. The selected isolates were five from the *Crotalaria* host group (i.e., Crot 99, Crot 100, Crot 224, SA 53-1a, and SA 53-1c) and six from the *Listia* host group (i.e., WSM 2598, WSM 3966, WSM 3960, XCT 8, XCT 13, and XCT 17). The expectations were that isolates from the same group would test positive or negative on the same carbon compounds. However, there were no clear specific patterns according to representative groups. This is because generally different isolates within a representative group revealed varied responses to similar carbon sources.

Interestingly, while A-Cycodextrin was not utilized by any of the selected *Methylobacterium* strains, D-Galactonic Acid Lactone and B-Hydroxybutyric Acid were utilized by all the selected representative *Methylobacterium* strains. Another interesting result was that Uridine was utilized by all the first three (Crot 99, Crot 100, and Crot 224) *Crotalaria* representative strains but not by the *Listia* representative strains or the second two (SA 53-1a, and SA 53-1c) *Crotalaria* strains that always grouped with *Listia* strains. On the other hand, D-Sorbital carbon was utilized by all the *Listia* and SA 53 strains but not by the first three *Crotalaria* strains. D-Trehalose was utilized by WSM and SA 53 representative isolates and not by the first *Crotalaria* and XCT representative isolates. Generally, a low or no utilization of D-Raffinose, L-Rhamnose, and Sucrose by the first three *Crotalaria* representative isolates compared to full utilization by the *Listia* representatives and the two SA 53 *Crotalaria* host isolates was observed. Thymidine, Phenylethylamine, Putrescine and 2-Aminoethanol were not utilized by all the isolates except Crot 99.

4.5.2. Growth responses to temperature, pH and NaCl concentrations

The twenty-nine isolates that were identified as *Methylobacterium* strains were used for growth response analyses. Growth was tested at five different temperatures (10 °C, 25 °C, 37 °C, 40 °C and 50 °C) using two different growth media (YMA and R2A agar) (Table 4.3 and 4.4). For both growth media, no growth was observed at the two extreme temperatures (10 °C and 50°C) for this study. Growth was observed at 25 °C, 37 °C, and 40 °C on both media. Interestingly, there were notable distinctions in *Methylobacterium* strains optimal growth temperature between YMA and R2A agar. Optimum growth of *Methylobacterium*

on YMA agar was recorded at 25 °C for 18 isolates compared to 14 and 7 isolates at 37 °C and 40 °C, respectively (Table 4.3). On the other hand, all *Methylobacterium* strains grew well at 37 °C on R2A agar. For the two extreme temperatures the results varied, no growth was recorded at 50 °C, while at 10 °C eight isolates presented slight growth and the rest did not grow at this temperature (Table 4.4).

Growth of *Methylobacterium* strains in different pH conditions (pH 3, 5, 8 and 10) was evaluated using Yeast Mannitol (YM) and R2A broth. No growth was observed at pH 3 (Table 4.5 and 4.6). At pH 5, most isolates showed notable growth in both YM (20 out of 29 isolates grew) and R2A broth (Table 4.5 and 4.6). All isolates showed maximum growth at pH 8 and pH 10 in both media (Table 4.5 and 4.6).

Growth requirement for NaCl was also tested using the two media (Yeast mannitol and R2A agar). Both media were supplemented with 0%, 1%, 2%, 3%, 4%, and 5% (w/v) NaCl concentrations (Table 4.7 and 4.8). For YMA, slight to no growth was observed at 4% and 5% NaCl concentrations. At 0-2% NaCl concentrations, full to moderate growth occurred for all isolates. At 3% NaCl concentration, most isolates grew except 6 that showed no growth (Table 4.7). Using R2A agar, slight-to no growth was observed at 4% and 5% NaCl concentrations, similar to that on YMA. At 0-3% NaCl concentrations, growth by all *Methylobacterium* isolates was observed (Table 4.8).

5. DISCUSSION

5.1. Phylogenetics of *Methylobacterium* strains in South Africa

Members within Crotalarieae tribe are nodulated by diverse rhizobia as was shown by diverse rhizobia that nodulated *Listia* and *Crotalaria* in this study. Out of the 92 bacterial isolates derived from *Listia* and *Crotalaria*, 29 were identified as *Methylobacterium*, 25 as *Bradyrhizobium* while the remaining isolates were identified either as *Neorhizobium*, *Agrobacterium*, *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Sphingomonas* or *Ochrobactrum* according to 16S rRNA housekeeping gene. *Leobordea*, a sister genus to *Listia* and *Lotononis* (Crotalarieae) also has been reported to nodulate with *Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, and *Methylobacterium* (Sy *et al*., 2001; Moulin *et al*., 2004; Renier *et al*., 2008; Ardley *et al*., 2013; Howieson *et al*., 2013). The findings presented here therefore suggest that species belonging to both *Crotalaria* and *Listia* are being nodulated by diverse rhizobia and not just by *Methylobacterium*.

Usually, *Methylobacterium* strains are characterized by pink pigmented colonies. This was true for 17 of the 29 isolates identified as *Methylobacterium*. These findings are in line with other studies (Green and Bousfield, 1982; Rice *et al*., 2000; Van Dien *et al.*, 2003; Ardley *et al*, 2014; Green and Ardley, 2018) that reported pink pigmentation colony as a characteristic for most members of *Methylobacterium*. However, the remaining 12 isolates (mainly from *Crotalaria*) produced non-pink pigmented cream colonies, most of which turned out to group with the *M. nodulans* reference strain, except the two SA53 isolates that grouped with *Methylobacterium* sp. 4-46 reference strain. Similarly, non-pink pigmented *Methylobacterium* were reported from *Crotalaria* which included the type strain *M. nodulans* originally isolated from root nodules of *C. podocarpa* (Sy *et al*.,2001). It can therefore be concluded that most *M. nodulans* strains are white in colour, suggesting that the "pink pigmentation" trait cannot be used as a defining characteristic for *Methylobacterium* species.

Phylogenetic analysis of the twenty-nine isolates identified as *Methylobacterium* using 16S rRNA gene revealed two potential groups. Microbial studies often rely on 16S rRNA gene sequences when taxonomically describing bacterial strains (Bukin *et al*., 2019). However, analysis of the 16S ribosomal RNA gene sequences reliably identifies species up to genus level (in most cases with a similarity estimation of > 90%) and provides limited resolution at the species level (Janda and Abbott, 2007). Consequently, the 16S ribosomal RNA gene

did not provide substantial information to conclude whether a potential new *Methylobacterium* species were delineated. Therefore, it was imperative to consider more housekeeping genes in addition to the 16S ribosomal RNA sequences. Single phylogenies of *recA, rpoB* and *atpD* genes were constructed and the findings established a clear separation pattern of the isolates into two cluster groups A and B. Accordingly, all the *Listia* isolates formed a cluster Group B and most of the *Crotalaria* isolates clustered in Group A for the *recA* and *rpoB* genes. These phylogenetic results, however, did not prove host legume specificity when it comes to clustering of strains, given that two of the *Crotalaria* isolates (SA53-1a and SA53-1c) grouped with *Listia* isolates in cluster Group B.

Only a few sequences the *recA* and *rpoB* genes of *Methylobacterium* isolates are captured in GenBank. Most strains in the database have been identified using only the 16S rRNA gene, and the demonstration of the ability of these genes to differentiate between potential species was therefore not very informative. The isolates from Group B clustered with the *Methylobacterium* sp. 4-46 isolate. This strain is considered to be an important reference strain as its genome sequence has been available for a while. Isolates in Group A clustered with the *M. nodulans* type strain. These findings are in line with another report, which showed a close association of *Methylobacterium* sp. 4-46 with strains from *Listia* as a host (Green and Ardley, 2018). In addition, the ANI values between the two whole genomes of *Methylobacterium* sp. 4-46 and *Methylobacterium* sp. WSM 2598 (a representative strain from *Listia*) was 98.8%, suggesting that the two strains belong to the same species (Konstantinidis and Tiedje, 2005; Goris *et al*., 2007; Jain *et al*., 2018). These findings agreed with those of Green and Ardley (2018) who showed that all *Listia* isolates grouped together with the two reference strains as part of their clade C2.

The phylogeny of the *atpD* gene was inconsistent with the rest of the gene phylogenies, as the *M. nodulans* type strain grouped with isolates from Group B. The resolution for this gene region was not very informative and the clusters formed had very low bootstrap support. To the best of my knowledge, this pattern has not been reported in any of the previous studies, where the isolates from these two legumes have been known to be host specific when it comes to phylogenetic clustering. As a result, two concatenated trees were presented, the first including the *atpD* gene for consistency of the examined isolates and the second excluding the *atpD* gene for consistency of both isolates and the reference strains.

All *Crotalaria* isolates except two isolates (i.e., SA 53-1a and SA 53-1c) formed part of group A and clustered together with the reference strain *M. nodulans*. ANI values between *M. nodulans* and *Methylobacterium* spp. 4-46 and WSM2598 strains were 83.3% and 83.5% respectively, which meant that *M. nodulans* belongs to a different but closely related species to the two other isolates with whole genomes available (Konstantinidis and Tiedje, 2005; Goris *et al*., 2007; Jain *et al*., 2018). Close relatedness between *M. nodulans*, *Methylobacterium* spp. 4-46 and WSM2598 strains was also evident as all isolates belonged to clade C2 as determined in a previous study (Green and Ardley, 2018). However, in that analysis *M. nodulans* had formed a separate cluster with *M. isbiliense* AR24^{T} away from *Methylobacterium* spp. 4-46, WSM2598 and other *Listia* strains (Green and Ardley, 2018).

Two of the *Crotalaria* isolates (SA 53-1a and SA 53-1c) consistently grouped with *Listia* isolates forming part of the Group B cluster and therefore formed part of the new species. This finding was consistent for all the single genes phylogenies, as well as the concatenated MLSA trees. The two isolates were isolated from ARC, Roodeplaat compared to the rest of the *Crotalaria* isolates, which were isolated from other areas (Mpumalanga, Kamiesberg, and Owen Sithole college of agriculture) in South Africa. It is, however, not clear why the two isolates consistently grouped with *Listia* instead of the other *Crotalaria* isolates. This was an interesting finding, because a case like this has never been recorded before. It may therefore necessitate the analysis of nodulation and symbiotic genes to try to explain the clustering of the two isolates with *Listia* isolates as well as the performance of cross inoculation studies. In accordance to expectations, most of the isolates grouped together with their closely related reference strains representing either *M. nodulans* or the new *Methylobacterium* sp 4-46 species. For example, *Listia* isolates grouped with *Methylobacterium* sp. 4-46 reference strain representing the new species while the *Crotalaria* isolates apart from the two isolates mentioned above grouped with the *M. nodulans* type strain.

5.2. Phenotypic characterization of *Methylobacterium* strains in South Africa

Phenotypic analyses were conducted on representative isolates from the two legume hosts (*Listia* and *Crotalaria*) to study the phenotype of the isolates in both Goup A and B. Unlike the phylogenetic analyses, phenotypic traits did not reveal notable and hence reliable patterns to differentiate between the two *Methylobacterium* species groups. For example, for carbon utilization tests, all the selected isolates utilized D-Galactonic Acid Lactone and B-

Hydroxybutyric Acid, while A- Cycodextrin was not utilized by any of the selected *Methylobacterium* isolates. This meant that some sole carbon substrates provide energy for both groups of *Methylobacterium* isolates. Different carbon substrates (Succinate and Glutamate) supported the growth of *Listia* isolates and strain ORS 2060 (from *Crotalaria podocarpa*) (Ardley *et al*., 2009).

Similar to A-Cycodextrin in this study, C1 carbon sources (i.e., Methylamine and Formaldehyde) as well as D+galactose, D-glucose and D-mannitol sole carbon sources were not utilized by any of the isolates (Ardley *et al*., 2009; Green and Ardley, 2018). However, D-mannitol in the current study was utilized by all isolates except Crot 100 and Crot 224. L-arabinose that was utilized by all Group B isolates except for SA53-1c, and all Group A isolates. These findings partly support, but also contradict those of Madhaiyan *et al*. (2007) and Ardley *et al*. (2009) as L-arabinose was only utilized by ORS 2060 but not by the *Listia* isolates. Uridine and D-Sorbitol carbon substrates utilization matched the patterns observed in phylogenetic findings. Accordingly, Uridine was utilized by the three isolates from Group A (Crot 99, Crot 100 and Crot 224) but not by any of the Group B isolates. A similar trend between *Listia* and *Crotalaria* ORS 2060 strains was observed for methanol where *Methylobacterium nodulans* ORS 2060 (group A in this study) utilized methanol but was not utilized by *Listia* strains (Group B in this study) (Ardley *et al*., 2009; Green and Ardley, 2018). On the other hand, all Group B isolates utilized D-sorbitol carbon substrate, but none of the three representatives from group A did. So far, D-sorbitol is the sole carbon source that is reported to be utilized by all group B isolates but not isolates that distinctly formed group A in this study. Taken together, carbon sources utilization revealed limited variations between the *Methylobacterium* strains considered for this study, all of which fall under clade C2 according to the phylogenetic analysis of Green and Ardley (2018). Since Green and Ardley (2018) and Madhaiyan *et al* (2007) were able to show carbon utilization distinctions between some carbon sources like Methylamine, Methanol and L-arabinose between strains belonging to different clades, it may be proposed that carbon sources utilization method is more reliable when isolates belonging to different lineages are compared.

Regarding the effect of temperature on growth of *Methylobacterium* isolates, the results presented here showed optimum growth of most isolates at temperatures between 25-32 °C for both YMA and R2A growth media. These findings generally agreed with other reports, which observed a similar temperature range for optimal growth for most *Methylobacterium*

isolates (Flournoy *et al*., 1992; Hornie *et al*., 1999; Doronina *et al*., 2000; Rice *et al*., 2000; Green and Ardley, 2018). Isolates from different host legumes did not show any variation in terms of growth temperature. Although temperature variations (e.g., either between the temperate and tropical climates or seasons like summer, winter, autumn and spring) may influence growth of particular microbial communities (Rasche *et al*., 2011; Muema *et al*., 2016), different plants are able to establish and thrive in close proximities thereby sharing similar habitats with regards to environmental conditions. This was the case for the two legumes in some parts in South Africa and may explain their ability, or that of their symbionts to grow optimally under similar temperature ranges.

With regards to pH of the growth medium, maximum growth for all *Methylobacterium* isolates tests was observed at pH 8 and 10 for both growth media. These findings are in agreement with Green and Ardley (2018) and Park *et al*. (2018), who reported good growth of *Methylobacterium* species at a pH between 7 to 10. Additionally, at pH 5 a notable growth of 73% of the isolates using YMA was observed while growth for most isolates was shown using R2A. Some *Methylobacterium* species can also grow at a pH lower than 5 (Park *et al*., 2018). The extreme acidic pH of 3 completely hindered the growth of *Methylobacterium* species on both media. There are, however, microbes that can survive at low pH conditions, especially under nutrients stressed conditions (Yang *et al*., 2012), which was not the case in the current study.

Just like the temperature and pH tests on growth of *Methylobacterium* species, their growth at different NaCl concentrations was similar. Moderate to good growth for most isolates was observed at NaCl concentrations between 0 % and 3%, with slight to no growth at 4-5% NaCl. Good growth at 3% NaCl contrasted with most reports that indicated that *Methylobacterium* strains cannot grow at salt concentrations more than 2% (Doronina *et al*., 2000; Green and Ardley, 2018; Park *et al*., 2018). Members of *Methylobacterium*, however, have been found to be able to tolerate stress conditions including antimicrobials in clinical studies (Doronina *et al*., 2000; Egamberdieva *et al*., 2015). In Australia, strains of pink pigmented *Methylobacterium* used as commercial inoculants for *Listia bainesii* can persist in acidic, sandy and infertile soils, while remaining symbiotically and serologically stable (Yates *et al*., 2007; Ardley *et al*., 2014). The previous findings could possibly explain why slight growth was observed between 4% and 5% NaCl concentrations in this study. Overall, temperature, pH and NaCl concentrations influenced growth of *Methylobacterium* species.

These influences were, however, not specific to the *Methylobacterium* isolates depending on their species group or the growth media used.

6. CONCLUSIONS

- Single gene phylogenies of 16S rRNA, *recA* and *rpoB* best delineated *Methylobacterium* isolates mainly relating them with the known and available genome reference strains of *M. nodulans* and *Methylobacterium* sp. 4-46 compared to the *atpD* gene phylogeny.
- Separation of isolates between different host legumes (i.e., *Crotalaria* and *Listia*) was not observed for all the *Crotalaria* isolates, as two of them (SA531a and SA531c) instead grouped with isolates obtained from *Listia*.
- Carbon utilization tests did not allow resolution of the two species groups observed in the phylogenies. These tests did, however, share similar patterns, as D-Galactonic Acid Lactone and B-Hydroxybutyric Acid were utilized by all the selected representative *Methylobacterium* isolates, while A-Cycodextrin was not utilized by any of the selected *Methylobacterium*. Since the *Methylobacterium* isolates examined were closely related, having fallen under clade C2 according to Green and Ardley (2018), it could be proposed that differentiation based on carbon utilization is more reliable when *Methylobacterium* isolates that fall within different clades are distinguished.
- Temperature, pH and NaCl concentration has a marked influence on the growth of *Methylobacterium* isolates, but these factors do not depend on host specificity of the isolates or the growth media used.

7. RECOMMENDATIONS

- Given the findings of the work presented here, I recommend future analyses of nodulation and nitrogen fixing symbiotic genes (e.g. nodABC, and nifH) of the two *Crotalaria* isolates (SA 53-1a and SA 531c) isolated from ARC-Roodeplaat and to compare these to those of other *Crotalaria* and *Listia* isolates. This may help explain why the two SA53 isolates consistently formed part of Group B, the new species cluster and not *M. nodulans* as was expected.
- This analysis would investigate whether there are symbiotic traits confined to *Listia* by determining the ability of rhizobial strains isolated from species of *Crotalaria* sp to nodulate *Listia* host, by examining the morphology structure of the resulting nodules.
- According to 16S rRNA, *recA* and *rpoB* gene phylogenies, the *M. nodulans* type strain consistently clustered with the *Crotalaria* isolates, while the *Methylobacterium* sp. 4-46 reference strain consistently clustered with *Listia* isolates which is also supported by literature. I therefore recommend that it would be prudent to embark on the process of formally describing the new *Methylobacterium* species arising from this study.
- The *Listia* group of isolates has at least two representative genomes (WSM 2598 and 4-46). To fully understand the genomic diversity of this group, further whole genome sequence analyses of its representative isolate/s may be necessary in the future, as it will assist in understanding how the *Listia* and *Crotaloria* isolates which form part of this species may differ.

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Appendices

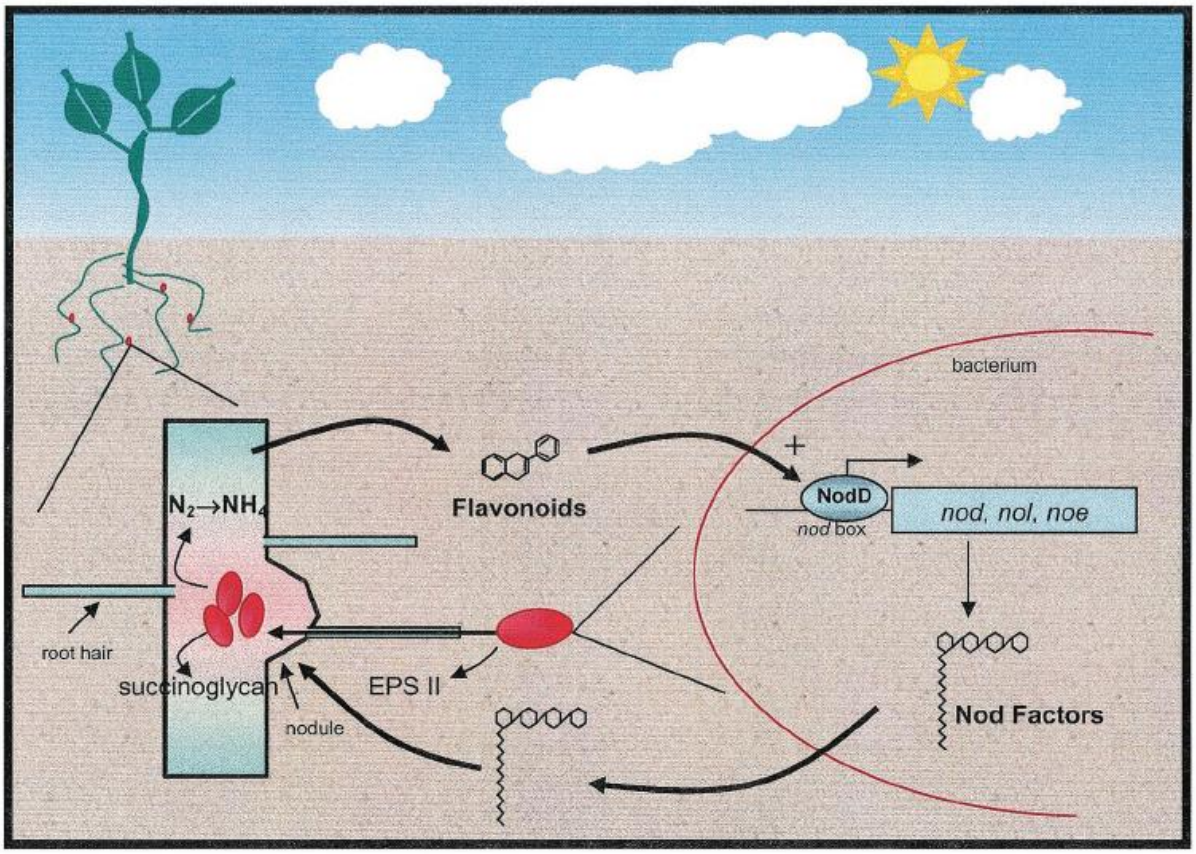

Figure 2.1. Rhizobium -legume symbiosis model (Gonzalez and Marketon, 2003).

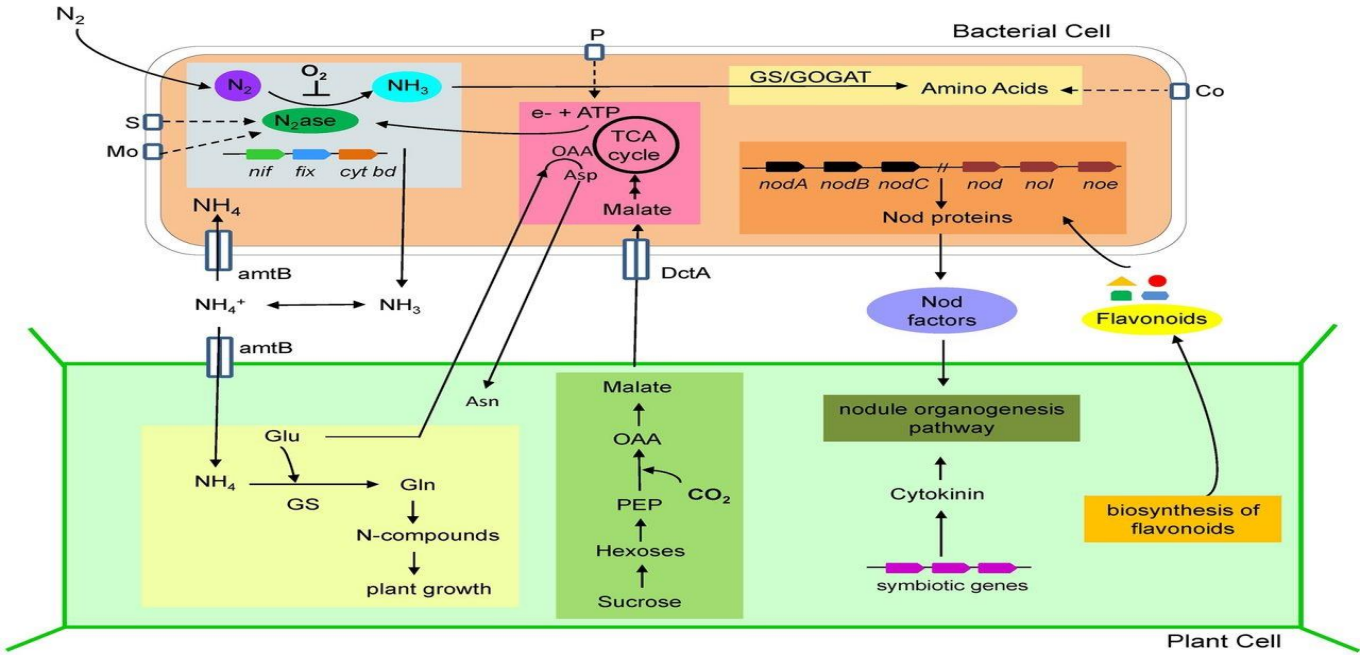

Figure 2.2: The relationship between a bacterial cell of a diazotroph and a nodulating plant cell during nitrogen fixation (Mus *et al.*, 2016).

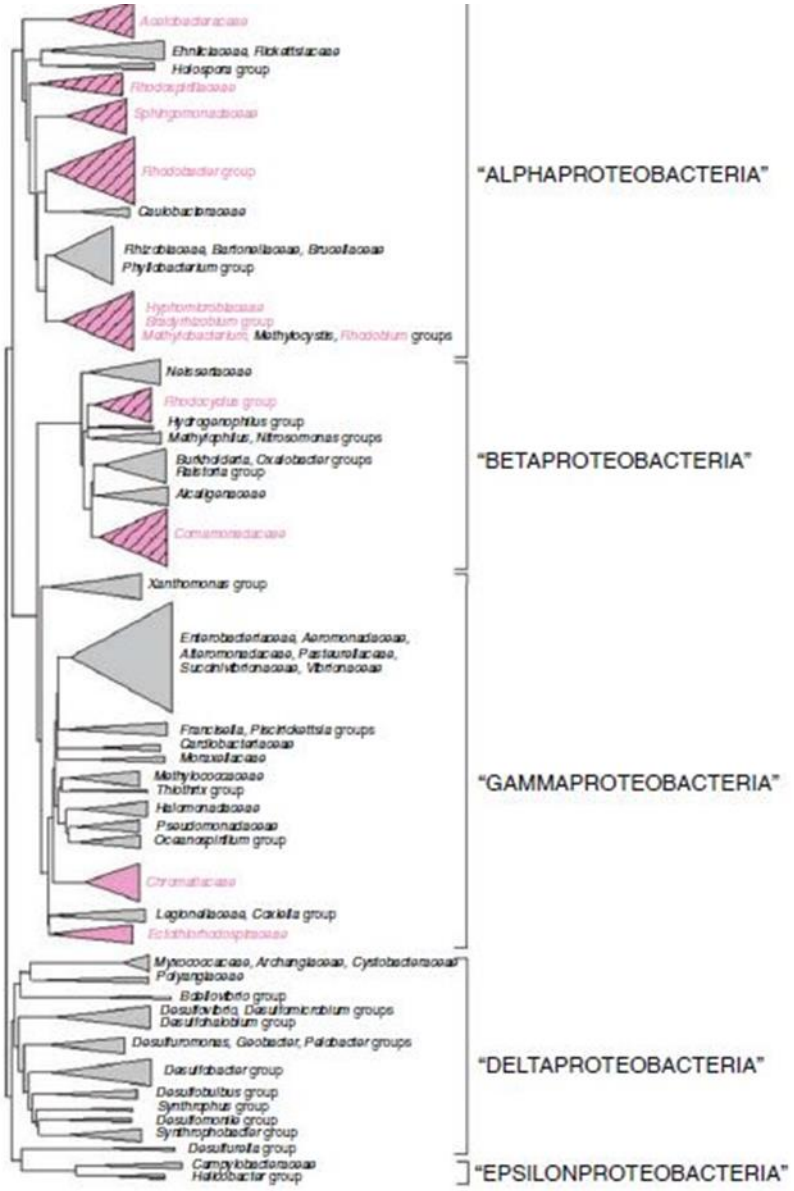

Figure 2.3: Simplified 16S rRNA based neighbour-joining phylogenetic tree, using type strain sequences of the genera proteobacteria (Garrity, 2011).

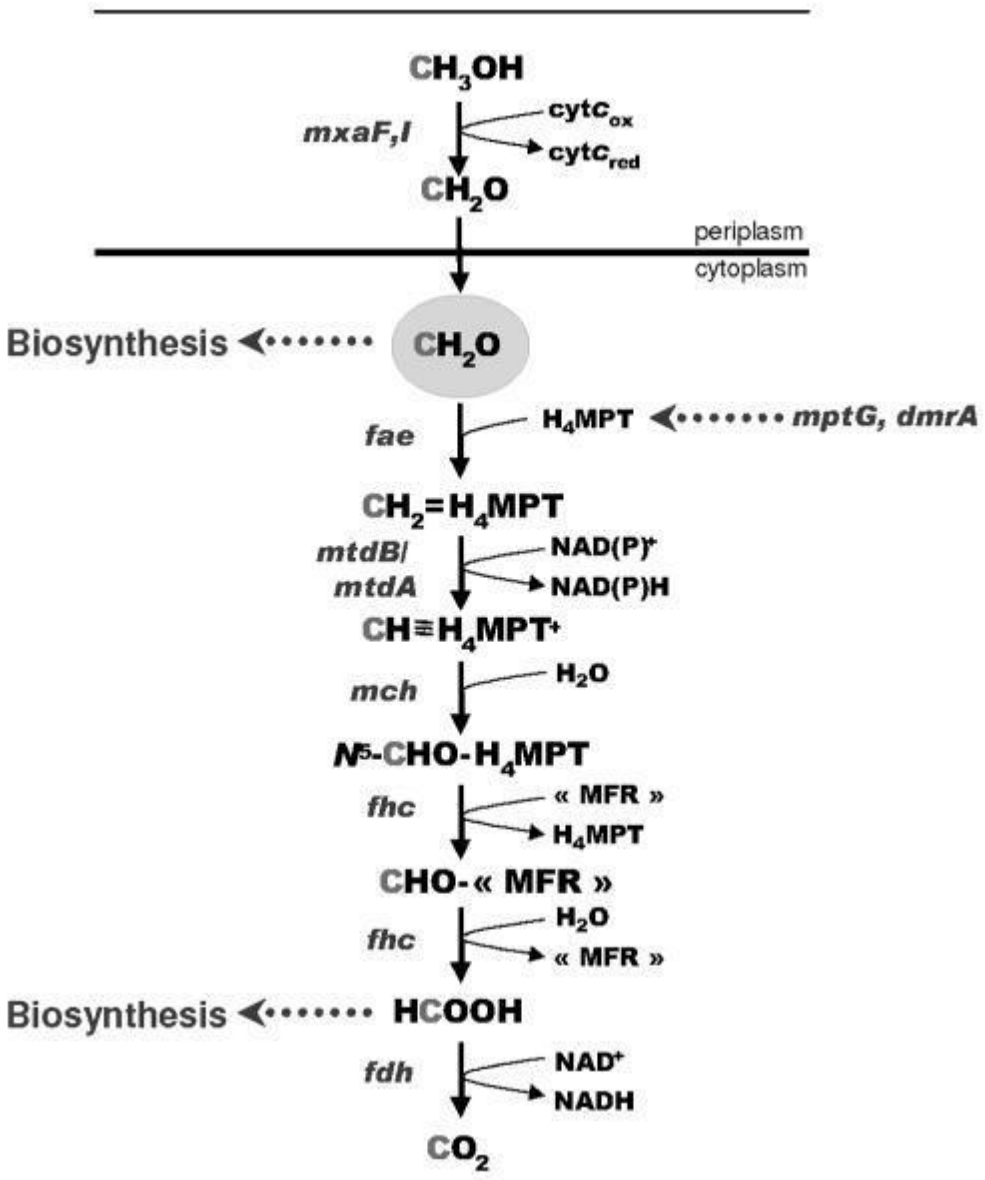

Figure 2.4: How carbon is converted in *Methylobacterium* when methylotrophic growth occurs (Sy *et al.*, 2005).

Figure 4.1: Phylogenetic tree based on *16S rRNA* (1500 bp) gene sequences, showing the taxonomic position of *Crotalaria* and *Listia* host specific strains. Bootstrap values are shown at the nodes. The 16S rRNA of *Methylobacterium salsuginis*, *Methylobacterium platani*, and *Methylobacterium populi* were used as outgroups. The GenBank accession numbers of each reference and outgroup sequences strains are shown in the parentheses.

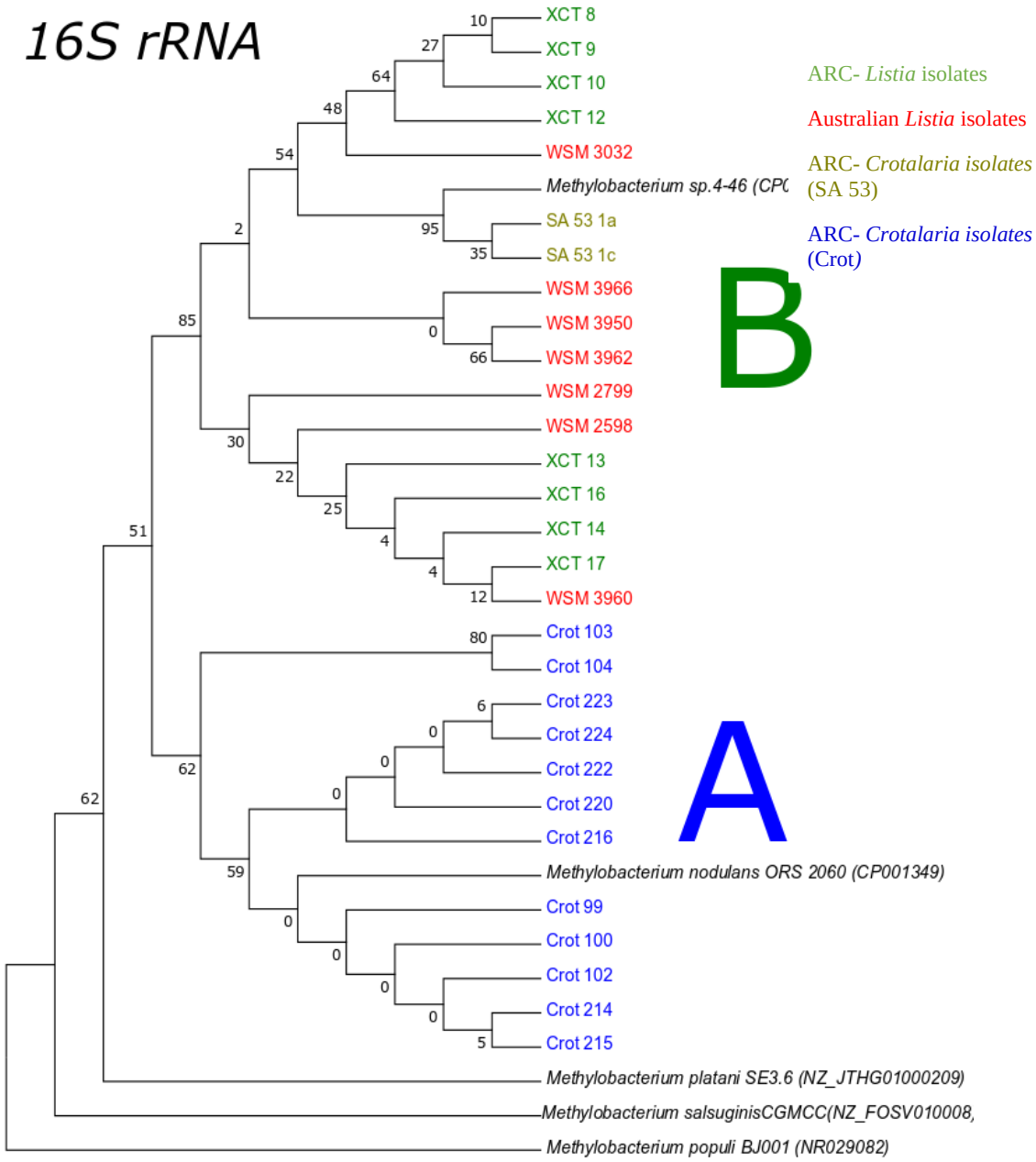

Figure 4.1: Phylogenetic tree based on *16S rRNA* (1500 bp) gene sequences, showing the taxonomic position of *Crotalaria* and *Listia* host specific strains. Bootstrap values are shown at the nodes. The 16S rRNA of *Methylobacterium salsuginis, Methylobacterium platani*, and *Methylobacterium populi* were used as outgroups. The GenBank accession numbers of each reference and outgroup sequences strains are shown in the parentheses.

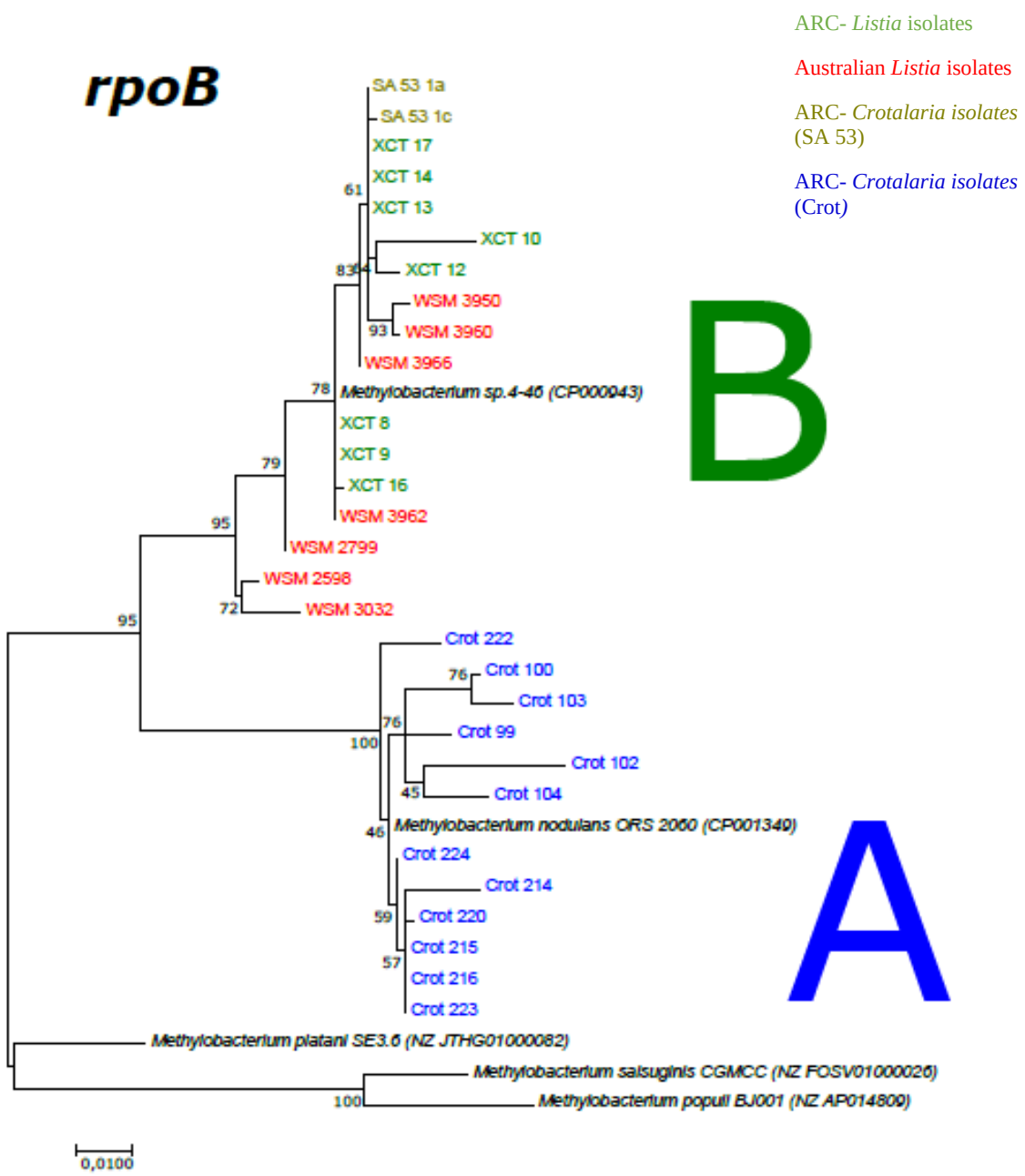

Figure 4.2: The Maximum-likelihood tree based on *rpoB* amino acyl sequences (700-800bp), showing the taxonomic positions of potential new species compared to strains ascribed to the genus *Methylobacterium*. Bootstrap values are shown at the nodes. The *rpoB* of *Methylobacterium salsuginis*, *Methylobacterium platani*, and *Methylobacterium populi* were used as outgroups. The GenBank accession numbers of reference and outgroup sequences strains are shown in the parentheses.

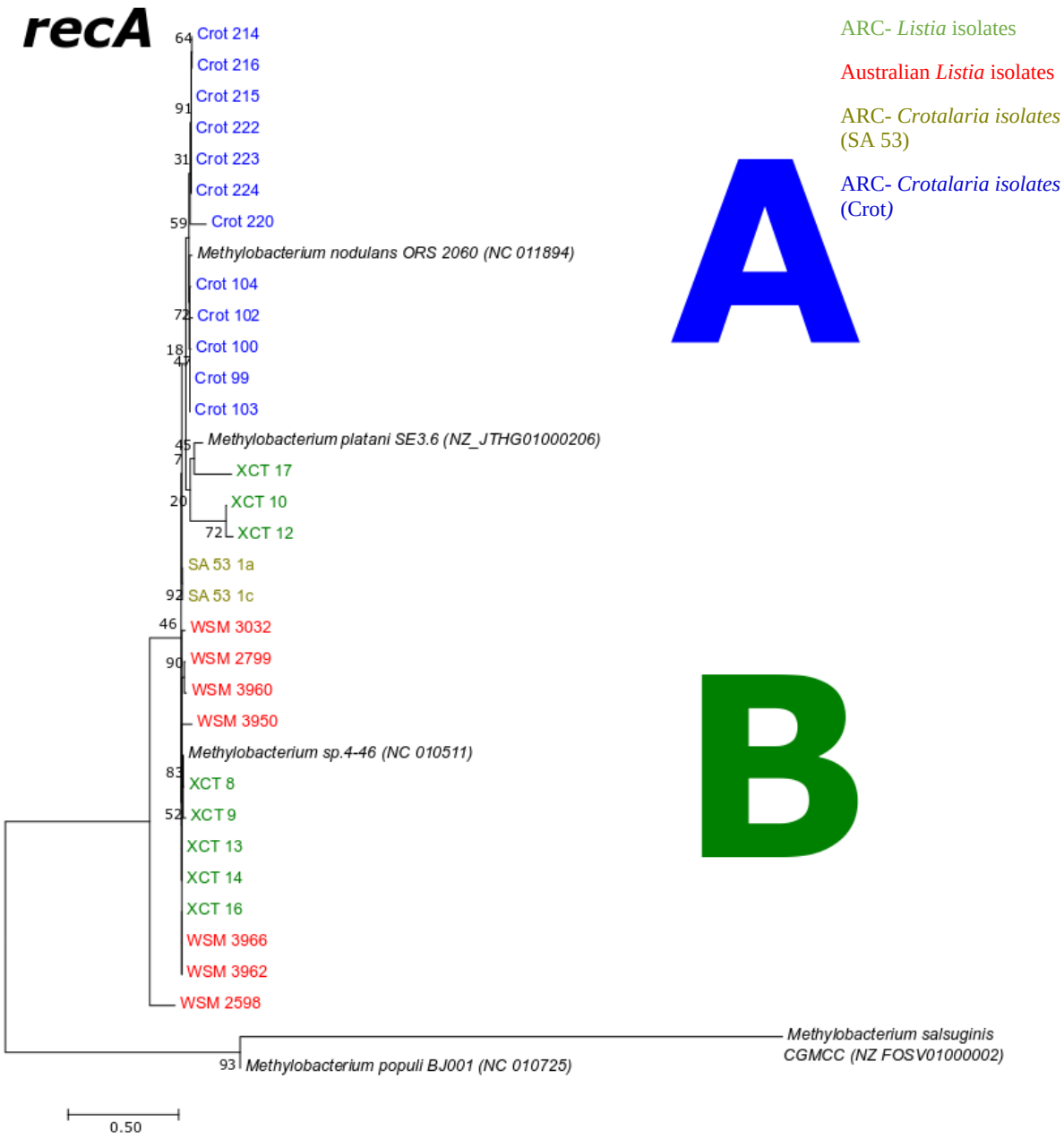

Figure 4.3: Phylogenetic tree based on *recA* amino acyl sequences (400-500bp), showing the taxonomic positions of potential new species compared to strains ascribed to the genus *Methylobacterium*. Bootstrap values are shown at the nodes. The *recA* of *Methylobacterium salsuginis*, *Methylobacterium platani*, and *Methylobacterium populi* were used as outgroups. The GenBank accession numbers of reference and outgroup sequences strains are shown in the parentheses.

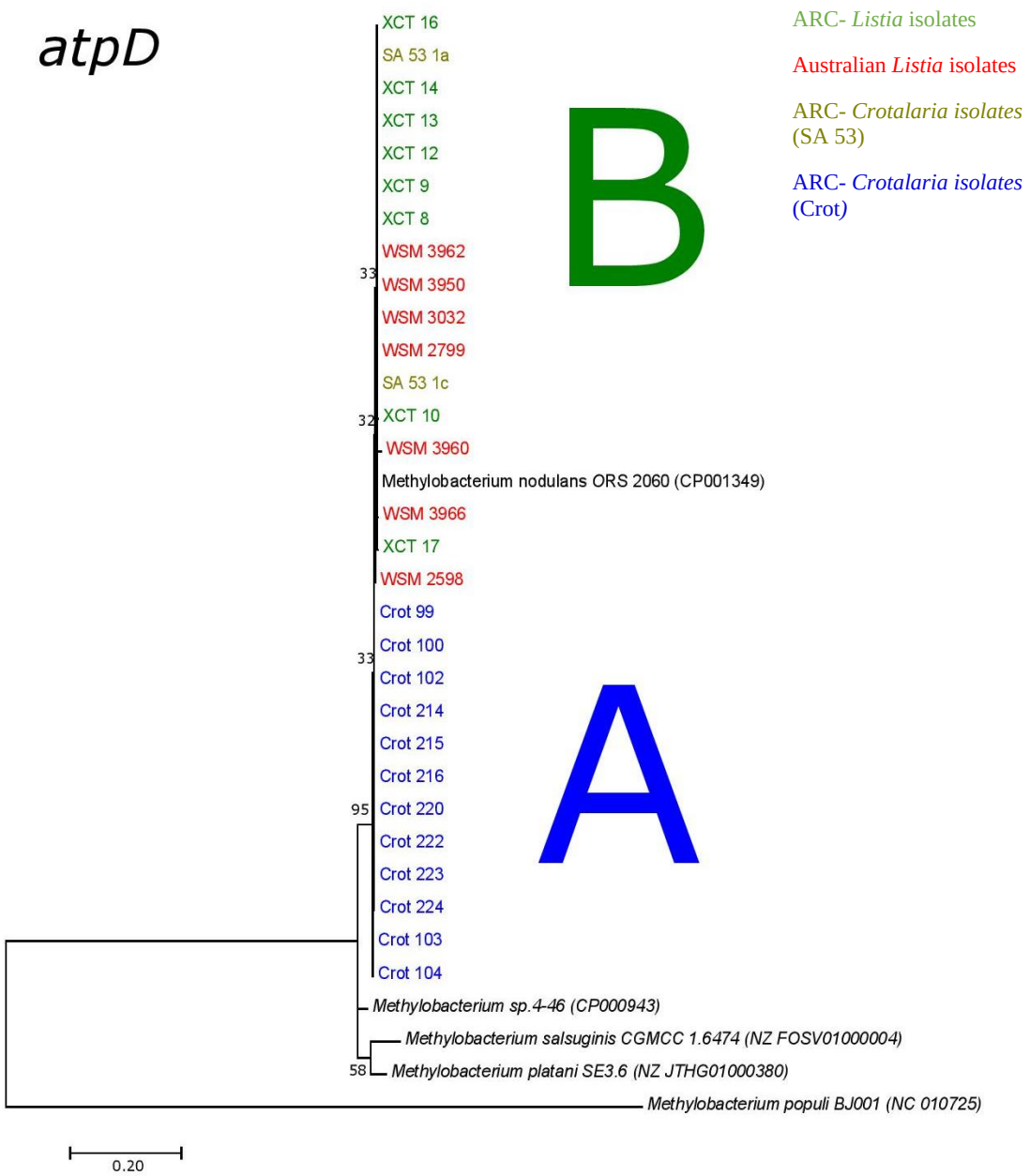

Figure 4.4: Phylogenetic tree based on *atpD* amino acyl sequences (500-600bp), showing the taxonomic positions of potential new species compared to strains ascribed to the genus *Methylobacterium*. Bootstrap values are shown at the nodes. The *atpD* of *Methylobacterium salsuginis*, *Methylobacterium platani*, and *Methylobacterium populi* were used as outgroups. The GenBank accession numbers of reference and outgroup sequences are shown in the parentheses.

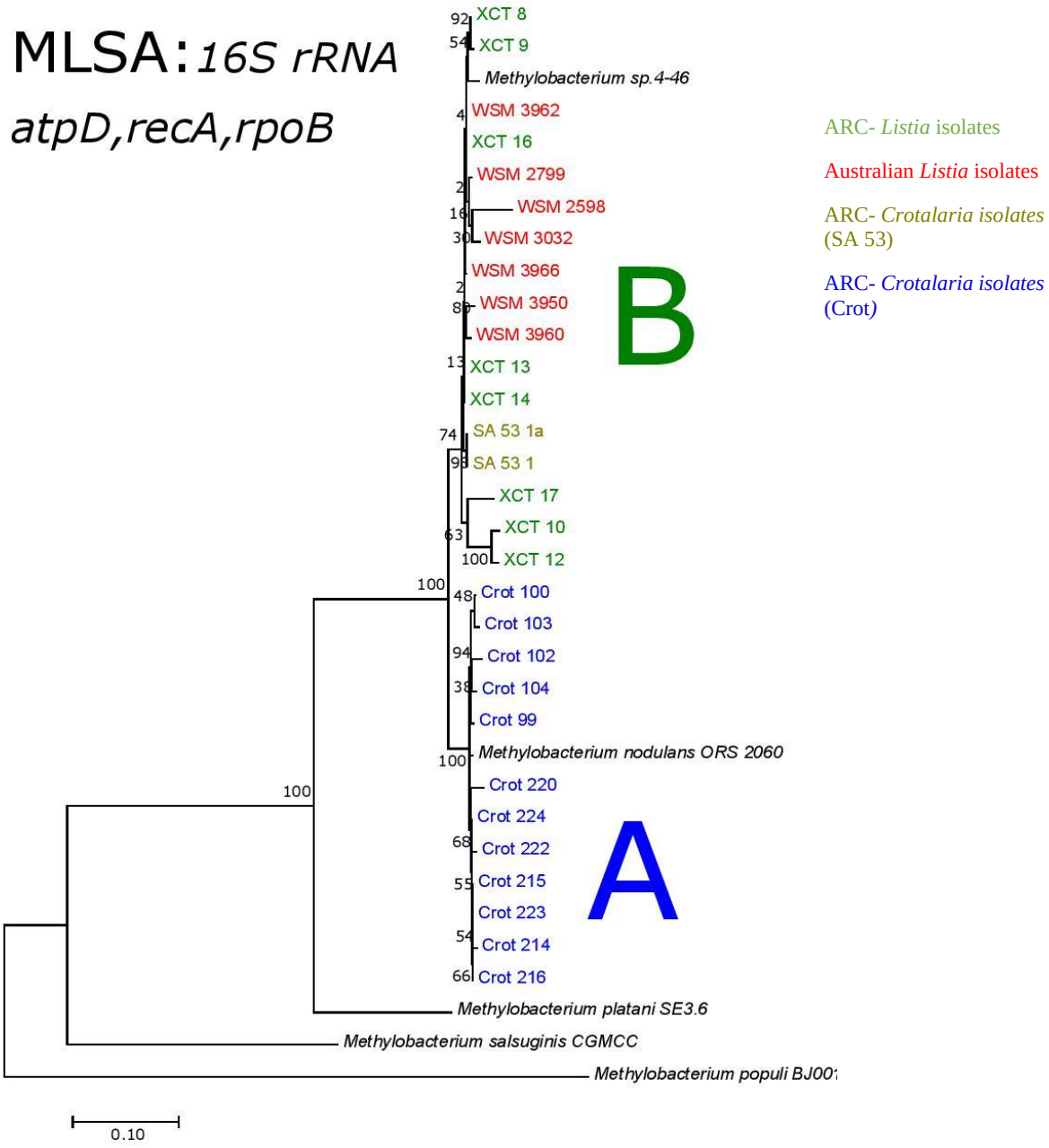

Figure 4.5: Multilocus Sequence Analysis/Concatenated phylogenetic tree consisting of the genes 16S rRNA, *atpD*, *recA*, and *rpoB* data sequences, showing taxonomic positions of the isolates in support of the respective four single phylogenetic trees. The maximum-likelihood tree was constructed using the General Reversible Model (Nei and Kumar, 2000). Bootstrap values are shown at the nodes. The *Methylobacterium salsuginis*, *Methylobacterium platani*, and *Methylobacterium populi* was selected as the outgroup.

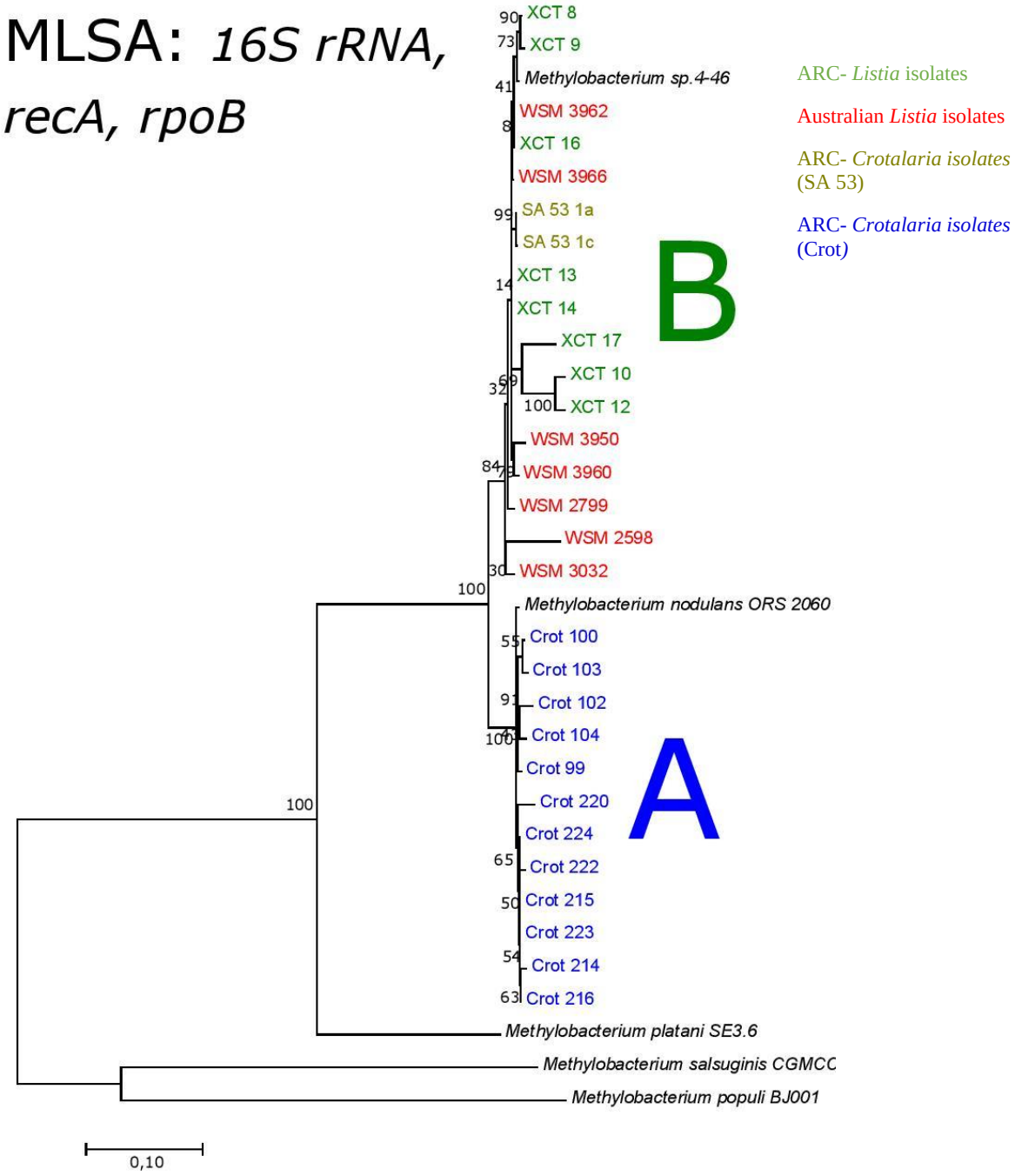

Figure 4.6: Multilocus Sequence Analysis/Concatenated phylogenetic tree made up of 16S rRNA, *recA*, and *rpoB* data sequences, showing taxonomic positions of the isolates in support of the four respective single phylogenetic trees. The maximum-likelihood tree was constructed using the General Reversible Model (Nei and Kumar, 2000), Bootstrap values are shown at the nodes. The *Methylobacterium salsuginis*, *Methylobacterium platani*, and *Methylobacterium populi* was selected as the outgroup.

Table 2.1: The presently defined nodulating rhizobia and the number of species each genus contains

Source: (Howieson and Dilworth, 2016; Lardi *et al.*, 2017; Beukes *et al.*, 2017)

Family	**Genus**	**Number of described species**
α-**Proteobacteria**		
Bradyrhizobiaceae	*Bradyrhizobium*	15
Brucellaceae	*Ochrobactrum*	2
Hyphomicrobiaceae	*Azorhizobium*	3
	Devosia	1
Methylobacteriaceae	*Methylobacterium*	1
	Microvirga	3
Phyllobacteriaceae	*Phyllobacterium*	1
	Aminobacter	1
	Mesorhizobium	29
Rhizobiaceae	*Rhizobium*	43
	Neorhizobium	3
	Sinorhizobium/Ensifer	13
	Shinella	1
β-**Proteobacteria**		
Burkholderiaceae	*Paraburkholderia*	17
	Cupriavidus	2

Table 2.2: Validly published *Methylobacterium* species

16s rRNA identity	Host	16S accession number	Reference
M. adhaesivum	Drinking water	AM040156.1	(Gallego *et al.,* 2005)
M. aerolatum	Air	EF174498.1	(Weon *et al.*, 2008)
M. aminovorans	Soil	AB175629.1	(Urakami *et al.*, 1993)
M. aquaticum	Drinking water	AJ635303.1	(Gallego *et al*., 2005)
M. brachiatum	Water samples food factories	AB175649.1	(Kato *et al.*, 2008)
M. brachythecii	leaves of *Brachythecium plumosum*	AB703239.1	(Tani and Sahin, 2013)
M. bullatum	leaf of *Funaria hygrometrica*	FJ268657.1	(Hoppe *et al.,* 2011*)*
M. cerastii	leaf of *Cerastium holosteoides*	FR733885.1	(Wellner *et al.,* 2013)
M. chloromethanicum	Soil, polluted environment	CP001298	(McDonald *et al.,* 2001)
M. dankookense	Drinking water	FJ155589.2	(Lee *et al.,* 2009)
M. dichloromethanicum	Active sludge	AB175631.1	(Dronina *et al.*, 2000)
M. extorquens	Forest and garden soil	AB175632	(Urakami and Komagata, 1984)
M. fujisawaense	Roots of Medicago sativa	AJ250801.1	(Green *et al.*, 1981)
M. gnaphalii	leaves of *Gnaphalium spicatum*	AB627071.1	(Tani *et al*., 2012)
M. gossipiicola	Cotton leaf	EU912445.1	(Madhaiyan *et al.*, 2012)
M. gregans	Fresh water samples food factories	AB252200.1	(Kato *et al*., 2008)
M. haplocladii	Leaf of *Haplocladium microphyllum*	AB698691.1	(Tani and Sahin, 2013)
M. hispanicum	Drinking water	AJ635304.1	(Gallego *et al.*, 2005)
M. iners	Air	EF174497.1	(Weon *et al*., 2008)
M. isbiliense	Drinking water	AJ888239.1	(Gallelo *et al.*, 2005)
M. jeotgali	shrimp jeotgal	DQ471331.1	(Aslam *et al.*, 2007)
M. komagatae	freshwater samples food factories	AB252201.1	(Kato *et al*., 2008)
M. longum	phyllosphere of *Arabidopsis thaliana*	FN868949.1	(Knief *et al.*, 2010)

Table 2.2 continued.

16s rRNA identity	Host	16S accession number	Reference
M. lusitanum	Sewage station	AB175635.1	(Dronina *et al*., 2000)
M. marchantiae	Phyllosphere of *Marchantia polymorpha*	FJ157976.2	(Schauer *et al.*, 2011)
M. mesophilicum	leaf of *L. perenne*	AB175636.1	(Austin and Goodfellow, 1979)
M. nodulans	Root nodules of *Crotalaria*	AF220763.1	(Jourand *et al.*, 2004)
M. organophilum	Lake samples	AB175638.1	(Patt *et al.*,1979)
M. oryzae	Stem of *Oryza sativa L.*	AY683045.1	(Madhaiyan *et al*., 2007)
M. oxalidis	Leaf of *Oxalis corniculata*	AB607860.2	(Tani *et al*., 2012)
M. persicinum	Fresh water samples food factories	AB252202.1	(Kato *et al*., 2008)
M. phyllosphaerae	Leaf of *Oryza sativa L.*	EF126746.2	(Madhaiyan *et al.*, 2007)
M. phyllostachyos	Bamboo leaf	EU912444.1	(Madhaiyan *et al.*, 2014)
M. platani	Leaf of *Platanus orientalis*	EF426729.1	(Kang *et al.*, 2007)
M. podarium	Human foot microflora	AF514774.1	(Anesti *et al.*, 2006)
M. populi	Poplar plantlets / the bark Populus x euramericana	CP001029	(Van Aken *et al.*, 2004)
M. pseudosasae	Bamboo leaf	EU912442.1	(Madhaiyan *et al.*, 2013)
M. pseudosasicola	Bamboo leaf	EU912439.1	(Madhayian *et al.*, 2014)
M. radiotolerans	Rice seeds	D32227.1	(Ito and Lizuka, 1971)
M. rhodesianum	Fermentor	AB175642.1	(Green *et al*., 1981)
M. rhodinum	*Alnus* rhizozphere	AB175644.1	(Heumann, 1962)
M. salsuginis	Seawater	EF015478.1	(Wang *et al.*, 2007)
M. soli	Forest soil	EU860984.1	(Cao *et al.*, 2011)
M. suomiense	Soil	AB175645.1	(Doronina *et al.*, 2002)
M. tardum	Fresh water	AB252208.1	(Kato *et al*., 2008)
M. tarhaniae	Arid soil	JQ864432.1	(Veyisoglu *et al.*, 2013)
M. thiocyanatum	Rhizosphere of *A. aflatunese*	U58018.1	(Wood *et al*., 1999)
M. thuringiense	surface of Cerastium holosteoides leaf	FR847847.1	(Wellner *et al.*, 2013)
M. trifolii	surface of Trifolium repens leaf	FR847848.1	(Wellner *et al.*, 2013)
M.variabile	Drinking water	AJ851087.1	(Gagello *et al.*, 2005)
M. zatmanii	Fermentor	AB175647.1	(Green *et al.*, 1981)

Table 3.1: Bacterial culture isolates used in this study

#	Isolates	Hosts	Place of collection and/or coordinates
1.	SA 4-2a1(1)	*Lotononis spp*	N7 4 km pas Klawer
2.	SA 4-2a1(2)	*Lotononis* spp.	N7 4 km pas Klawer
3.	SA 4-2b1	*Lotononis spp*	N7 4 km pas Klawer
4.	SA 4-2b2	*Lotononis spp*	N7 4 km pas Klawer
5.	WC29.6a	*Lotononis leptoloba*	N7 100 km before Springbok
6.	WC30.1a (1)	*Lotononis speciosa*	1 km N of Springbok to Pofadder
7.	WC30.1a (2)	*Lotononis speciosa*	1 km N of Springbok to Pofadder
8.	WC30.1d	*Lotononis speciosa*	1 km N of Springbok to Pofadder
9.	WC30.1i	*Lotononis speciosa*	1 km N of Springbok to Pofadder
10.	WC32.a1	*Lotononis benthamiana*	15 km S of Springbok
11.	WC32.b1	*Lotononis benthamiana*	15 km S of Springbok
12.	WC32.b2	*Lotononis benthamiana*	15 km S of Springbok
13.	WC32.c1	*Lotononis benthamiana*	15 km S of Springbok
14.	WC32.d1	*Lotononis benthamiana*	15 km S of Springbok
15.	WC32.d2	*Lotononis benthamiana*	15 km S of Springbok
16.	WC32.b2	*Lotononis benthamiana*	15 km S of Springbok
17.	WC32c	*Lotononis benthamiana*	15 km S of Springbok
18.	WC32.a2	*Lotononis benthamiana*	15 km S of Springbok
19.	WC32.a1	*Lotononis benthamiana*	15 km S of Springbok
20.	WC32.c1 (1)	*Lotononis benthamiana*	15 km S of Springbok
21.	WC32.c1 (2)	*Lotononis benthamiana*	15 km S of Springbok
22.	WC32.c2	*Lotononis benthamiana*	15 km S of Springbok
23.	WC31.3b	*Lotononis benthamiana*	15 km S of Springbok
24.	WC33.2b	*Lotononis acutiflora*	Kamiesberg pass from Kamieskroon
25.	WC33.2c	*Lotononis acutiflora*	Kamiesberg pass from Kamieskroon
26.	WC33.2d	*Lotononis acutiflora*	Kamiesberg pass from Kamieskroon
27.	WC33.2e	*Lotononis acutiflora*	Kamiesberg pass from Kamieskroon
28.	WC34.c	*Lotononis acutiflora*	Kamiesberg pass from Kamieskroon
29.	WC34.2a1	*Lotononis polycephala*	Kamiesberg pass from Kamieskroon
30.	WC34.2a2	*Lotononis polycephala*	Kamiesberg pass from Kamieskroon
31.	WC35.2d (1)	*Lotononis polycephala*	Kamiesberg pass from Kamieskroon
32	WC35.2d (2)	*Lotononid polycephala*	Kamiesberg pass from Kamieskroon
33.	WC35.2a (1)	*Lotononis Polycephala*	Kamiesberg pass from Kamieskroon
34.	WC35.2a (2)	*Lotononis polycephala*	Kamiesberg pass from Kamieskroon
36.	WC35.2c	*Lotononis polycephala*	Kamiesberg pass from Kamieskroon

Table 3.1 continued.

#	Isolates	Hosts	Place of collection and/or coordinates
37.	WC37.2b	*Lotononis delicatula*	Top of Kamiesberg Pass
38.	WC37.2c	*Lotononis delicatula*	Top of Kamiesberg Pass
39.	WC37.2e	*Lotononis delicatula*	Top of Kamiesberg Pass
40.	WC37.3a	*Lotononis branchyantha*	Top of Kamiesberg Pass
41.	WC37.3b	*Lotononis branchyantha*	Top of Kamiesberg Pass
42.	WC37.3c	*Lotononis branchyantha*	Top of Kamiesberg Pass
43.	WC37.4a	*Lotononis rostrata spp namaquensis*	Top of Kamiesberg Pass
44.	WC35.1d	*Crotalaria namaquensis*	Kamiesberg pass from Kamieskroon
45.	SA 44-235c	*Crotalaria spp.*	Owen Sithole College of Agriculture
46.	SA 44-25ca	*Crotalaria spp.*	Owen Sithole College of Agriculture
47.	SA 44-2b1 (1)	*Cotalaria spp.*	Owen Sithole College of Agriculture
48.	SA 44-25ca (2)	*Crotalaria* spp.	Owen Sithole College of Agriculture
49.	SA 44-2b2 (1)	*Crotalaria spp.*	Owen Sithole College of Agriculture
50.	SA 44-2b2 (2)	*Crotalaria* spp.	Owen Sithole College of Agriculture
51.	SA 44-2b2 (3)	*Crotalaria* spp.	Owen Sithole College of Agriculture
52.	WC34.3a	*Lotononis spp.*	Kamiesberg pass from Kamieskroon
53.	WC34 c	*Lotononis spp*	Kamiesberg pass from Kamieskroon
54.	SA 44-2b	*Crotalaria spp.*	Owen Sithole College of Agriculture
55.	SA53-1b	*Crotalaria lotoides*	ARC, Roodeplaat
56.	SA53-1a	*Crotalaria lotoides*	ARC, Roodeplaat
57.	SA53-1c (1)	*Crotalaria lotoides*	ARC, Roodeplaat
58.	SA53-1c (2)	*Crotalaria lotoides*	ARC, Roodeplaat
59.	SA53-2b1	*Crotalaria lotoides*	ARC, Roodeplaat
60.	SA53-2b3	*Crotalaria lotoides*	ARC, Roodeplaat
61.	WC35.1a	*Crotalaria namaquensis*	Kamiesberg pass from Kamieskroon
62.	WC35.1d2	*Crotalaria namaquensis*	Kamiesberg pass from Kamieskroon
63.	SA53 2b3/d3	*Crotalaria lotoides*	ARC, Roodeplaat
64.	XCT 8	*Listia bainesii*	South Africa (ARC)
65.	XCT 9	*Listia bainesii*	South Africa (ARC)
66.	XCT 10	*Listia bainesii*	East London (ARC)
67.	XCT 12	*Listia bainesii*	Kwazulu-Natal (ARC)
68.	XCT 13	*Listia bainesii*	Kwazulu-Natal (ARC)
69.	XCT 14	*Listia bainesii*	Kwazulu-Natal (ARC)
70.	XCT 16	*Listia bainesii*	South Africa (ARC)
71.	XCT 17	*Listia bainesii*	South Africa (ARC)
72.	Crot 99	*Crotalaria lotoides*	S: 25° 00’ 486” E: 30° 29’ 982”

Table 3.1 continued.

#	Isolates	Hosts	Place of collection and/or coordinates
73.	Crot 100	*Crotalaria lotoides*	S: 25° 00’ 486” E: 30° 29’ 982”
74.	Crot 102	*Crotalaria lotoides*	S: 25° 00’ 486” E: 30° 29’ 982”
75.	Crot 103	*Crotalaria lotoides*	S: 25° 00’ 486” E: 30° 29’ 982”
76.	Crot 104	*Crotalaria lotoides*	S: 25° 00’ 486” E: 30° 29’ 982”
77.	Crot 214	*Crotalaria sphaerocarpa*	S: 25° 42’ 412” E: 27° 57’ 406”
78.	Crot 215	*Crotalaria sphaerocarpa*	S: 25° 42’ 412” E: 27° 57’ 406”
79.	Crot 216	*Crotalaria sphaerocarpa*	S: 25° 42’ 412” E: 27° 57’ 406”
80.	Crot 220	*Crotalaria sphaerocarpa*	S: 25° 42’ 412” E: 27° 57’ 406”
81.	Crot 222	*Crotalaria sphaerocarpa*	S: 25° 42’ 412” E: 27° 57’ 406”
82.	Crot 223	*Crotalaria sphaerocarpa*	S: 25° 42’ 412” E: 27° 57’ 406”
83.	Crot 224	*Crotalaria sphaerocarpa*	S: 25° 42’ 412” E: 27° 57’ 406”
84.	WSM 3032	*Listia solitudinis*	University of Murdoch (South Africa)
85.	WSM 3950	*Listia bainessi*	University of Murdoch (South Africa)
86.	WSM 3960	*Listia bainessi*	University of Murdoch (South Africa)
87.	WSM 3962	*Listia bainessi*	University of Murdoch (South Africa)
88.	WSM 2598	*Listia bainessi*	University of Murdoch (South Africa)
89.	WSM 3674	*Listia bainessi*	University of Murdoch (South Africa)
90.	WSM 3686	*Listia bainessi*	University of Murdoch (South Africa)
91.	WSM 2799	*Listia listii*	University of Murdoch (South Africa)
92.	WSM 3966	*Listia bainesii*	University of Murdoch (South Africa)

Table 3. 2: PCR Primers used to amplify genes in this study

Primer	Sequence	Reference
27F	AGAGTTTGATCMTGGCTCAG	(Miller *et al*., 2013)
1492R	TACGGYTACCTTGTTACGACTT	
atpD 352F	GGCCGCATCATSAACGTCATC	(Galibert *et al*., 2001)
atpD 871R	AGAGCCGACACTTCMGARCC	
rpoB 83F	CCTSATCGAGGTTCACAGAAGGC	(Galibert *et al*., 2001)
rpoB 1061R	AGCGTGTTGCGGATATAGGCG	
recA F	CAGATCGAGCGCGCCTTCGGCAA	(Ardley *et al*., 2012)
recA R	ATCTGGTTGATGAAGATCACCAT	

Table 3.3: List of species and strains (with their genbank references) included in this study

		NCBI reference sequence			
Species Name	**Strain**	***16S rRNA***	***atpD***	***recA***	***rpoB***
Methylobacterium salsuginis	CGMCC	NZ_FOSV01000008	NZ_FOSV01000004	NZ_FOSV01000002	NZ_FOSV01000026
Methylobacterium platani	SE3.6	NZ_JTHG01000209	NZ_JTHG01000380	NZ_JTHG01000206	NZ_JTHG01000082
Methylobacterium populi	BJ001	NR029082	NC_010725	NC_010725	NZ_AP014809
Methylobacterium nodulans	ORS2060	CP001349	CP001349	NC_011894	NC_011894
Methylobacterium sp. 4-46	4-46	CP000943	CP000943	NC_010511	NC_010511

Table 4.1. The 16S rRNA representative results from Blastn searches

	Isolate	**Hosts**	***16S rRNA* BLST results**	**Identity %**
1.	SA 4-2a1(1)	*Lotononis spp*	*Neorhizobium*	94.85
2.	SA 4-2a1(2)	*Lotononis* spp.	*Neorhizobium*	92.28
3.	SA 4-2b1	*Lotononis spp*	*Sphingomonas*	91.18
4.	SA 4-2b2	*Lotononis spp*	*Sphingomonas*	92.49
5.	WC29.6a	*Lotononis leptoloba*	Didn't amplify	-
6.	WC30.1a (1)	*Lotononis speciosa*	*Bradyrhizobium*	95.24
7.	WC30.1a (2)	*Lotononis speciosa*	*Bradyrhizobium*	98.48
8.	WC30.1d	*Lotononis speciosa*	Didn't amplify	-
9.	WC30.1i	*Lotononis speciosa*	Didn't amplify	-
10.	WC32.a1	*Lotononis benthamiana*	*Bradyrhizobium*	94.13
11.	WC32.b1	*Lotononis benthamiana*	*Mesorhizobium*	92.47
12.	WC32.b2	*Lotononis benthamiana*	*Mesorhizobium*	94.58
13.	WC32.c1	*Lotononis benthamiana*	*Mesorhizobium*	97.30
14.	WC32.d1	*Lotononis benthamiana*	*Mesorhizobium*	98.03
15.	WC32.d2	*Lotononis benthamiana*	*Mesorhizobium*	96.47
16.	WC32.b2	*Lotononis benthamiana*	*Bradyrhizobium*	98.20
17.	WC32c	*Lotononis benthamiana*	*Mesorhizobium*	88.70
18.	WC32.a2	*Lotononis benthamiana*	*Bradyrhizobium*	98.05
19.	WC32.a1	*Lotononis benthamiana*	Didn't amplify	-
20.	WC32.c1 (1)	*Lotononis benthamiana*	*Bradyrhizobium*	95.36
21.	WC32.c1 (2)	*Lotononis benthamiana*	*Bradyrhizobium*	88.90
22.	WC32.c2	*Lotononis benthamiana*	*Bradyrhizobium*	98.46
23.	WC31.3b	*Lotononis benthamiana*	*Mesorhizobium*	94.25
24.	WC33.2b	*Lotononis acutiflora*	*Mesorhizobium*	98.31
25.	WC33.2c	*Lotononis acutiflora*	*Mesorhizobium*	96.70
26.	WC33.2d	*Lotononis acutiflora*	*Bradyrhizobium*	96.63
27.	WC33.2e	*Lotononis acutiflora*	*Bradyrhizobium*	93.46
28.	WC34.c	*Lotononis acutiflora*	*Bradyrhizobium*	91.51
29.	WC34.2a1	*Lotononis polycephala*	*Bradyrhizobium*	98.32
30.	WC34.2a2	*Lotononis polycephala*	*Rhizobium sp.*	99.80
31.	WC35.2d (1)	*Lotononis polycephala*	*Bradyrhizobium*	100.00
32	WC35.2d (2)	*Lotononid polycephala*	*Bradyrhizobium*	94.57
33.	WC35.2a (1)	*Lotononis Polycephala*	*Bradyrhizobium*	97.46
34.	WC35.2a (2)	*Lotononis polycephala*	*Bradyrhizobium*	97.45
36.	WC35.2c	*Lotononis polycephala*	*Bradyrhizobium*	99.03
37.	WC37.2b	*Lotononis delicatula*	Didn't amplify	-
38.	WC37.2c	*Lotononis delicatula*	Didn't amplify	-

Table 4.1. Continued

	Isolate	Hosts	*16S rRNA* BLST results	Identity %
40.	WC37.3a	*Lotononis branchyantha*	*Mesorhizobium*	98.30
41.	WC37.3b	*Lotononis branchyantha*	*Mesorhizobium*	88.70
42.	WC37.3c	*Lotononis branchyantha*	Didn't amplify	-
43.	WC37.4a	*Lotononis rostrata spp namaquensis*	*Mesorhizobium*	97.46
44.	WC35.1d	*Crotalaria namaquensis*	*Mesorhizobium*	82.85
45.	SA 44-235c	*Crotalaria spp.*	*Rhizobium sp.*	99.19
46.	SA 44-25ca (1)	*Crotalaria spp.*	*Ochrobatrum*	99.29
47.	SA 44-2b1 (1)	*Cotalaria spp.*	*Sinorhizobium*	85.60
48.	SA 44-25ca (2)	*Crotalaria* spp.	*Ochrobatrum*	95.20
49.	SA 44-2b2 (1)	*Crotalaria spp.*	*Bradyrhizobium*	92.49
50.	SA 44-2b2 (2)	*Crotalaria* spp.	*Bradyrhizobium*	91.18
51.	SA 44-2b2 (3)	*Crotalaria* spp.	*Agrobacterium*	90.03
52.	WC34.3a	*Lotononis spp.*	*Mesorhizobium*	82.85
53.	WC34 c	*Lotononis spp*	*Bradyrhizobium*	90.45
54.	SA 44-2b	*Crotalaria spp.*	*Bradyrhizobium*	95.96
55.	SA53-1b	*Crotalaria lotoides*	*Bradyrhizobium*	92.98
56.	SA53-1a	*Crotalaria lotoides*	*Methylobacterium*	98.98
57.	SA53-1c (1)	*Crotalaria lotoides*	*Methylobacterium*	98.87
58.	SA53-1c (2)	*Crotalaria lotoides*	*Bradyrhizobium*	91.18
59.	SA53-2b1	*Crotalaria lotoides*	*Mesorhizobium*	91.30
60.	SA53-2b3	*Crotalaria lotoides*	*Mesorhizobium*	93.23
61.	WC35.1a	*Crotalaria namaquensis*	*Bradyrhizobium*	94.79
62.	SA53 2b3/d3	*Crotalaria lotoides*	*Bradyrhizobium*	94.79
63.	XCT 8	*Listia bainesii*	*Methylobacterium*	97.71
64.	XCT 9	*Listia bainesii*	*Methylobacterium*	97.49

Table 4.1. continued.

	Isolate	Hosts	*16S rRNA* BLST results	Identity %
65.	XCT 10	*Listia bainesii*	*Methylobacterium*	96.50
66.	XCT 12	*Listia bainesii*	*Methylobacterium*	99.25
67.	XCT 13	*Listia bainesii*	*Methylobacterium*	99.75
68.	XCT 14	*Listia bainesii*	*Methylobacterium*	100.00
69.	XCT 16	*Listia bainesii*	*Methylobacterium*	98.97
70.	XCT 17	*Listia bainesii*	*Methylobacterium*	99.87
71.	Crot 99	*Crotalaria lotoides*	*Methylobacterium*	99.76
72.	Crot 100	*Crotalaria lotoides*	*Methylobacterium*	100.00
73.	Crot 102	*Crotalaria lotoides*	*Methylobacterium*	92.26
74.	Crot 103	*Crotalaria lotoides*	*Methylobacterium*	99.29
75.	Crot 104	*Crotalaria lotoides*	*Methylobacterium*	97.78
76.	Crot 214	*Crotalaria sphaerocarpa*	*Methylobacterium*	99.03
77.	Crot 215	*Crotalaria sphaerocarpa*	*Methylobacterium*	98.30
78.	Crot 216	*Crotalaria sphaerocarpa*	*Methylobacterium*	99.52
79.	Crot 220	*Crotalaria sphaerocarpa*	*Methylobacterium*	96.54
80.	Crot 222	*Crotalaria sphaerocarpa*	*Methylobacterium*	98.76
81.	Crot 223	*Crotalaria sphaerocarpa*	*Methylobacterium*	99.76
82.	Crot 224	*Crotalaria sphaerocarpa*	*Methylobacterium*	97.96
83.	WSM 3032	*Listia solitudinis*	*Methylobacterium*	100.00
84.	WSM 3950	*Listia bainesii*	*Methylobacterium*	99.76
85.	WSM 3960	*Listia bainessi*	*Methylobacterium*	99.78
86.	WSM 3962	*Listia bainesii*	*Methylobacterium*	100.00
87.	WSM 2598	*Listia bainesii*	*Methylobacterium*	99.47
88.	WSM 3674	*Listia bainesii*	*Microvirga*	99.76
89.	WSM 3686	*Listia bainesii*	*Microvirga*	99.71
90.	WSM 2799	*Listia listii*	*Methylobacterium*	99.43
91.	WSM 3966	*Listia bainesii*	*Methylobacterium*	96.75
92.	WSM 2693		Didn’t amplify	-

Table 4.2: Pairwise comparisons of genomic Average Nucleotide Identity (gANI) values[a] of two sequenced strains currently ascribed to the genus *Methylobacterium* and used as reference strains in this study, as well as the *Methylobacterium* sp. WSM 2598. Cut-off value for species delineation is 96.5%.

Genome	*M*. sp4_46	WSM 2598	*M. nodulans*
M. sp 4_46	100	98,79	83,3
WSM 2598	98,79	100	83,5
M. nodulans	83,3	83,5	100

[a]gANI values were calculated in pairwise comparison using the Jspecies software.

Table: 4.3. Temperature test results on Yeast Mannitol agar.

Isolates	Temperature				
	10 º C	**25 º C**	**37 º C**	**40 º C**	**50 º C**
Crot 99	No growth	Full growth	Slight growth	Growth	No growth
Crot 100	No growth	Growth	Full growth	Full growth	No growth
Crot 102	No growth	Full growth	Full growth	Full growth	No growth
Crot 103	No growth	Growth	Growth	Growth	No growth
Crot 104	No growth	Growth	Growth	Growth	No growth
Crot 214	No growth	Full growth	Growth	Slight growth	No growth
Crot 215	No growth	Full growth	Full growth	Slight growth	No growth
Crot 216	No growth	Slight growth	Slight growth	Slight growth	No growth
Crot 220	No growth	Full growth	Full growth	Full growth	No growth
Crot 222	No growth	Slight growth	Full growth	Growth	No growth
Crot 223	No growth	Full growth	Full growth	Growth	No growth
Crot 224	No growth	Full growth	Full growth	Slight growth	No growth
WSM 3032	No growth	Slight growth	Growth	Growth	Slight growth
WSM 3950	No growth	Full growth	Full growth	Slight growth	No growth
WSM 3960	No growth	Full growth	Full growth	Growth	No growth
WSM 3962	No growth	Full growth	Growth	Slight growth	Slight growth
WSM 2598	No growth	Full growth	Full growth	Full growth	No growth
WSM 2799	No growth	Slight growth	Growth	Growth	Slight growth
WSM3966	No growth	Slight growth	Growth	Slight growth	No growth
XCT 8	No growth	Growth	Slight growth	Slight growth	No growth
XCT 9	No growth	Growth	Slight growth	Slight growth	No growth
XCT 10	No growth	Full growth	Growth	Slight growth	No growth
XCT 12	Slight growth	Full growth	Full growth	Full growth	No growth
XCT 13	No growth	Full growth	Full growth	Full growth	No growth
XCT 14	No growth	Growth	Growth	Growth	No growth
XCT 16	No growth	Full growth	Growth	Growth	No growth
XCT 17	No growth	Full growth	Full growth	Full growth	No growth
SA53-1a	No growth	Slight growth	No growth	No growth	No growth
SA53-1c	No growth	Full growth	Growth	Growth	No growth

Table: 4.4: Temperature test results on R2A agar

Isolates	Temperatures (R2A agar)				
	10 º C	**25 º C**	**37 º C**	**40 º C**	**50 º C**
Crot 99	No growth	Full growth	Full growth	Growth	No growth
Crot 100	No growth	Growth	Full growth	Growth	No growth
Crot 102	No growth	Growth	Full growth	Growth	No growth
Crot 103	No growth	Growth	Full growth	Slight growth	No growth
Crot 104	No growth	Full growth	Full growth	Growth	No growth
Crot 214	No growth	Full growth	Full growth	Slight growth	No growth
Crot 215	Slight growth	Growth	Full growth	Growth	No growth
Crot 216	No growth	Growth	Full growth	Slight growth	No growth
Crot 220	No growth	Growth	Full growth	Slight growth	No growth
Crot 222	No growth	Full growth	Full growth	Growth	No growth
Crot 223	No growth	Full growth	Full growth	Growth	No growth
Crot 224	No growth	Full growth	Full growth	Growth	No growth
WSM 3032	No growth	Full growth	Full growth	Slight growth	No growth
WSM 3950	No growth	Full growth	Full growth	Growth	No growth
WSM 3960	No growth	Full growth	Full growth	Full growth	No growth
WSM 3962	No growth	Growth	Full growth	Growth	No growth
WSM 2598	No growth	Full growth	Full growth	Slight growth	No growth
WSM 2799	Growth	Full growth	Full growth	Full growth	No growth
WSM3966	No growth	Full growth	Full growth	Growth	No growth
XCT 8	Slight growth	Full growth	Full growth	Growth	No growth
XCT 9	No growth	Growth	Full growth	Growth	No growth
XCT 10	Slight growth	Full growth	Full growth	Growth	No growth
XCT 12	Slight growth	Full growth	Full growth	Growth	No growth
XCT 13	Growth	Full growth	Full growth	Growth	No growth
XCT 14	Slight growth	Full growth	Full growth	Growth	No growth
XCT 16	Slight growth	Full growth	Full growth	Growth	No growth
XCT 17	Slight growth	Full growth	Full growth	Growth	No growth
SA53-1a	No growth	No growth	Full growth	Growth	No growth
SA53-1c	Slight growth	Growth	Full growth	Growth	No growth

Table 4.5: pH test results on Yeast Mannitol broth. Growth = cloudy on a scale of 1-5

Isolates	YMA (pH)			
	3	**5**	**8**	**10**
Crot 99	0	3	3	3
Crot 100	0	3	3	3
Crot 102	0	0	5	3
Crot 103	0	0	3	3
Crot 104	0	0	3	2
Crot 214	0	0	5	3
Crot 215	0	3	5	5
Crot 216	0	5	5	5
Crot 220	0	3	5	5
Crot 222	0	3	3	3
Crot 223	0	3	5	5
Crot 224	0	0	3	3
WSM 3032	0	3	5	5
WSM 3950	0	3	3	2
WSM 3960	0	3	5	5
WSM 3962	0	3	3	2
WSM 2598	0	5	5	3
WSM 2799	0	5	5	3
WSM3966	0	3	3	4
XCT 8	0	1	3	2
XCT 9	0	0	4	5
XCT 10	0	3	5	5
XCT 12	0	0	5	5
XCT 13	0	0	5	5
XCT 14	0	4	5	5
XCT 16	0	5	5	5
XCT 17	0	0	5	3
SA53-1a	0	3	5	5
SA53-1c	0	3	3	3

Table 4.6: pH test results on R2A broth. Growth = cloudy on a scale of 1-5

Isolates	YMA (pH)			
	3	**5**	**8**	**10**
Crot 99	0	5	5	5
Crot 100	0	5	5	3
Crot 102	0	5	5	5
Crot 103	0	5	5	5
Crot 104	0	3	5	3
Crot 214	0	3	5	3
Crot 215	0	5	5	3
Crot 216	0	5	5	3
Crot 220	0	5	5	5
Crot 222	0	5	5	5
Crot 223	0	3	5	5
Crot 224	0	3	5	5
WSM 3032	0	3	5	5
WSM 3950	0	5	5	5
WSM 3960	0	3	5	5
WSM 3962	0	5	5	5
WSM 2598	0	5	5	5
WSM 2799	0	5	5	5
WSM3966	0	5	5	5
XCT 8	0	5	3	5
XCT 9	0	5	3	5
XCT 10	0	5	5	5
XCT 12	0	5	5	5
XCT 13	0	5	5	5
XCT 14	0	5	5	5
XCT 16	0	5	5	5
XCT 17	0	3	5	5
SA53-1a	0	5	5	5
SA53-1c	0	5	5	5

Table 4.7: Salt (NaCl) tolerance test results on YMA

Isolates	NaCl concentration					
	0 % (w/v)	**1 % (w/v)**	**2 % (w/v)**	**3 % (w/v)**	**4 % (w/v)**	**5 % (w/v)**
Crot 99	Full growth	Full growth	Full growth	Growth	Growth	Slight growth
Crot 100	Full growth	Growth	Growth	Slight growth	Slight growth	No growth
Crot 102	Full growth	Full growth	Full growth	Growth	Slight growth	Slight growth
Crot 103	Full growth	Full growth	Full growth	Slight growth	No growth	No growth
Crot 104	Full growth	Full growth	Growth	Growth	Slight growth	Slight growth
Crot 214	Full growth	Full growth	Full growth	Full growth	Growth	Slight growth
Crot 215	Full growth	Full growth	Growth	Full growth	Growth	Growth
Crot 216	Full growth	Growth	Growth	No growth	No growth	No growth
Crot 220	Full growth	Full growth	Growth	Growth	Slight growth	No growth
Crot 222	Full growth	Slight growth	Slight growth	Slight growth	No growth	No growth
Crot 223	Full growth	Full growth	Growth	Growth	Slight growth	No growth
Crot 224	Full growth	Full growth	Slight growth	Growth	Slight growth	Growth
WSM 3032	Full growth	Slight growth	Slight growth	No growth	Slight growth	No growth
WSM 3950	Full growth	Growth	Slight growth	Slight growth	Slight growth	No growth
WSM 3960	Full growth	Growth	Slight growth	No growth	No growth	No growth
WSM 3962	Full growth	Slight growth	Slight growth	No growth	No growth	No growth
WSM 2598	Full growth	Full growth	Full growth	Growth	Slight growth	No growth
WSM 2799	Full growth	Full growth	Full growth	Growth	Growth	Growth
WSM 3966	Full growth	Growth	Slight growth	Slight growth	Slight growth	Slight growth

Table 4.7 continued.

Isolates	NaCl concentration					
	0 % (w/v)	1 % (w/v)	2 % (w/v)	3 % (w/v)	4 % (w/v)	5 % (w/v)
XCT 8	Full growth	Slight growth	Slight growth	Growth	No growth	No growth
XCT 9	Full growth	Growth	Slight growth	Slight growth	Slight growth	No growth
XCT 10	Full growth	Full growth	Full growth	Growth	Growth	Growth
XCT 12	Full growth	Full growth	Growth	Growth	Growth	Slight growth
XCT 13	Full growth	Full growth	Full growth	Growth	Growth	Growth
XCT 14	Full growth	Full growth	Growth	Growth	Growth	Growth
XCT 16	Full growth	Full growth	Growth	Growth	Growth	Growth
XCT 17	Full growth	Full growth	Growth	No growth	No growth	No growth
SA53-1a	Full growth	Slight growth	No growth	No growth	No growth	No growth
SA53-1c	Full growth	Full growth	Full growth	Growth	Growth	Slight growth

Table 4.8: NaCl tolerance test using R2A agar.

Isolates	NaCl concentration R2A agar					
	0 % (w/v)	1 % (w/v)	2 % (w/v)	3 % (w/v)	4 % (w/v)	5 % (w/v)
Crot 99	Full growth	Growth	Slight growth	Slight growth	No growth	No growth
Crot 100	Full growth	Growth	Slight growth	Slight growth	No growth	No growth
Crot 102	Full growth	Full growth	Growth	Full growth	Growth	Slight growth
Crot 103	Full growth	Growth	Slight growth	Slight growth	No growth	No growth
Crot 104	Full growth	Full growth	Growth	Slight growth	No growth	No growth
Crot 214	Full growth	Full growth	Slight growth	Slight growth	No growth	No growth
Crot 215	Full growth	Full growth	Growth	Growth	Growth	Growth
Crot 216	Full growth	Full growth	Full growth	Full growth	Growth	Growth
Crot 220	Full growth	Full growth	Full growth	Growth	Slight growth	No growth
Crot 222	Full growth	Growth	Slight growth	Slight growth	No growth	No growth
Crot 223	Full growth	Full growth	Full growth	Growth	No growth	No growth
Crot 224	Full growth	Full growth	Full growth	Full growth	Growth	Slight growth

Table 4.8 continued.

Isolates	NaCl concentration R2A agar					
	0 % (w/v)	**1 % (w/v)**	**2 % (w/v)**	**3 % (w/v)**	**4 % (w/v)**	**5 % (w/v)**
WSM 3032	Full growth	Slight growth	slight growth	Slight growth	No growth	No growth
WSM 3950	Full growth	Slight growth	Slight growth	Slight growth	No growth	No growth
WSM 3960	Full growth	Slight growth	Slight growth	No growth	No growth	No growth
WSM 3962	Full growth	Slight growth	Slight growth	Slight growth	No growth	No growth
WSM 2598	Full growth	Full growth	Growth	growth	Slight growth	Slight growth
WSM 2799	Full growth	Full growth	Full growth	Growth	Growth	Growth
WSM 3966	Full growth	Slight growth	Slight growth	Slight growth	No growth	No growth
XCT 8	Full growth	Slight growth	Slight growth	Slight growth	Slight growth	No growth
XCT 9	Full growth	Growth	Full growth	Growth	Growth	Growth
XCT 10	Full growth	Growth	Full growth	Growth	Growth	Growth
XCT 12	Full growth	Full growth	Full growth	Full growth	Growth	Growth
XCT 13	Full growth	Full growth	Full growth	Full growth	Growth	Growth
XCT 14	Full growth	Full growth	Growth	Full growth	Growth	Growth
XCT 16	Full growth	Full growth	Growth	Growth	Growth	Slight growth
XCT 17	Full growth	Full growth	Slight growth	No growth	Growth	No growth
SA53-1a	Full growth	Full growth	Growth	Growth	Slight growth	Growth
SA53-1c	Full growth	Full growth	Growth	Slight growth	No growth	No growth

Table 4.9: Utilization of carbon compounds by the Methylobacterium representative isolates.

Carbon source/Water	*Methylobacterium* representative isolates									
	Crot 99	**Crot 100**	**Crot 224**	**WS M 2598**	**WS M 3966**	**WS M 3960**	**XCT 8**	**XCT 17**	**SA 53-1a**	**SA 53-1c**
Water	-	-	-	-	-	-	-	-	-	-
A-Cyclodextrin	-	-	-	-	-	-	-	-	-	-
Dextrin	+	-	-	-	-	+	-	-	-	+
Glycogen	+	-	-	-	+	+	-	-	+	+
Tween 40	+	-	-	+	+	+	+	-	+	+
Tween 80	+	+	-	+	+	+	+	-	+	+
N-Acetyl-D-Glucosamine	-	-	-	+	+	+	-	-	+	+
Adonitol	-	-	-	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	-
D-Arabitol	-	-	-	-	+	+	-	-	+	+
D-Cellobiose	-	+	-	+	+	+	+	+	+	-
i-Erythritol	-	-	-	-	-	-	-	-	+	-
D-Fructose	-	+	-	+	+	-	-	-	+	+
L-Fucose	-	-	-	+	+	-	+	+	+	+

Table 4.9 continued.

Carbon source/Water	Crot 99	Crot 100	Crot 224	WS M 2598	WS M 3966	WS M 3960	XCT 8	XCT 17	SA 53-1a	SA 53-1c
D-Galactose	-	-	-	+	+		+	+	+	-
Gentibiose	-	-	-	+	+	+	+	+	+	+
A-D-Glucose	+	+	-	+	+	+	+	+	+	+
m-Inositol	-	-	-	+	+	+	+	+	+	+
A-D-Lactose	-	-	-	+	+	+	-	-	+	+
Lactulose	-	-	-	+	+	+	+	+	+	+
Maltose	+	-	-	-	+	-	+	+	+	+
D-Mannitol	+	-	-	+	+	+	+	+	+	+
D-Mannose	+	-	-	+	+	-	+	+	+	-
D-Melibiose	+	-	-	-	-	-	+	+	+	-
B-Methyl-D-Glucoside	-	-	-	-	-	+	-	-	+	+
D-Psicose	+	-	-	-	-	+	-	-	-	-
D-Raffinose	+	-	-	+	+	+	+	+	+	+
L-Rhamnose	-	+	-	+	+	+	+	+	+	+
D-Sorbitol	-	-	-	+	+	+	+	+	+	+
Sucrose	+	-	-	+	+	+	+	+	+	+
D-Trehalose	-	-	-	+	+	+	-	-	+	+

Table 4.9 continued.

Carbon source/Water	Crot 99	Crot 100	Crot 224	WSM 2598	WSM 3966	WSM 3960	XCT 8	XCT 17	SA 53-1a	SA 53-1c
Turanose	+	-	-	+	+	-	+	+	+	+
Xylitol	+	-	-	-	+	+	-	-	+	+
Pyruvic Acid Methyl Ester	+	+	-	+	+	+	+	+	+	+
Succinic Acid Mono-Methyl Ester	+	+	+	+	+	-	+	+	+	+
Acetic Acid	+	+	+	+	+	-	+	+	-	-
Cis-Aconitic Acid	+	+	+	-	-	+	+	+	+	+
Citric Acid	+	+	+	-	-	-	+	+	+	-
Formic Acid	+	+	+	+	+	+	+	+	-	+
D-Galactonic Acid Lactone	+	+	+	+	+	+	+	+	+	+
D-Galacturonic Acid	+	-	-	+	+	+	+	+	+	-
D-Gluconic Acid	+	+	-	+	+	+	+	+	+	+
Glucosamic Acid	+	+	-	-	-	+	-	-	+	-
D-Glucuronic Acid	+	+	-	+	+	-	+	-	+	-
A-Hydroxybutyric Acid	+	+	-	+	+	+	-	+	+	+
B-Hydroxybutyric Acid	+	+	+	+	+	+	+	+	+	+
Y-Hydroxybutyric Acid	+	+	-	+	+	-	+	+	+	+

Table 4.9 continued.

Carbon source/Water	Crot 99	Crot 100	Crot 224	WS M 2598	WS M 3966	WS M 3960	XCT 8	XCT 17	SA 53-1a	SA 53-1c
p-Hydroxyphenlyacetic Acid	+									
Itaconic Acid	+	+	-	+	+	-	-	-	-	+
A-Ketoglutaric Acid	+	+	-	-	+	-	-	+	+	-
A-Ketovaleric Acid	+	+	-	+	+	+	+	+	-	+
D, L-Lactic Acid	+	+	-	+	+	+	+	+	+	-
Malonic Acid	+	+	-	+	+	+	+	+	+	+
Propionic Acid	+	+	-	+	+	-	+	-	-	+
Quinic Acid	+	+	-	+	+	+	+	+	+	+
D-Saccharic Acid	+	+	-	+	+	-	+	-	-	+
Sebacic Acid	+	+	-	+	+	+	-	-	+	+
Succinic Acid	-	+	+	+	+	+	-	-	-	+
Bromosuccinic Acid	+	+	-	+	+	-	+	+	-	+
Succinamic Acid	+	+	+	+	+	+	+	+	-	+
Glucuronamide	+	+	+	+	+	-	+	+	+	+
L-Alaninamide	+	-	+	+	+	-	+	+	+	-
D-Alanine	+	+	-	+	+	+	+	+	+	+
L-alanine	+	-	-	+	+	+	-	-	-	-

Table 4.9 continued.

Carbon source/Water	Crot 99	Crot 100	Crot 224	WSM 2598	WSM 3966	WSM 3960	XCT 8	XCT 17	SA 53-1a	SA 53-1c
L-Alanyl-Glycine	+	+	+	+	+	-	-	+	-	+
L-Asparagine	+	-	+	+	+	+	-	+	+	+
L-Aspartic Acid	+	+	-	+	+	+	+	+	-	+
L-Glutamic Acid	+	+	+	+	+	-	+	+	+	+
Glycyl-L-Aspartic Acid	-	+	+	-	+	+	-	+	+	+
Glycyl-L-Glutamic Acid	-	-	-	+	+	-	-	+	+	-
L-Histidine	+	-	-	+	+	-	-	+	+	-
Hydroxy-L-Proline	+	+	-	+	+	-	-	+	-	+
L-Leucine	-	-	-	-	-	-	-	-	-	+
L-Ornithine	+	+	-	-	+	-	-	+	+	-
L-Phenylalanine	+	+	-	+	+	-	-	+	+	+
L-Proline	+	+	+	-	+	+	-	+	+	-
L-Pyroglutamic Acid	+	+	-	+	+	+	+	+	+	+
D-Serine	+	+	-	+	+	+	+	-	-	+

Table 4.9 continued.

Carbon source/Water	Crot 99	Crot 100	Crot 224	WSM 2598	WSM 3966	WSM 3960	XCT 8	XCT 17	SA 53-1a	SA 53-1c
L-Serine	+	+	-	+	+	-	+	-	-	+
L-Threonine	+	-	-	+	+	-	+	+	+	+
D, L-Carnitine	+	-	-	+	+	+	-	-	+	-
Y-Aminobutyric Acid	+	-	-	+	+	+	-	-	+	+
Urocanic Acid	+	-	-	-	-	+	-	+	+	+
Inosine	+	+	+	+	+	-	-	-	-	+
Uridine	+	-	+	-	-	-	-	-	-	-
Thymidine	+	+	+	-	-	-	-	-	-	-
Phenylethylamine	+	-	-	-	-	-	-	-	-	-
Putrescine	+	-	-	-	-	-	-	-	-	-
2-Aminoethanol	+	-	-	-	-	-	-	-	-	-
2,3-Butanediol	+	-	-	-	-	-	-	-	-	+
Glycerol	+	-	-	+	+	+	-	-	-	+
D, L, A-Glycerol Phosphate	+	-	-	-	-	-	-	-	+	+
A-D-Glucose-1-Phosphate	+	-	-	+	+	+	-	-	-	-
D-Glucose-6-Phosphate	+	-	-	+	-	-	-	-	-	-
D-Glucose-6-Phosphate										