The diversity of root nodule bacteria associated with indigenous *Lotononis* spp. as determined by sodium dodecyl-sulphate polyacrylamide gel electrophoresis and 16S rDNA sequencing

by

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Submitted in partial fulfilment of the requirements for the degree of
MAGISTER SCIENTIA (MICROBIOLOGY)

in the

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Pretoria, South Africa

June 2003

BEDANKINGS

Ek wil graag my innige dank en waardering betuig aan die volgende persone:

Prof P.L. Steyn van die Departement Mikrobiologie en Plantpatologie, Universiteit van Pretoria Baie dankie vir Prof se oneindige vertroue in my, waardevolle ondersteuning, inspirasie en insette. Dit was vir my 'n groot voorreg om onder Prof se leiding deel te wees van hierdie projek.

Dr. Julian Jaftha vir sy volgehoue ondersteuning, waardevolle raad en leiding. Sy hulp in laboratorium en skryf van die verhandeling is van onskatbare waarde.

Prof Ben-Erik van Wyk (Departement Plantkunde, Rand Afrikaanse Universiteit) vir al sy hulp met die identifisering van lokaliteite en *Lotononis* spesies. Dankie vir Prof se bereidwillige insette in die projek en vriendelike hulp.

Elsa van Wyk en Magda Nel vir al hul insette en hulp met die verberging en lokaliteit bepalings van plant monsters vir herbarium doeleindes.]

Dr. M Van der Linde en Dr. H Boraine van die departement Statistiek, Universiteit van Pretoria vir hulle hulp met statistiese verwerking van resultate.

Almal wat saam met my in die laboratorium gewerk het – Carinne Joubert, Marleen Kock en Michelle Lindeque.

Marleen Kock, Raynard MacDonald, Bridgitta Steyn en Marinda Oosthuizen vir hul vriendskap, waardevolle insette, hulp en belangstelling.

Al my wonderlike vriende vir al die moed inpraat en ondersteuning, die lys is oneindig lank.

Aan my sussie, Ubré en haar man James. Dankie vir julle belangstelling in my werk, gebede en liefde.

My dierbare ouers vir al hulle vertroue, liefde en oneindige ondersteuning. Vreeslik baie dankie vir die inspirerende voorbeeld wat julle altyd uitstraal en alles wat julle deur al die jare vir my geleer en opgeoffer het.

Aan my Skepper wat elke dag se pad vir my uitlê.

No matter how hard he struggles toward . . . the goal of building a system capable of identifying and grouping all kinds that exist, . . .the bacterial taxonomist is always painfully aware of the shortcomings of his efforts and the enormous amount of work remaining to be done. At best, he can only contribute to a progress report.

R. E. Gordon, W. C. Haynes and C. N. Pang, 1973.
The Genus Bacillus

Hierdie verhandeling word opgedra aan my dierbare ouers; Pieter en Bettie Le Roux.

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SUMMARY

With the high rate of human population growth in Africa, it is imperative that food production be increased through improving yields and bringing more land under cultivation. Nitrogen is a key element required for plant growth and with the low input of fertilizers into African farming-systems, it is necessary to harness biological nitrogen fixation (BNF) to its fullest extent. To maximize nitrogen fixation in crop and pasture legumes under various conditions and cropping systems requires intensified research.

In this study the diversity of root nodule bacteria associated with *Lotononis* species in South Africa was investigated. To our knowledge, no past attempts have been made to investigate the diversity of rootnodule-bacteria associated with the genus *Lotononis.*

Eighty nodulated plants representing thirty-two *Lotononis* spp. were collected from all the main geographical and climatological regions in South Africa. Isolates obtained from rootnodules were purified and characterized with sodium dodecyl-sulphate gel polyacrylamide gel electrophoresis (SDS-PAGE), supplemented at the genomic level with 16S rDNA sequence data of selected strains.

Initial screening (SDS-PAGE) showed that most isolates obtained from the same plant species, grouped into various clusters within the dendrogram. A few of the isolates from similar host plants seemed to cluster with high similarity. It is thus reasonable to conclude that host specificity in the symbiotic interaction is less applicable for most species of *Lotononis*. When considering the geographical origins of the isolates, their diverse nature was clearly illustrated. It was showed that isolates from similar geographical regions were evenly distributed throughout the dendrogram. Some of the isolates obtained from arid environments formed closely related electrophoretic groups.

It was subsequently shown that root-nodule bacteria associated with *Lotononis* species are not restricted to a particular rhizobial genus, but that heterogeneity is evident. Some of the isolates were also related to genera outside the *Rhizobiaceae*, namely *Methylobacterium* and *Burkholderia*.

Die diversiteit van wortel-knoppiesbakterieë geassosieerd met inheemse *Lotononis* spp soos bepaal deur natriumdodesielsulfaatpoliakrielamiedjelelektroforese en 16S rDNS volgordebepaling

deur

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OPSOMMING

Gegewe die hoë tempo van menslike bevolkingsgroei in Afrika, is dit noodsaaklik dat voedselproduksie verhoog moet word deur opbrengste te verhoog en dat meer areas vir verbouing aangewend moet word. Stikstof is 'n sleutelelement benodig vir plantegroei en met die lae insette van bemestingstowwe in Afrika boerderysisteme, is dit noodsaaklik om biologiese stikstofbinding tot die uiterste in te span. Intensiewe navorsing is noodsaaklik om maksimum stikstofbinding in gewas- en weidingspeulplante onder verskeie toestande en verbouingstelsels te verseker.

In hierdie studie was die diverse omvang van wortel-knoppiebakterieë geassosieerd met *Lotononis* spp in Suid-Afrika ondersoek. Dit was verwag dat die ekstreme aard van die

habitatte van *Lotononis* spp duidelik in die diversiteit van wortel-knoppiesbakterieë geassosieerd met hierdie plante gereflekteer sou word. Sover ons kennis strek was hierdie 'n pionierstudie gewees.

In hierdie studie is tagtig genoduleerde plante wat twee en dertig *Lotononis* spp verteenwoordig versamel van al die uiteenlopende geografiese en klimaatstreke in Suid-Afika. Die isolate uit die wortelknoppies is gesuiwer en gekarakteriseer deur natriumdodesielsulfaatpoliakrielamiedjelelektroforese (SDS-PAGE), aangevul deur genomiese data (16S rDNS volgordebepaling) van geselekteerde isolate.

Inisiële sifting (SDS-PAGE) het getoon dat die meeste isolate wat van dieselfde plantspesie verkry is, gegroepeer het in verskeie groepe van die dendrogram. 'n Kleiner aantal van die isolate het wel 'n hoë graad van gasheerspesifisiteit getoon. Dit was redelik om te aanvaar dat gasheerspesifisiteit minder belangrik is by die simbiotiese interaksie van meeste *Lotononis* spesies en wotel-knoppiebakterieë. Ten opsigte van die geografiese oorsprong van die isolate was hul diverse aard duidelik. Isolate van ooreenstemmende geografiese oorsprong het geneig om kontinu versprei te wees regdeur die dendrogram. Van die isolate van ariede omgewings het egter geneig om hoë elektroforetiese verwantskappe te toon.

Verder is gevind dat die wortel-knoppiebakterieë geassosieerd met *Lotononis* spesies heterogeen was en nie beperk was tot oorwegend spesifieke rhizobiale genusse nie. Daar was ook gevind dat van die isolate verwant is aan genusse buite die *Rhizobiaceae*, nl *Methylobacterium* en *Burkholderia*.

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LIST OF ABBREVIATIONS

α	alpha
Å	armstrong
AR	arid
ARC	Agricultural research council
ARDRA	amplified ribosomal DNA restriction analysis
β	beta
BNF	biological nitrogen fixation
bp	basepair
bv	biovar
°C	degrees Celsius
C:18	carbon 18
C/N	carbon to nitrogen
ClustalX	cluster analysis version X
CSN	cultivar specific nod genes
cv	chemovar
d	day(s)
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5'-triphosphate
EDTA	ethylenediaminotetra-acetic acid
EPS	extracellular polysaccharide
ERIC	enterobacterial repetitive intergenic consensus
ESG	extra slow growing
g	grams
% G+C	percentage guanine and cytosine content
GSN	genotype specific nod genes
h	hour
HPLC	high performance liquid chromatography
kg	kilogram
kV	kilovolt

rRNA	ribosomal ribonucleic acid
SA	savanna
SCE	staircase electrophoresis analysis
SDS	sodium dodecyl-sulphate
SDS-PAGE	sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SE	semi-arid
SEM	scanning electron microscopy
sp	species
ssu	small subunit
ST	strandveld
STB	sample treatment buffer
SYM	symbiotic
T	type strain
TEM	transmission electron microscopy
TY	tryptone yeast extract medium
UHQ	ultra-high quality
μ	micro
μl	microliters
μM	micromolar
UPGMA	unweighted pair group method using arithmetic averages
USDA	United States Department of Agriculture – ARS National Rhizobium
UV	ultraviolet
V	volume
v/v	volume per volume
YMA	yeast extract mannitol agar
YMB	yeast extract mannitol broth

CHAPTER 1

CHAPTER 1

INTRODUCTION

The demand for fixed nitrogen as a nutrient for world crop and pasture production has increased rapidly during the last century as a result of the projected exponential growth of the human population and will undoubtedly increase in future. The attributes of both symbiotic nitrogen fixation in legumes and the industrialized Haber-Bosch process are essential to meet this increased demand (Rockefeller Foundation, 1997). The advantageous aspects of biological nitrogen fixation as brought about by rhizobia have led to numerous research studies. These include investigating identity and diversity of the associated bacterial symbionts, aiming at the identification of more efficient, stress tolerant and compatible micro symbionts.

The process of biological nitrogen fixation and plant growth stimulation and the involved symbionts commonly known as rhizobia has been studied for a long period of time. Additionally novel bacterial genera, other than those encompassing the rhizobia, have recently been identified as active rhizosphere inhabitants capable of forming active symbiosis with their leguminous hosts. This can be attributed to more and more nodule bacteria being characterized as the range of host plants from different environments under investigation are increasing worldwide. Coupled with this, the diversity and our understanding of these bacteria are equally broadened. The stringency of host specificity coupled with environmental factors involved in the symbiotic process led to the general acceptance that this process is restricted to species contained in the *Rhizobiaceae*. However, genera such as *Methylobacterium* (Sy *et al.*, 2001), *Burkholderia* (Trân Van *et al.*, 2000; Baldani *et al.*, 2000; Cruz *et al.*, 2001 and Estrada-de Los Santos *et al.*, 2001), *Herbaspirillum* (Cruz *et al.*, 2001; Baldani *et al.*, 2000) and *Ralstonia* (Chen *et al.*, 2001) have recently been shown as capable of biological nitrogen fixation and successful symbioses. The identification of rhizobia within the β-subclass of the *Proteobacteria*, shows that the ability to establish a symbiosis with legumes is more widespread in bacteria than generally accepted to date. Such symbiosis is not a sporadic phenomenon, since *Ralstonia* appears to be the favourite partners of *Mimosa pudica* and *M. diplotricha*

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in Taiwan (Chen *et al.*, 2001). Consequently, the term rhizobium could now be considered a generic term grouping phylogenetically diverse bacteria with the ability to establish a legume symbiosis.

Species of the plant genus *Lotononis* are widely distributed over a broad range of diverse geographic and climatological regions. The genus has drawn the attention of scientists concerned with pasture legumes over the past 50 years due to its ability to maintain itself in association with grasses under moderate to heavy grazing conditions in tropical climates. The bacteria which effectively nodulate *Lotononis bainesii* and which are used for commercial inoculants in South Africa are pigmented and differ in this aspect from other rhizobia (B. W. Strijdom, personal communication). A recent study showed the rhizobial symbionts of *L. bainesii* to be closely related to *Methylobacterium nodulans* (Jaftha *et al.*, 2002). The aim of this study was therefore to investigate the diversity and taxonomy of the root nodule associated bacteria of the genus *Lotononis* in South Africa. The root nodule bacteria of *Lotononis* spp. in South Africa have not been studied in detail.

CHAPTER 2

CHAPTER 2
LITERATURE REVIEW

2.1 BIOLOGICAL NITROGEN FIXATION

2.1.1 Nodulation and biological nitrogen fixation

Biological nitrogen fixation has commanded the attention of scientists concerned with plant mineral nutrition for more than 100 years. The importance of this process in managed and natural ecosystems has sustained a substantial research effort that has expanded markedly in recent years. This can be ascribed particularly to an increase in energy costs of fertilizer production, and ecological concern about excessive usage of fertilizer in agriculture and forestry.

Nitrogen is a crucial mineral element needed for the sustenance of life where it forms the core of all known proteins. Nitrogen fixation is second only to photosynthesis in terms of importance for growth and development of plants (Kahindi *et al.*, 1997). Soil nitrogen, originating from decomposing plant residues and microorganisms as well as lightning, is normally insufficient for extensive production of leguminous crops and therefore the importance of legume-rhizobia interaction cannot be emphasized enough. It is known that most leguminous plants can obtain between 50 and 80% of their total nitrogen requirements from biological nitrogen fixation (Zahran, 2001).

During the process of biological nitrogen fixation both chemical and genetic interactions are involved (see detailed discussion in section 2.1.2). Rhizobia in natural soil respond positively to exudates from plant roots. They react chemotactic towards sugars, amino acids and other nutrients, like most soil saprophytes (Triplett, 1990). Rhizobia show a high chemotactic affinity towards specific flavonoid compounds that are released by the roots of leguminous plants (Kape *et al*, 1991). These flavonoids are also responsible for the induction of transcription of nodulation

(*nod*) genes of rhizobia. This interaction is mediated by the *nodD* gene, which is the only *nod* gene to be constitutively expressed (Long, 1989). In the presence of an appropriate flavonoid, the NodD protein activates the transcription of all the other *nod* genes by binding to a conserved 60-bp region of DNA sequence in each *nod* gene promotor, a region known as the *nod* box (Fisher *et al.*, 1989).

The *nodD* product (NodD) associates with the cytoplasmic membrane of rhizobia and appears to interact with the flavonoids in the root exudates (Recourt *et al.*, 1989). This molecular recognition is an important determinant of host-rhizobia specificity (Spainck *et al.*, 1989).

Common *nod*-gene products are oligo-polysaccharides, collectively termed Nod-factors. Nod-factors are essential for the curling and branching of legume root hairs, and is a rapid response after inoculation of rhizobia that stimulates cortical cell divisions and the invasion of plant cells by rhizobia. Invasion occurs via the induction of an infection tread that penetrates into the plant tissue and continues to grow and ramify in the root cortex. The infection thread eventually invades a focus of dividing plant cells and rhizobia are released into these cells following packaging within a plant membrane (so-called peribacteriod membrane), which separates the bacteria from the contents of the host cell (Dixon, 1967). The rhizobia keeps on dividing and when the cytoplasm is almost saturated with bacteria, the bacteria enlarge and change shape, and due to its altered morphology it is referred to as the bacteriod. A specific plant signal coupled with the appropriate physiological environment is thought to stimulate the expression of a different set of genes, the nitrogenase genes or *nif* genes enabling nitrogen fixation to occur within the mature nodule. Multiple copies of the nitrogenase structural genes are found in various species of rhizobia (Martinez *et al.*, 1990), which are responsible for the synthesis and processing of nitrogenase, various cofactors, electron donors, bacteriod differentiation and heme processing.

2.1.2 Rhizobia and legume host specificity

(i) Introduction

The existence of communication between plants and microbes on a molecular level is a well-documented phenomenon in various fields of plant-microbe research. This is especially evident when considering the interaction between plants and plant pathogens. The interaction between plants and microbes normally involves signal molecules being exchange between both organisms. In most instances the signal molecule from the microbe would interact with that of the plant, eliciting a certain response. Such responses include hypersensitive reactions that might lead to the death of plant tissue and morphogenesis leading to altered cell structures. This relationship is referred to as a gene-for-gene interaction (Triplett and Sadowsky, 1992).

Bacterial host specificity in symbiotic nitrogen fixation became apparent by observation that certain rhizobial isolates were capable of forming effective nodules on some host legumes (Nod^+, Fix^+) whilst forming ineffective nodules on others (Nod^+, Fix^-). (Nod^+) would thus refer to those rhizobia capable of inducing nodular structures on a certain host plant, whilst (Fix^+) indicates that rhizobia are capable of successful fixation of atmospheric nitrogen in symbiosis with a particular host plant. It should thus be noted that specificity is not only confined to nodulation but also extends to other characters such as the ability to form effective (N_2-fixing) nodules. This feature was further supported due to the inability of some effective symbionts to form nodules on legumes other than the original host. It is clear that the degree of host specificity fluctuates among the rhizobia (Young and Johnston, 1989), with some strains having broad host ranges, capable of nodulating various leguminous species, whilst others are restricted to a much narrower host range. For example; the slower-growing bradyrhizobial species which typically infect and fix nitrogen in tropical legumes versus the faster-growing *Rhizobium* species typically found in symbiosis with temperate zone legumes. The latter exhibit narrow or specialized host range: for example, *Rhizobium meliloti* is symbiotic with *Medicago, Melilotus* and *Trigonella*. In contrast bradyrhizobia exhibit a non-specialized host range which has been regarded as evolutionary primitive and ancestral (Stanley and Cervantes, 1991).

(ii)   Host specificity as determined by the rhizobial partner

Rhizobial host specific determinants include three major types. The interaction of *NodD* with the specific flavonoids of the host plant represents the first level of host-specific recognition (Schultze and Kondorosi, 1998). The second is the production of lipochitooligosaccharides (LCO's), that is determined by the common *nod* genes *nodABC*. Lastly, several genetic loci exist whose expression is directly responsible for discrimination between different host plants (Triplett and Sadowsky, 1992).

The specificity of each different *NodD* product is determined at the amino acid level with the flavonoid specificity primarily located at the less highly conserved N-terminal part of the *NodD* product (van Rhijn and Vanderleyden, 1995). The feature of host specificity as determined by the differential sensitivity of *nodD* towards specific flavonoids has been proven by point mutations within the *nodD* sequence which may change the sensitivity to additional inducers (Burn *et al.*, 1987; Bulawa and Wasco, 1991). It was also previously shown that the transfer of *nodD* to a different rhizobium renders the recombinant sensitivity towards new flavonoids (Spainck *et al.*, 1987).

The second molecular entity determining host specificity is that of lipochitooligosaccharides (LCO's) or extracellular polysaccharides signal molecules. These are also referred to as so-called Nod-factors being the products of some nodulation genes. Polysaccharides contribute greatly to the composition of the rhizobial cell surface and it would be reasonable to expect it to be involved in recognition events and plant infection mechanisms (Stanley and Cervantes, 1991). Various exopolysaccharide (*exo*) genes have been identified by their effect on production of extracellular or capsular polysaccharides. Mutations within some of these genes have shown to influence the quantity and quality of EPS produced as well as the infection phenotypes on various hosts (Chen *et al.*, 1985). The Nod factors are considered to be the main *Rhizobium* nodulation signal molecules. These molecules are LCO-like compounds and are the products of common nodulation genes, *nodABC*. Purified fractions of these molecules are capable of inducing plant responses similar to that observed during the early steps of nodulation. Lipochitooligosaccharides from several species of rhizobia are able to elicit root hair deformation and nodule

primordia in a host-specific way (Lerouge *et al.*, 1990). Different *nod* genes are involved in the modification of LCO structure within different rhizobial species. The Nod factor composition seems to reflect adaptation to the specific host plant since rhizobia belonging to different taxonomic groups produce structurally similar lipochitooligosaccharides (LCO's) when isolated from the same host plant species (Lorquin *et al.*, 1997).

Various loci involved in host specificity are known for several rhizobial species: the common *nod* genes, host-specific *nod* genes (*hsn*) and the genotype- or cultivar-specific *nod* genes (GSN and CSN respectively) [Triplett and Sadowsky, 1992].

Discrimination between different genotypes within a particular legume species is mediated through bacterial sequences referred to as genotype-specific nodulation (GSN) genes whereas in the case of cultivated varieties these genes are called cultivar-specific nodulation (CSN) determinants (Triplett and Sadowsky, 1992). One such characterized gene is *nodX*, known to be responsible for the same rhizobial species having preferred genotypes of the same legume species (Lie, 1978; Lie, 1984). After this report several other genes with similar function have been reported for various rhizobial species (Triplett and Sadowsky, 1992). One such gene cluster has been identified within the rhizobial species *R. meliloti* (now *S. meliloti*) [Horvath *et al.*, 1986]. These genes are involved in host-specific nodulation and have been designated *hsnABCD/nodFEGH*. In the case of *R. meliloti, nodFEGH* are required for nodulation of specific host plants within different legume genera. Gene clusters with similar function were identified in *B. japonicum* referred to as *nodV* and *nodW* and appears to be essential for the nodulation of siratro, mungbean and cowpea.

Taken together, results from several studies investigating rhizobia-legume host specificity suggest a hierarchy to nodulation determinants (Spainck, 1992; Ehrhardt *et al.*, 1992; Heidstra *et al.*, 1994; etc.). While some host-range determinants such as Nod-factors and flavonoids affect nodulation at the legume genus level, others such as GSN and CSN determinants affect nodulation of a single genotype/cultivar within a given host species.

(iii) Host specificity as determined by the host plant

Root hair deformation, the development of pre-infection threads and the division of cortical cells are some of the host plant responses towards Nod signals and events during early nodulation. Subsequently the enhanced or induced expression of host plant genes is being triggered. Most of these genes identified seem to be involved in the synthesis of cell wall proteins emphasizing the importance of alterations in cell wall biochemistry during infection and nodulation (Schultze and Kondorosi, 1998).

The obvious first step of host specific regulation is determined by the type/structure of isoflavonoid being excreted by the host's roots. These would contribute towards activating the appropriate rhizobial *nodD* genes.

One of the earliest cases of genetic specificity as determined by the host plant was coupled with the discovery of cultivar-specific nodulation gene *nodX* (see discussion in previous section). It was shown that the *nodX* product interacts with a locus in the host plant, designated the *sym-2* locus. Interaction between these two gene loci enabled the appropriate symbiotic partners to form a successful symbiosis (Gotz *et al.*, 1985).

Spatial and temporal expression of genes involved in specificity are characteristic for marking the different stages involved in rootnodule formation. The best characterized of these genes include the Enod genes, (Enod2, Enod5, Enod10, Enod11, Enod12 etc.), first identified in soybean (*Glycine max*). Regulation and function in terms of host specificity has been reported for several of these genes (Dehio and De Bruijn, 1992; Bauer *et al.*, 1996). The Enod12 gene was shown to be responsible for cortical cell division and its expression is induced and enhanced by cytokinins, a chemical compound associated with some Nod factors (Bauer *et al.*, 1996). Other reports indicated similar stimulation of expression for other enod genes (Dehio and De Bruijn, 1992; Bauer *et al.*, 1996).

Soybean genotypes restricting nodulation by specific strains or serogroups of *Bradyrhizobium* have been reported (Triplett and Sadowsky, 1992). The genes *Rj₁,*

Rj₂, *Rj₃* and *Rj₄* were identified as restricting nodulation by bradyrhizobia to various degrees.

The molecular and genetic characters of the plant symbiotic partner is less understood and studied than the bacterial counterpart, and would in future reveal more discoveries concerning host specificity as determined by the host plant.

2.2 BIOLOGICAL NITROGEN FIXATION SYSTEMS IN NATURAL ECOSYSTEMS.

2.2.1 Introduction

The process of biological nitrogen fixation occurs in almost every natural environment including the sea, encompassing a wide range of nitrogen-fixing systems. The importance of nitrogen fixation in nature can best be seen in non-climax ecosystems. At early stages of primary succession on freshly weathering substrata where there is little organic matter in the soil, nitrogen is the nutrient most limiting productivity. Nitrogen-fixing organisms have a major role to play in the accumulation of nitrogen in microbial and plant biomass and in soil organic matter. Although these systems are biologically diverse, there are commonalities such as physiology and genetics involved in N_2-fixation, affecting the optimum exploitation of these systems and their importance in agriculture. However, nitrogen fixation within these systems, is restricted to procaryotes and has never been found in eucaryotic organisms (Rockefeller foundation, 1997). The procaryotes in the Eubacteria and Archaebacteria kingdoms that are able to fix nitrogen are metabolically diverse, but is nevertheless restricted to a small number of the total number of species. To group all organisms capable of biological nitrogen fixation into a single category is convenient from the molecular and physiological perspective but more cumbersome when their ecological roles and biodiversity are considered. These bacteria represent free-living-, associative- and symbiotic nitrogen-fixing bacteria. The understanding of these systems are constantly undergoing change as novel entities and bacteria involved in this process emerge.

2.2.2. Biological nitrogen fixation in free-living systems

The free-living system encompasses all those diazotrophs that are "completely free-living", or in loose association as a result of rhizosphere or phyllosphere colonization. These organisms are genetically diverse and although current data are incomplete because the ability to fix nitrogen was not always rigorously tested, 16 families and over 46 genera of procaryotes, excluding the cyanobacteria, are represented (Mulongoy *et al.*, 1990) encompassing both heterotrophic and autotrophic organisms. Our understanding of the biodiversity among the free-living procaryotes is complicated due to the inability to reliably culture them, as a result, many of these organisms remain undiscovered (Ward *et al.*, 1990). Non-symbiotic nitrogen-fixing bacteria are phylogenetically extremely diverse, having representatives in nine subdivisions of the Eubacteria and in four of the Archaebacteria, whereas symbiotic nitrogen-fixers are only found in four subdivisions of the Eubacteria (Kahindi *et al.*, 1997).

(i)   Heterotrophic systems

Most of the best known free-living organisms capable of nitrogen fixation belong to this category. These include organisms such as *Azomonas* spp., *Azospirillum* spp., *Azotobacter* spp., *Bacillus* spp., *Beijerinckia, Clostridium* spp., *Herbaspirillum, Klebsiella,* etc. When these organisms are associated with the rhizospheres of leguminous and other plants, fixing nitrogen, they normally promote plant growth in most cases. They have therefore been grouped as plant growth-promoting bacteria (Berge *et al.*, 1991). This aspect is not only restricted towards the physical promotion of plant growth or the ability to fix nitrogen, but in most cases properties such as enhancement of crop yield via growth hormone production (Riggs *et al.*, 2001), the suppression of soilborne plant pathogens or degrading a diversity of pesticides (Santos *et al.*, 2001). Research focussing on the diversity of plant-associated bacteria is continuously contributing towards the discovery of new beneficial plant-microbe interactions.

Studies of dominant diazotrophs or plant growth-promoting rhizobacteria associated with various commercial crops have been performed to establish applicability and

their beneficial attributes towards agriculture. Many of these free-living organisms have been isolated from the rhizospheres of roots of all the major cereal crops, in both Africa and Latin America. Diazotrophs such as *Burkholderia tropicalis, B. brasilensis, Herbaspirillum seropedicae, H. rubrisubalbicans* have been recognized as valuable rhizosphere inhabitants of banana and pineapple plants (Cruz *et al.,* 2001). Similarly, *Burkholderia cepacia* allows growth promotion of common bean (Peix *et al.,* 2001) and maize (Di Cello *et al.,* 1997). Although the ability to fix nitrogen is not reported to be a common feature among the known species of the genus *Burkholderia,* it was shown previously that the genus is very rich in diazotrophic species (Santos *et al.,* 2001). Nitrogen fixation genes have recently been identified and characterized in the *Burkholderia* genus (Miner *et al.,* 2001, Santos *et al.,* 2001) and seemingly horizontal gene transfer is the best explanatory reason for this phenomenon. The horizontal transfer of nitrogen fixing genes have long been recognized as commonly occurring events between unrelated bacteria (Young, 2001).

Cilicate bacteria are generally placed in the species *Bacillus circulans* and are widely used in biological fertilizers and biological leaching. The bacteria can form conspicuous amounts of extracellular polysaccharides in nitrogen-free media or in presence of substrates with large C/N ratios (Lian *et al.,* 2001). These extracellular polysaccharides proved to act as bacteria-to-plant signal molecules (see discussion in 2.1.2 section ii) similar to LCO's being excreted by rhizobial symbionts (Prithiviraj et al., 2000). The capability of *Bacillus circulans* as a potential inhibitor of other microorganisms and to fix nitrogen led to the identification of these bacteria as plant growth-promoting towards maize. *B. circulans* colonize maize roots heavily both *in situ* and *in vitro* and coupled with its substantially higher rate of nitrogen fixation compared with most other maize root colonizers, makes it an attractive possibility for agricultural application (Berge *et al.,* 1991). The closely related *Paenibacillus azotofixans* are also associated with the rhizoplane of maize as an active nitrogen fixer. Bacilli, especially nitrogen-fixing strains have been known as active colonizers of grass and wheat roots (Seldin *et al.,* 1998).

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(ii)   Autotrophic systems

Free-living nitrogen fixing organisms include autotrophic organisms (cyanobacteria), photosynthetic organisms (purple and non-sulphur bacteria) and chemoautotrophs (*Thiobacillus*).

The filamentous and unicellular cyanobacteria are common inhabitants of tropical soils. The filamentous species could form large amounts of biomass in flooded rice fields, where they significantly promote growth and yield (Roger *et al.,* 1987). The total amount of nitrogen fixed by cyanobacteria in rice paddies is normally moderate (5-25 kg N/ha per annum), but with efficient management such as fertilization with phosphorus could rise substantially (Roger and Ladha, 1992).

2.2.3   Cyanobacterial symbiosis

Almost every division of plants and lichens is capable of forming a symbiosis with cyanobacteria (Giller and Wilson, 1991).   Although most of these symbiotic relationships have major ecological roles, the only one notable for its importance in agriculture as that formed by *Azolla*. The genus consists of aquatic ferns, found free-floating on water surfaces. It forms a symbiotic relationship with the cyanobacterium *Anabaena azollae* where effective nitrogen fixation occurs (Elkan, 1992).   The effectiveness of this symbiosis can be compared with that of the legume-*Rhizobium* symbiosis when conditions are optimal. It was shown that *A. azollae* might contribute significantly towards the nitrogen requirements of rice (Lumpkin and Plucknett, 1982).

2.2.4   Frankia symbiosis

Members representing *Frankia* are actinomycetes, having at some stage in their lifecycle a filamentous habit, reflecting morphological resemblance to some fungi. The genus is known to form nitrogen-fixing symbiosis with some 279 angiosperm species (Baker and Mullin, 1992), of which most are shrubs and trees found in temperate climates. Despite various attempts to isolate *Frankia* it was only isolated in 1978 for the first time, years after Beijerinck isolated rhizobia for the first time. The

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slow growth rate of *Frankia* on non-selective media and the frequent occurrence of contamination hampered the isolation and identification of organisms from actinorhizal nodules.

Although the plant symbionts belong to a wide range of families, the bacterial symbionts are reasonably homogeneous at genus level, even though there exists considerable heterogeneity between the relatively few strains isolated in pure culture (Lechevalier, 1994).

An important feature of *Frankia* is its capability to fix nitrogen at normal oxygen concentrations at rates sufficient to support growth in culture. Nitrogen fixation in such cultures is inhibited by the addition of combined nitrogen. Molecular techniques are implemented to assess the biodiversity of the *Frankia* genus.

2.3   THE USE OF POLYPHASIC TAXONOMY IN BACTERIAL SYSTEMATICS

2.3.1   Introduction

Identification and classification, or groupings of various entities, are important and common human activities practiced since earliest times. In dealing with a large number of objects or pieces of information some convenient system of orderly arrangement is needed for the purpose of storage and retrieval. Essentially taxonomy forms a communication network for scientists sharing the same fields of interest.

In bacteriology, classification is a means of summarizing our knowledge of the prokaryotes and cataloguing that knowledge. As this information is constantly and rapidly expanding, so classifications evolve and increase in importance, with contemporary schemes reflecting our state of knowledge about the organisms concerned.

Van Berkum and Eardly (1998) described three interrelated areas of bacterial systematics as follows:

- Classification: the arrangement of organisms into taxonomic groups based on their similarities.

- Nomenclature: the assignment of names to the taxonomic groups according to international rules.

- Identification: the process of determining whether a new isolate belongs to one of the established and named groups.

The results of characterization studies are classification schemes presumed to reflect natural relationships. Comparative analyses of DNA and gene encoded products (molecular systematics) can be used to develop a phylogenetic classification scheme. By definition, therefore, taxonomy is the science and practice of classification of distinct groups and is useful for identification purposes (Prescott, *et al.*, 1996).

The central goal of molecular evolutionary systematics is the reconstruction of evolutionary history through the study of patterns of molecular genetic diversity in natural populations. In its search for evolutionary relationships among organisms, molecular systematics encompasses the field of taxonomy, which focuses on the classification and naming of species. This phylogeny of a group of organisms is traditionally diagrammed as a hierarchical tree to reflect putative evolutionary relationships.

2.3.2   Polyphasic taxonomy

Another development of bacterial taxonomy is polyphasic taxonomy (Colwell, 1970), which aims to integrate different types of data and information (phenotypic, genotypic and phylogenetic) on microorganisms to essentially indicate a consensus type of taxonomy (Vandamme *et al.*, 1996). This approach allows more reliable and better resolution of the interrelationship among microorganisms and is an attempt to synthesize the real landscape and a step toward a synthetic taxonomy (Vandamme *et al.*, 1996). Therefore, polyphasic taxonomy aims to incorporate all genotypic, phenotypic and phylogenetic information.

(i)   Genotyping

Genotypic analysis would correspond to all methods aiming at the investigation of nucleic acids (DNA or RNA) within the cell. The central dogma surrounding molecular biology makes genotyping the pre-dominant approach towards classification of organisms. Phylogenetic relationships are also determined making use of genotypic information. The basic taxonomic unit, the bacterial species, is defined using a range of techniques (mainly genomic), and is the single most important entity around which taxonomy centres.

The percentage of guanosine plus cytosine (% G+C) is a well-developed and defined technique when classifying bacteria. Today it is almost consensus and standard to include this technique when describing bacterial taxa. Within a well-defined species the range should be in the order of 3% and 10% within a genus (Stackebrandt and Liesack, 1993).

Species level discrimination by definition involves a DNA-DNA hybridization value of 70% or more and with 5 °C or less difference in ΔTm of the DNA-DNA hybrid. DNA-DNA hybridization reflects the sequence similarity between two entire genomes, a technique widely valued in the description of new bacterial taxa (Wayne *et al.,* 1987).

There has been an increasingly reliance on ribosomal gene sequence data for studying phylogenetic relationships, due to these sequences being present in all bacteria, remaining functionally constant and having highly conserved as well as less conserved regions. In some cases, especially when multiple strains are investigated, DNA-rRNA hybridization proves a valuable tool.

Together with all the emerging new tools in taxonomy came the application of various DNA-based typing methods. These techniques are of special interest when determining relationships below the species level. The earliest of these techniques included restriction enzyme digestion of whole-genome DNA and the analysis of the resultant fragments on agarose gels (Botstein *et al.,* 1980; Vandamme *et al.,* 1996).

Today recognized as a common technique called restriction fragment length polymorphism (RFLP). The technique is less applicable when dealing with plasmid analysis, since plasmids are not readily kept within cells and most strains often belong to only a few types. Restriction fragment length polymorphism has also been applied towards amplified rDNA genes. Arbitrary primers have been used to determine variation within species by generating Random Amplified Polymorphic DNA (RAPD) [Williams *et al.*, 1990). However, analysis with primers for conserved repeat sequences present in bacterial genomes has been more extensively used. These include pairs of primers for amplification of DNA regions between Repetitive Extragenic Palindromic (REP) and Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences (Versalovic *et al.*, 1991).

(ii)   Phenotyping

Phenotypic methods include techniques not directly involving the genetic material (DNA or RNA) of the organism of concern. Classical examples of the entities involved in phenotypic classification are morphological, physiological and biochemical characters. Morphology encompasses both cellular and colonial characteristics of the organisms. The physiological and biochemical features taken into account for taxonomical purposes include growth temperatures, optimum pH preferences, substrate utilization, salt concentration tolerance, the activity of various enzymes, etc..

The use of computerized analysis (numerical analysis) of phenotypic data allowed the comparison of large numbers of phenotypic traits for large numbers of strains. Numerical analysis allows researchers to construct data matrices showing degrees of similarity from the interpretation of phenotypic data as dendrograms. Automated systems such API and Biolog tend to replace classical phenotypic analysis. These systems mostly contain a battery of dehydrated reagents and the addition of a standardized inoculum initiates the reaction. Results are normally interpreted according to the manufacture's instructions.

Some of the phenotypic tools most widely applied in bacterial systematics is SDS-PAGE analysis of whole cellular proteins and fatty acid analysis. The same level of

discrimination than that obtained by DNA-DNA hybridization could be achieved by comparison of electrophoretic groupings of whole cellular protein extracts when comparing organisms (Kersters and de Ley, 1975; Kersters, 1985; Priest and Austin, 1993; Vauterin *et al.,* 1993). SDS-PAGE analysis is in particular a sensible approach when comparing large groups of organisms, with strains that share 90% to 100% genomic DNA sequence homology showing identical protein patterns. Furthermore this technique is more affordable and rapid than DNA-DNA hybridization and has the advantage that clusters are formed from complete similarity matrices.

It has been reported that bacterial species can be identified with a high degree of precision on the basis of their fatty acid composition (Jarvis and Tighe, 1994). Under standardized conditions, large numbers of organisms can rapidly and at low costs be compared. Fatty acid profiles is an excellent tool for recognition and identification of strains belonging to the same species but is unlikely to be reliable as a source of detailed phylogenetic information.

2.4 TAXONOMY OF THE RHIZOBIA

2.4.1 Introduction

Rhizobia are aerobic, motile, Gram-negative, non-sporulating bacteria, belonging to the alpha subdivision of the *Proteobacteria* and are found in great abundance in soil ecosystems. These bacteria are capable of forming a symbiotic relationship with members of the *Fabaceae* resulting in the formation of specialized structures, the nodules, on roots and/or stems of the plant partner in which the fixation of atmospheric nitrogen takes place.

The first root-nodule bacterium was isolated and cultured by Beijerinck, naming it *Bacillus radicicola.* The credibility of the taxonomical status of these isolates however, was questioned for a number of years. Subsequently, it led to the establishment of the *Rhizobium* genus in 1929. *Rhizobium* species have been defined in terms of plant cross-inoculation groups. However, this approach is generally

recognized as inadequate since cross-inoculation groups are not mutually exclusive and due to the fact that host specificity involves various genes that are in many cases plasmid borne and interchangeable between related strains. The emergence of polyphasic taxonomy (Colwell, 1970), incorporating a combination of tools and techniques applied in classification and identification, allowed taxonomists to project rhizobial taxonomy in a more refined view.

The crown-gall inducing bacterium, *Agrobacterium*, was first isolated in 1907 and was named *Bacterium tumefaciens* (van Berkum and Eardly, 1998). The genus *Agrobacterium* was proposed by Conn (1942) after arguing that *Bacterium tumefaciens* showed very high similarities to the previously isolated *Alcaligenes radiobacter* and other legume nodule bacteria. The proposed genus *Agrobacterium*, would include both soil saprophytes and pathogens. Currently the genus encompasses five different species: *A. tumefaciens* (Smith and Townsend, 1907), *A. radiobacter* (Holmes and Roberts, 1981), *A. rhizogenes* (Riker *et al.*, 1930), *A. larrymoorei* (Bouzar and Jones, 2001) and *A. vitis* (Ophel and Kerr, 1990).

Phylogenetic analysis using the sequence of the conserved 16S rDNA gene is a common and widely applied tool when classifying bacteria. The availability of these sequences over the past few years led to a sufficient database that can be applied in rhizobial taxonomy. Fox *et al.*, (1992) argued that the sequence of the 16S rDNA gene might be insufficient to discriminate between closely related species and that actual culture work should be included in order to insure trustworthy results. Nevertheless, in the case of rhizobial taxonomy, the three main genera/branches (Figure 1.1), *Azorhizobium*, *Bradyrhizobium* and *Rhizobium* are clearly distinguished with 16S rDNA information and in most cases is an adequate tool for discriminating between closely related species.

Rhizobia that were originally isolated from soybean (*Rhizobium fredii*) were assigned to the genus *Sinorhizobium*. However, this proposed genus was rejected after 16S rDNA sequences revealed *Rhizobium fredii* to be closely related to *Rhizobium meliloti* (Jarvis *et al.*, 1992). Upon further investigation it became apparent that *Rhizobium meliloti* should be assigned to the *Sinorhizobium* genus.

The phylogeny of the slower-growing bradyrhizobia enjoyed less attention than the faster-growing rhizobia. Only one "species", *Rhizobium japonicum*, was originally recognized. Jordan (1982) proposed the latter to be named *Bradyrhizobium japonicum* upon the description of the genus *Bradyrhizobium*. Cross-inoculation groups once again caused confusion as *Bradyrhizobium japonicum* was capable of forming nodules on cowpea whilst cowpea strains proved to nodulate soybean. It led to the description of a second species within the genus, namely *B. elkanii* (Kuykendall *et al.*, 1992).

Determining rhizobial relationships are currently adjusting to general bacterial taxonomy, making use of genomic, phenotypic and phylogenetic features moving away from sole nodulation properties. The considerable rate at which molecular techniques are developing together with the investigation of more and more different legume hosts would provide a platform for flexible and changing taxonomy within the rhizobia. The currently recognized species of rhizobia are summarized in Table 1.1.

2.4.2 Current rhizobial taxonomy

Small subunit rDNA phylogeny showed several subbranches occurring within the *Rhizobiaceae* (Figure 1.1), representing the different genera *Rhizobium* (Frank, 1889), *Bradyrhizobium* (Jordan, 1982), *Azorhizobium* (Dreyfus *et al.*, 1988), *Sinorhizobium* (Chen *et al.*, 1988; de Lajudie *et al.*, 1994), *Mesorhizobium* (Jarvis *et al.*, 1997), *Allorhizobium* (de Lajudie *et al.*, 1998), *Agrobacterium* (Conn, 1942).

As discussed previously, rhizobial taxonomy based on symbiotic association is unreliable, and the taxonomy of rhizobia has undergone major changes. Young *et al.* (2001) proposed the emendation of the genera *Agrobacterium*, *Rhizobium* and *Allorhizobium* into a single genus, *Rhizobium*. The genus *Agrobacterium* is closely related to *Rhizobium* and the amalgamation of these two genera has often been proposed (Young *et al.*, 2001), whilst *Allorhizobium undicola* proved to be an outlying branch of the *Agrobacterium-Rhizobium* cluster based on 16S rDNA phylogeny (de Lajudie *et al.*, 1998b). Young and co-workers (2001) argued that no consistency existed in phylogenetic differentiation between these three genera and

that no unique phenotypic circumscriptions could be assigned to them and that they should encompass the single genus *Rhizobium*.

(i)   The genus *Rhizobium*

R. leguminosarum

The genus *Rhizobium* (Frank, 1889) is defined by the type species *R. leguminosarum* nodulating members of the legume genus *Vicia*. Two previously described *Rhizobium* species, *R. trifolii* and *R. phaseoli*, were shown to be too closely related to *R. leguminosarum*, not meeting the criteria to be viewed as two separate species. Currently it is accepted as proposed by Jordan (1984) that these two species would represent two different biovars of *R. leguminosarum*. Type strain *R. leguminosarum* nodulating members of the genus *Vicia* encompasses the biovar *viciae*; whilst biovar *trifolii* refers to those members capable of nodulating the legume genus *Trifolium*, and biovar *phaseoli* being the symbionts of *Phaseolus* species. The host-restricted nature of the cross-inoculation groups within these three biovars indicated that discrimination between them are more likely their plasmids rather than their chromosomal backgrounds (Young, 1996).

R. tropici

Within biovar *phaseoli,* however, some uncertainty still existed, since bean strains originating from Mexico and South America were shown to be phylogenetically extremely diverse. One particular distinct phylogenetic lineage led to the proposal of *R. tropici* (Martinez-Romero *et al.,* 1991).

R. etli

The species *R. etli* (Segovia *et al.,* 1993) was proposed after genetic and phenotypic characters of isolates nodulating both alfalfa and beans indicated a group distinct from *R. leguminosarum* bv. *phaseoli.* Full length small subunit (SSU) rRNA sequencing and DNA-DNA reassociation analysis supported these findings.

R. galegae

Fast-growing root nodule bacteria isolated from the roots of the legume species *Galega orientalis* and *G. officinalis* were initially classified as belonging to the *R.*

leguminosarum lineage. Strains isolated from each host species were capable of forming ineffective nodules on other *Galega* species, whilst for all other legumes genera tested, no nodulation was observed (Young, 1996). A polyphasic approach proved them to be distinct from any previously described rhizobial genus. Lindström, (1989) subsequently proposed the species *R. galegae*.

R. gallicum and R. giardinii

An investigation of the diversity of *R. leguminosarum* strains of French origin (Laguerre *et al.,* 1993a) made use of DNA-DNA hybridization using whole plasmids as probes. It became clear that two distinct groups differing from *R. leguminosarum, R. etli* and *R. tropici* could be distinguished. DNA-DNA hybridization and partial sequencing of the 16S rDNA gene led to the assignment of two new genomic species (Laguerre *et al.,* 1993b). These genomic species showed the highest phylogenetic relationship towards *R. etli, R. galegae* and *R. loti* (now *M. loti*). These two genomic species were assigned to *R. gallicum* and *R. giardinii* after further phenotypic differentiation and 16S rDNA sequence analysis by Amarger *et al.* (1997).

R. hainanense

R. hainanense was proposed after DNA hybridization studies, 16S rDNA sequencing, diagnostic test and symbiotic performance tests was done on fast-growing strains isolated from various tropical legumes in the Hainan Province, China (Chen *et al.,* 1997). The type strain was only capable of inducing nodules on its original host, *Desmodium sinuatum*, and the universal host *Vigna unguiculata*.

R. mongolense

The species *R. mongolense* (van Berkum *et al.,* 1998) was proposed after two genomic species, not representing any previously described rhizobial species, were identified as the symbionts of *Medicago ruthenica*. Using various taxonomical tools these genomic species were shown to be closely related to species within the *Rhizobium* genus, contradicting the conventional idea that *Sinorhizobium* species are normally the predominant symbionts of the *Medicago* genus.

R. huautlense

The most commonly known symbiont of *Sesbania* species has been shown to belong to *Azorhizobium caulinodans*. However, previous reports (Rinaudo *et al.* 1991) indicated that a small proportion belongs to the genus *Rhizobium*. *Sesbania aculeata* found on the Asian continent appeared to have close phylogenetic affinities with *R. galegae*. The investigation of isolates obtained from *Sesbania herbacea* by 16S rDNA sequences and electrophoretic alloenzyme types led to the identification of a new species, *R. huautlense* (Wang *et al.*, 1998).

R. yanlingense

Tan *et al.* (1999) identified rhizobia isolated from wild legumes in north-western regions in China as *Rhizobium* and *Mesorhizobium*. A more recent study (Tan *et al.*, 2001) investigated one specific cluster from this previous study with the addition of new isolates in order to clarify the taxonomy of the specific cluster. With this cluster previously shown related to the genus *Rhizobium*, the nearest DNA homology found between other known *Rhizobium* spp. was 42% and the new species *R. yanlingense* was proposed.

R. sullae

The plant species *Hedysarum coronarium* is a member of a genus consisting of more then 100 species and is an important agricultural crop in Spain and Italy commonly known as sulla (Squartini *et al.*, 2002). The rhizobia isolated from sulla root nodules where previously referred to as *R. hedysari* and were shown to exhibit a high degree of host specificity. Previous work done on sulla isolates includes determination of G + C content and metabolic properties (Struffi *et al.*, 1998), genetics (Espuny *et al.*, 1987; Mozo *et al.*, 1988), cellular fatty acid analysis (Tighe *et al.*, 1994) etc. Squartini and co-workers (2002) investigated sulla isolates by making use of 16S rDNA phylogeny, analyses of polymorphism of the *rrn* operon by ARDRA, DNA-DNA hybridization and staircase electrophoresis of low-molecular-mass (LLM) RNA molecules. Small subunit RNA gene phylogeny revealed sulla isolates to be related to *Rhizobium* species and in particular *R. gallicum* (98.4%), *R. mongolense* (97.7%) and *R. leguminosarum* (97.7%). These results were further supported when the LMM RNA profiles of sulla isolates grouped with that of members of *Rhizobium*. Based on these results the species *R. sullae* was proposed (Squartini *et al.*, 2002).

Table 1.1 **Classification of members of the *Rhizobiaceae* capable of forming symbiosis with their hosts.**

Species	Host plant(s)	Reference
Rhizobium		Frank, 1889
R. leguminosarum		Frank, 1889; Jordan, 1984
biovar viciae	*Pisum sativum, Vicia, Lathyrus, Lens*	Frank, 1889; Jordan, 1984
biovar trifolii	*Trifolium pratense*	Frank, 1889; Jordan, 1984
biovar phaseoli	*Phaseolus vulgaris*	Frank, 1889; Jordan, 1984
R. tropici		
Type II A	*Phaseolus vulgaris, Leucaena leucocephala*	Martinez-Romero *et al.*, 1991
Type II B	*Phaseolus vulgaris, Leucaena leucocephala*	Martinez-Romero *et al.*, 1991
R. etli		Segovia *et al.*, 1993
biovar phaseoli	*Phaseolus vulgaris*	Segovia *et al.*, 1993; Hernandez-Lucas *et al.*, 1995
biovar mimosae	*Mimosa affinis, Leucaena leucocephala, Phaseolus vulgaris L.*	Wang *et al.*, 1999
R. hainanense	*Desmodium sinuatum* and other arid plants	Chen *et al.*, 1997
R. gallicum		Amarger *et al.*, 1997
biovar gallicum	*Phaseolus vulgaris*	Amarger *et al.*, 1997
biovar phaseoli	*Phaseolus vulgaris*	Amarger *et al.*, 1997
R. mongolense	*Medicago ruthenica*	van Berkum *et al.*, 1998
R. galegae		Lindström, 1989
biovar orientalis	*Galega orientalis*	Nick, 1998
biovar officinalis	*Galega officinalis*	Nick, 1998
R. giardinii		Amager *et al.*, 1997
biovar giardinii	*Phaseolus vulgaris*	Amager *et al.*, 1997
biovar phaseoli	*Phaseolus vulgaris*	Amager *et al.*, 1997
R. huautlense	*Sesbania herbacea*	Wang *et al.*, 1998
R. yanglingense	*Coronilla varia, Gueldenstaedtia, Amphicarpaea trisperma*	Tan *et al.*, 2001
R. sullae	*Hedysarum coronarium L.*	Squartini *et al.*, 2002
Mesorhizobium		Jarvis *et al.*, 1997
M. loti	*Lotus corniculatus*	Jarvis *et al.*, 1982
M. chacoense	*Prosopis alba, P. chilensis, P. flexuosa*	Velázquez *et al.*, 2001
M. huakuii	*Astragalus sinicus, Acacia*	Chen *et al.*, 1991
M. ciceri	*Cicer arietinum*	Nour *et al.*, 1994
M. tianshanense	*Glycyrrhiza pallidiflora,* other tropical plants	Chen *et al.*, 1995

Table 1.1 (continued)

Species	Host plant(s)	Reference
Mesorhizobium (continued)		
M. mediterraneum	*Cicer arietinum*	Nour *et al.*, 1995
M. plurifarium	*Acacia, Prosopis*	de Lajudie *et al.*, 1998
M. amorphae	*Amorpha fruticosa*	Wang *et al.*, 1999
Sinorhizobium		Chen *et al.*, 1988; de Lajudie *et al.*, 1994
S. meliloti	*Medigaco, Melilotus, Trigonella*	de Lajudie *et al.*, 1994; Jordan, 1984
S. fredii		de Lajudie *et al.*, 1994; Scholla *et al.*, 1984
chemovar fredii	*Glycine max*	Scholla *et al.*, 1984
chemovar siensis	*Glycine max*	Scholla *et al.*, 1984
S. saheli	*Sesbania* spp.	de Lajudie *et al.*, 1994
biovar acaciae	*Acacia* spp.	Boivin and Giraud, 1999
biovar sesbaniae	*Sesbania* spp.	Boivin and Giraud, 1999
S. terangae		de Lajudie *et al.*, 1994; Trüper and de Clari, 1997
biovar acaciae	*Acacia* spp.	Lortet *et al.*, 1996
biovar sesbaniae	*Sesbania* spp.	Lortet *et al.*, 1996
S. medicae	*Medicago*	Rome *et al.*, 1996
S. kostiense	*Acacia, Prosopis*	Nick *et al.*, 1999
S. arboris	*Acacia, Prosopis*	Nick *et al.*, 1999
S. morelense	*Leucaena leucocephala*	Wang *et al.*, 2002
Azorhizobium		Dreyfus *et al.*, 1988
A. caulinodans	*Sesbania rostrata*	Dreyfus *et al.*, 1988
Bradyrhizobium		Jordan, 1982
B. japonicum	*Glycine max*	Jordan, 1984; Kirchner *et al.*, 1992
B. elkanii	*Glycine max*	Kuykendall *et al.*, 1992
B. liaoningense	*Glycine max*	Xu *et al.*, 1995
Allorhizobium		de Lajudie *et al.*, 1998
A. undicola	*Neptunia natans*	de Lajudie *et al.*, 1998
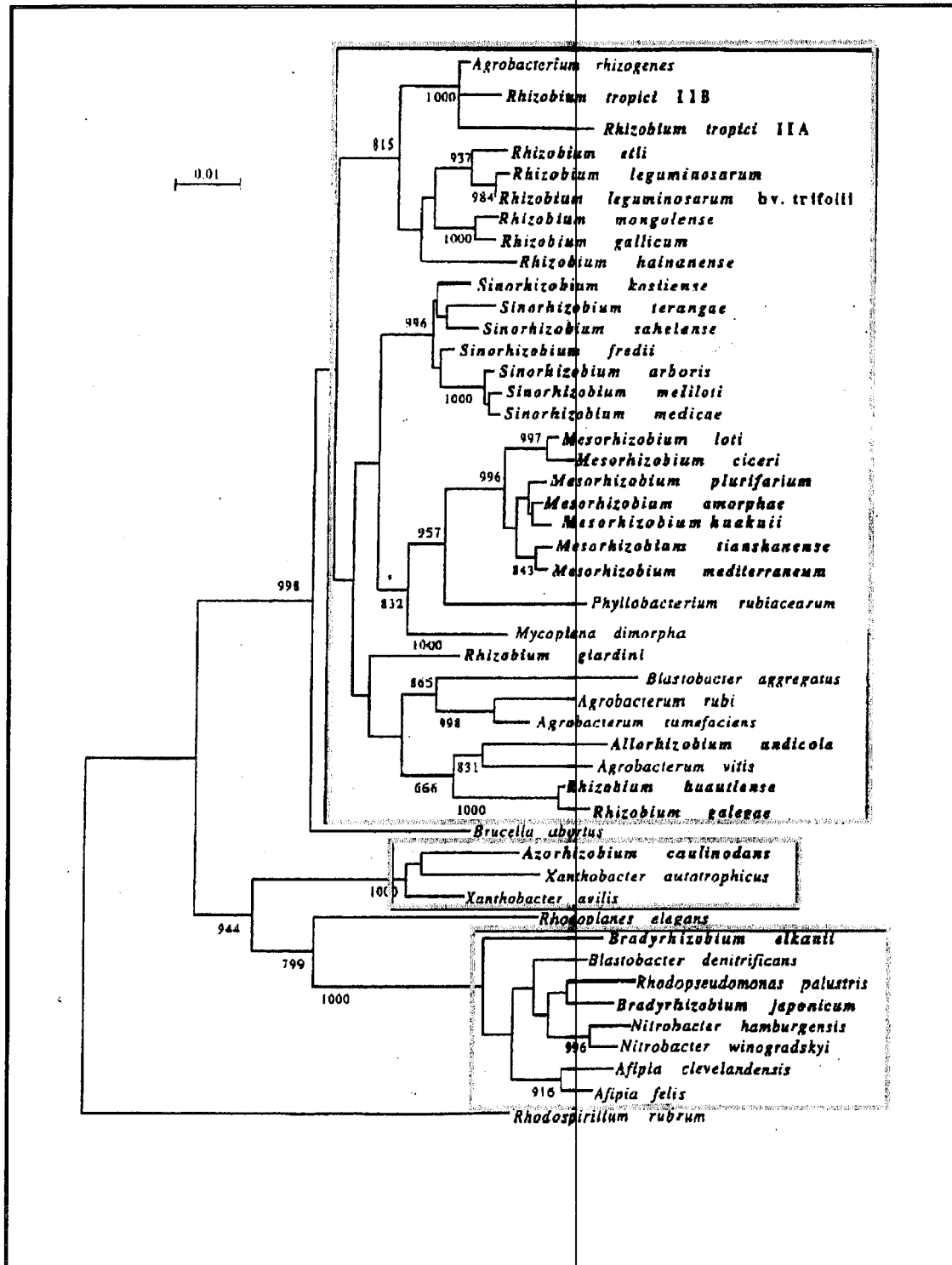
Figure 1.1 Phylogenetic relationships among members of the *Rhizobiaceae* (indicated in bold) and closely related bacteria as determined by their 16S rRNA gene sequence. The three main branches within the *Rhizobiaceae* are blocked (Zakhia and de Lajudie, 2001). The species *B. llaoningense, R. yanglingense, R. sullae,* and *M. chacoense* are not included in the tree.

(ii) The genus *Sinorhizobium*

Chen *et al.* (1988) first proposed the genus *Sinorhizobium* arguing that *R. fredii* and other related soybean isolates were phenotypically too different from other rhizobia. A later study by de Lajudie *et al.* (1994) on isolates obtained from *Acacia* and *Sesbania* species, revealed that on the basis of 16S rDNA gene sequences, the isolates all grouped within the *R. meliloti-R. fredii* branch. Supported by polyphasic results they proposed a new genus, *Sinorhizobium*, to group these isolates.

S. meliloti

The distinctive nature of the formerly *Rhizobium meliloti* (Jordan, 1984) from other members in the genus led to the reclassification of this species as *S. meliloti*. Making use of techniques such as multilocus enzyme electrophoresis (MLEE) and RFLP analysis of the rRNA operons, Eardly *et al.* (1990) could distinguish between two subgroups within the species. The evident genetic heterogeneity led to the thinking that these might represent two different species. It was also shown that *S. meliloti* could be distinguished from its genus members by electrophoretic protein profiles, DNA-DNA hybridization data and 16S rDNA sequences (de Lajudie *et al.*, 1994). *S. meliloti* is classically described as the symbiotic partner of the three legume genera *Medicago, Melilotus* and *Trigonella* (Rome *et al.*, 1996).

S. medicae

Rome *et al.* (1996) investigated a number of *Medicago* isolates by DNA polymorphism of regions being carried on plasmids or on chromosomes of the isolates. It was clear that the isolates represented two distinct genetic divisions. DNA-DNA hybridization suggested that these two groups could be seen as two different genomic species. One of the groups corresponded to strains of *S. meliloti* whilst the other showed very low homology to any *S. meliloti* strains. The species *S. medicae* was proposed for the latter group (Rome *et al.*, 1996).

S. fredii and *S. xinjiangensis*

Several authors reported the isolation of fast-growing rhizobia from the root-nodules of soybean (*Glycine max*) in China before (Chen *et al.*, 1988; Keyser *et al.*, 1982). Since these fast-growing soybean-nodulating rhizobia resemble physiological

properties similar to that of other species within *Rhizobium* it was initially proposed that these strains represents a new *Rhizobium* species. They were assigned to the species *Rhizobium fredii* (Scholla *et al.*, 1984). Scholla *et al.* (1984) further stated that on the basis of various entities including DNA-DNA hybridization two distinct subgroups were evident within *R. fredii*. This led to the identification of two chemovars, chemovar *fredii* and chemovar *siensis*. The taxonomical status of these isolates remained unsatisfactory for a while. Chen *et al.* (1988) proposed that the new genus *Sinorhizobium* include *R. fredii* being called *S. fredii* comb. nov. and the species *S. xinjiangensis* (Chen *et al.*, 1988). *S. fredii* also encompassed the two chemovars, cv. *fredii* and cv. *siensis* previously proposed for *R. fredii*.

S. saheli and *S. terangae*

The genus *Sinorhizobium*, in its more generally accepted taxonomical sense, was defined when the two new species, **S. saheli** and **S. terangae** (de Lajudie *et al.*, 1994), were described. *S. saheli* strains are capable of nodulating *Sesbania* species occurring in the Sahel area in Africa. They have also been found in association with *Acacia*, *Leucaena* and *Neptunia* species. A numerical taxonomical approach proved them a distinct lineage from other species within the *Sinorhizobium* genus. *S. terangae* is the symbiont of various *Sesbania* and *Acacia* species originating in Senegal, Western Africa. In many instances these isolates share common legume hosts with *S. saheli*.

S. arboris and *S. kostiense.*

The rhizobial symbionts of *Acacia senegal* and *Prosopis chilenses* originating from Kenya and Sudan showed upon classification to belong to *Sinorhizobium* (Nick *et al.*, 1999a). The authors included previous work on pulsed-field gel electrophoresis of restricted total DNA (Haukka and Lindström, 1994) and partial 16S rDNA sequences (Haukka *et al.*, 1996) in their extended analysis. Previous results were complimented with DNA-DNA hybridization, rep-PCR genomic fingerprinting and %G+C content determinations. A small proportion of the strains under investigation corresponded to *S. saheli* whilst others were related to *S. terangae*. The majority of strains grouped into two distinct genotypic and phenotypic groups. Based on the differences, Nick *et al.* (1999b) assigned the two groups to two separate species, *S. arboris* and *S. kostiense.*

S. morelense

The rhizobial isolates associated with *Leucaena leucocephala* represented various genomic groups upon investigation of RFLP analysis of 16S rDNA, multilocus enzyme electrophoresis (MLEE), plasmid electrophoresis and Southern hybridization of *nifH* and *nodDAB* genes (Wang *et al.* 1999c). One of these genomic groups, designated rDNA type 11, was closely related to *R. giardinii*. *R. giardinii* represents a distantly related phylogenetic branch to other *Rhizobium* species (Amarger *et al.*, 1997). The taxonomical status of the rDNA type 11 group was further investigated making use of 16S rDNA sequencing, DNA-DNA hybridization and phenotypic analysis (Wang *et al.*, 2002). These results revealed that the rDNA type 11 group showed higher phylogenetic relationship to the genus *Sinorhizobium* and *Ensifer adhaerens* than to *R. giardinii* and subsequently led to the proposal of the new species *S. morelense* (Wang *et al.*, 2002).

(iii)   The genus *Mesorhizobium*

Most of the species within the genus were former members of the *Rhizobium* genus. However, properties such as the position of their flagella, the location of symbiotic genes, 16S rDNA phylogeny and DNA homology clearly separated them from the other fast-growing rhizobia. The genus *Mesorhizobium* reflects that some members of the genus have growth rates slower than that of *Sinorhizobium* and *Rhizobium* but faster than that of *Bradyrhizobium*.

M. loti

Upon investigation of the rhizobial symbionts associated with various *Lotus* species Jarvis *et al.* (1982) showed that these strains resembled a widely divergent group of organisms based on various traits such as bacteriod ultrastructure, internal antigens, extracellular polysaccharide composition, SDS-PAGE analysis of whole cell protein extracts, growth rate, etc.. Several of these criteria indicated that these fast-growing isolates were clearly distinguishable from other *Rhizobium* species, and they were assigned to *R. loti*. 16S rDNA sequence similarities between *R. loti* and other *Rhizobium* and *Agrobacterium* species are around 93.5% (Jarvis *et al.*, 1997), supporting the proposal that *R. loti* and related rhizobia should be assigned to a

separate genus. The genus *Mesorhizobium* was then proposed encompassing the species *R. loti* being referred to as *M. loti.*

M. huakuii

Being a very large legume genus, *Astragalus* contains between 1,500 to 2,000 different species, with a few agriculturally important species. Analysis of *A. sinicis* isolates originating from China revealed a distinct DNA homology group (Chen *et al.*, 1991). Similarities of 16S rDNA sequences indicated that these isolates belonged to the species *M. loti.* These isolates, however, carried their symbiotic genes on plasmids and not on their chromosomes as in the case of the other *Mesorhizobium* species. These isolates were identified as a new species, *M. huakuii* (Chen *et al.*, 1991).

M. mediterraneum

The exact taxonomical placement of chickpea rhizobia was in dispute for a long time. While some scientists claimed them to be a unique group of organisms on the basis of host-specificity, serological and antigenic traits and polymorphism of the nitrogenase genes; others argued that depending on their generation time, these isolates should either be classified as *Rhizobium loti* or *Bradyrhizobium* sp. (Jarvis *et al.*, 1982). In an attempt to clarify this issue Nour *et al.* (1995), performed a genomic and phenotypic investigation on chickpea isolates. The authors reported that regardless of the generation time, all isolates grouped within the *Rhizobium* genus. It was shown that five distinct genomic species were predominant, one of which showed no close relatedness to any other *Rhizobium* species. This specific genomic species were named *R. mediterraneum.* Being closely related to the former *R. loti* (now *M. loti*) these species was transferred to *Mesorhizobium*, and *M. mediterraneum* was proposed (Jarvis *et al.*, 1997).

M. tianshanensis

Making use of numerical taxonomy, Chen *et al.* (1995) analyzed a number of strains isolated from various leguminous species growing in the northwestern region of the People's Republic of China. The results revealed that a large number of the faster-growing isolates obtained from common legume species corresponded to the *Rhizobium* genus. On the other hand a significant number of the isolates seemed to be

a unique group with members not showing relatedness to either the *Rhizobium* or *Bradyrhizobium* genera. These findings led to the isolation of more strains from the same host species. Upon DNA base composition analysis, DNA-DNA hybridization, 16S rDNA sequencing, megaplasmid profiles and cross-inoculation analysis it became apparent that these isolates should be considered a new species. They were assigned to the species *Rhizobium tianshanensis* (Chen *et al.*, 1995). On the basis of extensive analysis Jarvis *et al.* (1997) proposed *R. tianshanensis* to be transferred to the *Mesorhizobium* genus, naming it *M. tianshanensis*.

M. plurifarium

Electrophoretic patterns of whole cellular proteins, auxanographic tests, rRNA-DNA hybridization, 16S rDNA sequencing, DNA base composition and DNA-DNA hybridization was done on a collection of tropical rhizobia, isolated from the root nodules of *Acacia* species in Senegal (de Lajudie *et al*, 1994). From the results, it was clear that these isolates constituted two defined groups, one being *S. terangae*, whilst the identity of the others were referred to as the gel electrophoretic cluster U, the latter group being closely related to the former *R. huakuii* (now *M.* huakuii). A more in depth report on the investigation of the taxonomy of electrophoretic cluster U (de Lajudie *et al.*, 1998a), using additional techniques such as REP-PCR, 16S rDNA sequencing and DNA-DNA hybridization led to the description of the new species *M. plurifarium*.

M. amorphae

The legume shrub, *Amorpha fruticosa*, native to south-eastern and mid-western United States has various agricultural applications including soil erosion control, wind breakage, green manure, biological control, etc. (Wang *et al.*, 1999b). Since these plants are being introduced to many new soils in eastern countries, it was decided to investigate the rhizobial symbionts of *A. fruticosa* (Wang *et al.*, 1999b). A polyphasic approach indicated a unique group based on their phylogenetic position, subsequently being named *M. amorphae*.

M. chacoense

A new species within the *Mesorhizobium* genus was described after determining the diversity of rhizobia associated with *Prosopis chilensis* occurring in diverse

geographical regions in central Argentina. Staircase electrophoresis analysis (SCE) of low-molecular-weight RNA, 16S rDNA sequencing, total cellular protein analysis, DNA base composition and DNA-DNA hybridization coupled with various biochemical tests led to the description of the species *M. chacoense* (Velázquez *et al.*, 2001).

(iv)   The genus *Allorhizobium*

A. undicola

The water-associated annual legume *Neptunia natans* is indigenous to waterlogged areas in Senegal. Isolates obtained from *N. natans* proved to nodulate common legumes used in cross-inoculation studies ineffectively. Furthermore *N. natans* isolates were originally reported as being fast-growing rhizobia (Dreyfus *et al.*, 1984). A polyphasic approach aimed at the classification of these isolates distinguished them as a new genus *Allorhizobium*, containing only a single species (monospecific), *A. undicola* (de Lajudie *et al.*, 1998b). Phylogenetically the closest neighbour to *A. undicola* is *Agrobacterium vitis*.

(v)   The genus *Agrobacterium*

Originally, *Agrobacterium* species were defined in terms of their pathogenic effects. Those strains responsible for crown gall were placed in *A. tumefaciens* and those causing hairy-root (rhizogenic) reactions in *A. rhizogenes*. Strains causing cane gall on *Rubus* spp. encompassed *A. rubi* whilst the non-pathogens constituted the species *A. radiobacter*. Phenotypic and genetic data, however, indicated that phytopathogenic effects might be insufficient for classification due to its plasmid-mediated nature. Taxonomic studies on *Agrobacterium* divided the genus into two major groups on the basis of biochemical tests that did not correspond to the phytopathogenic effect (Holmes and Roberts, 1981). This became evident since *A. tumefaciens* and *A. radiobacter* could not be differentiated except for the presence or absence of tumor inducing (Ti) plasmids. As a result it was more desirable to base nomenclature for the agrobacteria on chromosomal relatedness rather then plasmid-encoded characters. This would not be possible without posing some major problems. For instance, based on phylogenetic identity, *A. rhizogenes* is only distantly related to the other members

of *Agrobacterium*, but shows a notable high correspondence to *Rhizobium tropici*. It was subsequently suggested that the latter two isolates might be considered two different biovars of the same species, where plasmid-encoded characters would differentiate between them (Young, 1996).

The investigation of various *Agrobacterium* species on the basis of numerical taxonomy (Keane *et al.*, 1970; Holmes *et al.*, 1981; etc.) divided the genus into three genetically and phenotypically distinct clusters, excluding *A. rubi*. These clusters represented the already identified biovars or biotypes within the genus with each biovar/biotype having a separate species status.

A. tumefaciens and *A. radiobacter*

Biotype 1 represents all non-pathogenic species responsible for crown gall tumors and was classified as *A. tumefaciens* (Smith and Townsend, 1907). The species could be differentiated from the other members of the genus on the basis of phenotypic characterization (Holmes and Roberts, 1981), low DNA-DNA hybridization values and comparison of 16S rDNA sequences (Sawada *et al.*, 1993; Willems and Collins, 1993). The high DNA homology that exists between *A. tumefaciens* and *A. radiobacter* (80%-87%) suggests that these two species encompass a single species. Since the only difference between the two species is their plasmid content, biotype 1 is now circumscribed to include the non-pathogenic *A. radiobacter*.

A. rhizogenes

Biovar type 2 strains are assigned to *A. rhizogenes*, causing abnormal root growth on various plants. This particular species' taxonomical placement remains questionable as DNA homology values of between 28% and 47% are obtained compared with other members of the genus, and only 94% 16S rDNA sequence homology.

A. rubi and *A. vitis*

A. rubi were originally isolated from the cane galls of *Rubus* species. The species could clearly be differentiated from *A. tumefaciens* on the basis of physiology and pathogenecity. The type species shows low DNA–DNA homology values to the other species within the genus (Kersters and De Ley, 1984; Ophell and Kerr, 1990).

In a numerical study investigating grapevine isolates (Holmes and Roberts, 1981) it became apparent that *A. rubi* type strain grouped with various grapevine isolates clearly distinct from biovars 1 and 2. Upon DNA homology analysis, phenotypic tests and serological reaction of grapevine isolates, Ophell and Kerr, (1990), proposed a new species, *A. vitis*, to group all biovar 3 isolates.

A. larrymoorei

The isolation of *Agrobacterium* species from tumors on weeping fig trees (*Ficus benjamina* L.) and subsequent analysis of their differential oxidation of carbon substrates and fatty acid content proved them clearly different from the previously described *A. tumefaciens*, *A. rhizogenes*, *A. vitis* and *A. rubi*. The production of three unusual opines was also observed (Bouzar *et al.*, 1995). Phylogenetic data based on the similarities of 16S rDNA sequences with other agrobacteria suggested sufficient difference for the proposal of a new species. Bouzar and Jones (2001) proposed the species *A. larrymoorei* based on their previous data combined with DNA relatedness to other members of the genus.

(vi)   The genus *Azorhizobium*

The monospecific genus *Azorhizobium*, contains the species *A. caulinodans*, created for the rhizobia isolated from the stem nodules of the legume *Sesbania rostrata* (Dreyfus *et al.*, 1988). These bacteria clearly belonged to a separate genus after numerical analysis of phenotypic characters, protein comparisons, DNA-DNA and DNA-rRNA hybridizations, separated them from the two known genera, *Rhizobium* and *Bradyrhizobium*, at the time (Dreyfus *et al.*, 1988). Compared to the rest of the *Rhizobiaceae* novel features exhibited by the *Azorhizobium* genus includes *in vitro* nitrogen fixation and assimilation of growth under very low oxygen partial tension of 3% (Zakhia and de Lajudie, 2001). Phylogenetically, the genus shows very high relatedness to species of the genera *Xanthobacter* and *Aquabacter*, and their inclusion in the single genus *Xanthobacter* has been under investigation but not proposed due to their phenotypic diversity (Raincy and Wiegel, 1996).

(vii)   The genus *Bradyrhizobium*

For a long time ***B. japonicum*** was the only recognized species of this genus, which also contained all the slower-growing rhizobia species including all soybean symbionts.

Bradyrhizobium elkanii

Comparative DNA-DNA relatedness of soybean isolates showed that heterogeneity was evident within this group of rhizobia, and that three DNA-DNA homology groups (I, Ia and II) could be identified (Hollis *et al.,* 1981). Isolates representing Group II showed only 30% homology to the type strain *B. japonicum* and it was suggested that these organisms might have a separate species status. For Group II the results of a numerical study using tools such as RFLP analysis, fatty acid and antibiotic resistance profiles, extracellular polysaccharide (EPS) and cytochrome composition and 16S rDNA data, Kuykendall *et al.* (1992) proposed a new species, *Bradyrhizobium elkanii.*

Bradyrhizobium liaoningense

Isolates obtained from the root nodules of soybeans of various provinces of the People's Republic of China were classified as extra slow-growing (ESG) isolates with a generation time between 16 and 24 hours (Xu *et al.,* 1995). Based on numerical taxonomy analysis, %G+C content, DNA-DNA hybridization, partial 16S rDNA sequencing and nitrogen and carbon content of cell components, these ESG isolates were proposed as *Bradyrhizobium liaoningense* (Xu *et al.,* 1995).

(viii)   The genus *Phyllobacterium* (Knösel, 1984)

This genus consists of the two species *P. myrsinacearum* and *P. rubiacearum.* The species are responsible for hypertrophies in plants and being pathogenic strains are also included in the *Rhizobiaceae.* Separate species status for these two strains are questionable since their 16S rDNA sequences are similar (Yanagi and Yamasoto, 1993) and the absence of DNA homology data makes it difficult to define their taxonomic status. The genus shows high homology to the genus *Mesorhizobium.*

2.5 NOVEL BACTERIA CAPABLE OF SYMBIOSIS AND BIOLOGICAL NITROGEN FIXATION

2.5.1 Introduction

The discovery of organisms, other than those belonging to the *Rhizobiaceae*, capable of forming successful symbiosis with their legume hosts is a quite recent event compared with the very long time that this process has been studied. All the rhizobia described so far belonged to the α-subclass of the Proteobacteria, forming three distinct phylogenetic branches. Various bacteria in the other proteobacterial subclasses have been known to be capable of nitrogen fixation, where it was generally recognized as a common feature. This should not be surprising since the transfer of genes responsible for nitrogen fixation is a commonly occurring event. However, the transfer of genes essential for the rhizobial type symbiosis, for instance common nodulation genes, is a very uncommon phenomenon for other proteobacterial subclasses. Recently the three main branches within the rhizobia was supplemented with a fourth branch, containing the non-pigmented methylotrophic isolate *Methylobacterium nodulans* (Sy *et al.*, 2001). More surprisingly was the discovery of the β-subclass proteobacterial genus, *Burkholderia*, found in a rhizobial type association with *Aspalathus* and *Machaerium* species (Moulin *et al.*, 2001).

2.5.2 Novel bacterial species exhibiting rhizobial type symbiosis

(i)   *The genus Methylobacterium*

The genus *Methylobacterium* is composed of a variety of pink-pigmented bacteria capable of growth on one-carbon sources such as formate and methanol as sole carbon source. In a study concerning amine-utilizing bacteria, den Dooren de Jong (1927) described pink, methylamine utilizing species, *Protaminobacter rubrum,* which De Vries and Derx (1953) later found to be very similar to bacterial isolates obtained from leaf nodules and leaf surfaces.

Upon investigation of the rhizobial symbionts of various *Crotalaria* species, Sy *et al.* (2001) revealed that two very distinct groups of rhizobia were evident, the one being broad-host-range *Bradyrhizobium* species, and the other of unknown taxonomical status. Furthermore, these authors showed that the rhizobial symbionts of *Crotalaria* species were capable of facultative utilization of methanol as sole carbon source. This feature is unique for members of known rhizobial species. 16S rDNA sequence identity of the isolates assigned them to the *Methylobacterium* genus. Nodulation ability coupled with the detection of *Nod* A gene confirmed the nodulation capability of these isolates. These methylotrophic "rhizobia" led to the creation of the new species: ***Methylobacterium nodulans***. In contrast to the highly red pigmented members of the genus, *Methylobacterium nodulans* appeared to be non-pigmented.

A so-called "red strain of rhizobium" isolated from root nodules of *Lotononis bainesii* in South Africa was described by Norris (1958). The supposition that this strain simply represented a pigmented slower-growing rhizobial strain led to no further characterization of the isolates. Jaftha *et al.* (2002) determined the identity of similar isolates associated with *Lotononis bainesii* in South Africa. 16S rDNA sequencing, partial *nif*H sequencing and substrate utilization patterns showed these isolates to be closely related to *Methylobacterium nodulans*.

(ii) ***The genus Burkholderia***

The genus *Burkholderia* comprises 19 species, some being common soil and rhizosphere inhabitants whilst others are plant and human pathogens. Diazotrophic traits within the genus is well documented (Baldani *et al.*, 2000; Santos *et al.*, 2001; Cruz *et al.*, 2001; etc.).

A recent report (Moulin *et al.*, 2001) argued that the root nodule bacteria associated with *Aspalathus* and *Machaerium* species are very distantly related to any known rhizobia. The phylogenetic identity of one particular strain (STM678), making use of the 16S rDNA sequence homology, indicated that the strain did not correspond to any of the four rhizobial branches, and more interestingly neither to any members of the α-subclass of the *Proteobacteria*. The closest phylogenetic neighbour to strain

STM678 was the β-subclass proteobacterium *Burkholderia kururiensis* (96,9%), followed by two other *Burkholderia* species. These results were supported when partial sequencing of the 23S rDNA gene and the *dnaK* gene, encoding a chaperon heat shock protein, showed similar results. Verification of the nodulating capability of strain STM678 was done by reintroducing STM678 in the roots of a suitable host plant. The host plant (*Macroptilium atropurpureum*) indeed produced nodules, and STM678 was identified as the symbiont after re-isolation from these nodules. Due to the fact that *M. atropurpureum* was not the original host from which STM678 was isolated the nodules formed were ineffective. The gene *nifH*, encoding dinitrogenase reductase, was detected, indicating that STM678 would probably be capable of forming effective nodules on its appropriate host species. Genes essential for successful symbiosis, involved in signal exchange and initiation of nodulation (*Nod* genes), were found to be present within strain STM678.

(iii) *The genus Ralstonia*

The genus *Ralstonia* was proposed by Yabuuchi *et al.* (1995) to incorporate the previous species, *Alcaligenes eutrophus, Pseudomonas solanacearum* and *Pseudomonas pickettii*. Today various species has been identified within the genus, mostly isolated from environmental and human clinical samples (Chen *et al.*, 2001). Incorporating species that are opportunistic human pathogens as well as species of important biotechnology implications makes the *Ralstonia* genus an unusual genus.

Upon a polyphasic taxonomical approach of eight *Mimosa* root nodule isolates, Chen and co-workers revealed that the isolates corresponded to members of the *Ralstonia* genus (Chen *et al.*, 2001). These isolates were shown to effectively nodulate *Mimosa* species and to possess *nif* genes. This was the first report where members of the β-subclass of the Proteobacteria were shown as nodulating symbionts of leguminous hosts. Subsequently Chen *et al.* (2001) created the new species *R. taiwanensis*.

2.6 THE PLANT GENUS LOTONONIS

2.6.1 Introduction

Members of the *Fabaceae* are the third largest family within the flowering plants. Compared with the other large families within this group they are generally found in a diverse range of environments. Currently the family encompasses approximately 650 genera and nearly 20,000 species. Members of the *Fabaceae* range from forest giants to tiny ephemerals and show great diversity in their methods of acquiring the essentials of growth and in their modes of reproduction and defense (Polhill *et al.;* 1981). Although the family extends in all terrestrial habitats from the equator to the edges of dry and cold deserts, it has much of its diversity centered in areas of varied topography with seasonal climates. The versatility of the *Fabaceae* emphasizes the considerable ecological, agricultural and scientific importance of the family.

The genus *Lotononis* (DC.) Eckl. & Zeyh, (tribe: *Crotalarieae*), is the third largest genus of the *Fabaceae* in southern Africa (van Wyk, 1987) and comprises an assemblage of 150 species. The habit of *Lotononis* shows extreme diversity progressing from woody groups to presumably more derived herbaceous plants as a result of adaptation to diverse climatological and geographical habitats (Figure 1.2). The majority of species, however, are herbaceous perennials with flowering shoots developing from a persistent woody base (van Wyk, 1991).

2.6.2 Geographical distribution of *Lotononis*

The approximate geographical distribution of the genus *Lotononis* is given in Fig. 1.3. The distribution includes the whole of the African continent and the adjoining extreme southern parts of the Mediterranean region. Six species occur in the northern part of the distribution range, five species in the central part and 144 species in the southern part.

Most of the species are restricted to an endemic distribution and that only a few (mostly annuals) are widely distributed. The geographical distribution of *Lotononis*

indicates that in the case of annual habits an adaptation to erratic or seasonal rainfall patterns developed (van Wyk, 1991). For the genus as a whole, 11 centres of endemism are identified, as shown in Figure 1.3.

The remarkable species density of *Lotononis* in southern Africa led to the identification of so-called 'centres of richness': the south-western Cape, the north-western Cape, the eastern Cape and the Drakensberg (van Wyk, 1991). The south-western Cape is, however, substantially richer with a sudden decrease in richness towards the dry central interior. A total of 140 *Lotononis* species occur in the flora of South Africa. The diversity of *Lotononis* in South Africa is not restricted to the Cape region as in typical Cape genera such as *Aspalathus* and *Muraltia*, but the eastern afromontane areas and the north-western Cape make a significant contribution. The diversity pattern supports the concept of southern Africa as a survival centre for mesic and temperate floristic elements that have subsequently evolved in relative isolation (van Wyk, 1991).
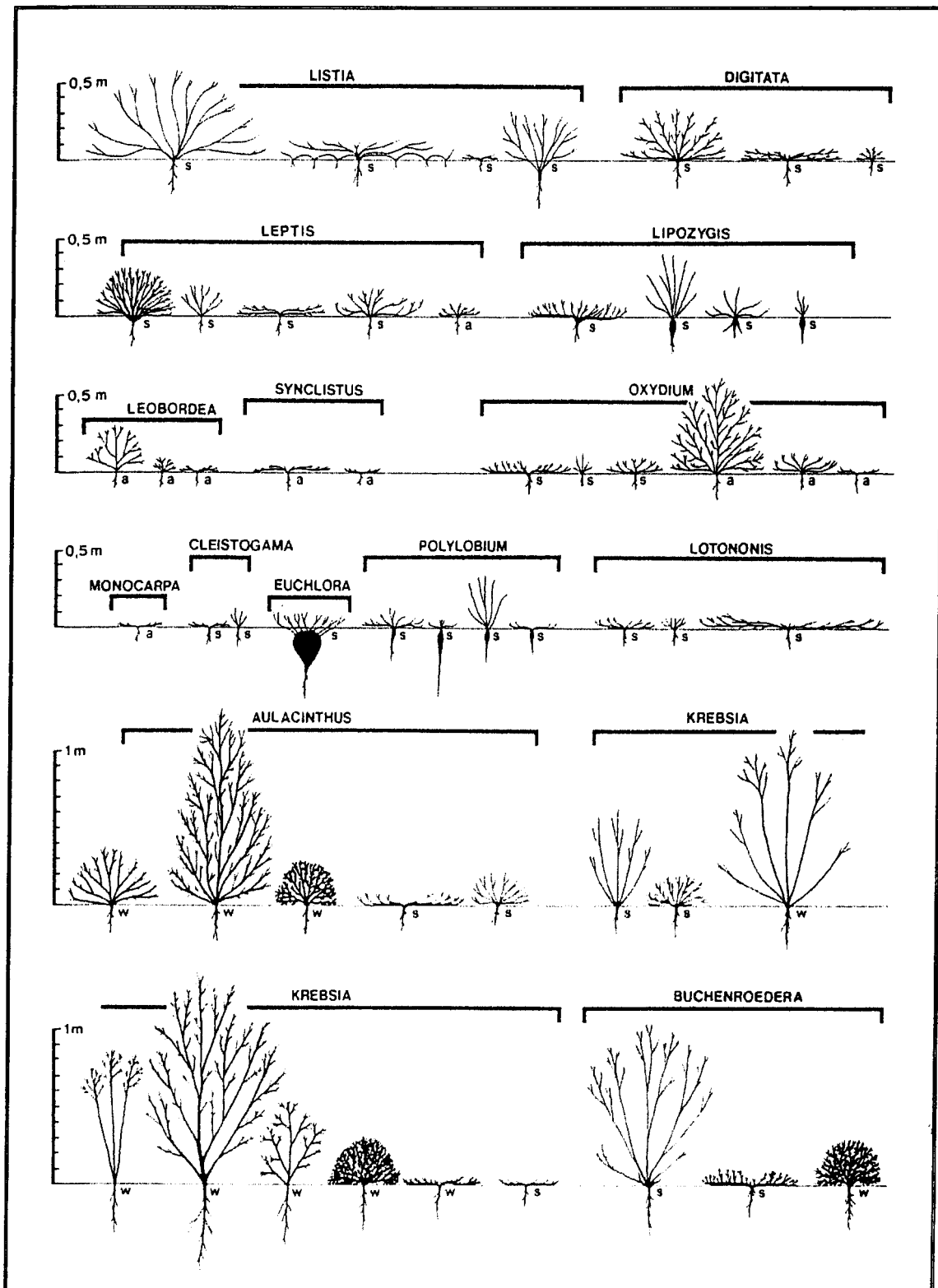
Figure 1.2. Schematic summary of variation in the habit of the sections of *Lotononis*. a, annuals; s, suffrutescent perennials; w, woody shrubs. Different lengths of plants are indicated on vertical scale bars. (van Wyk, 1991)
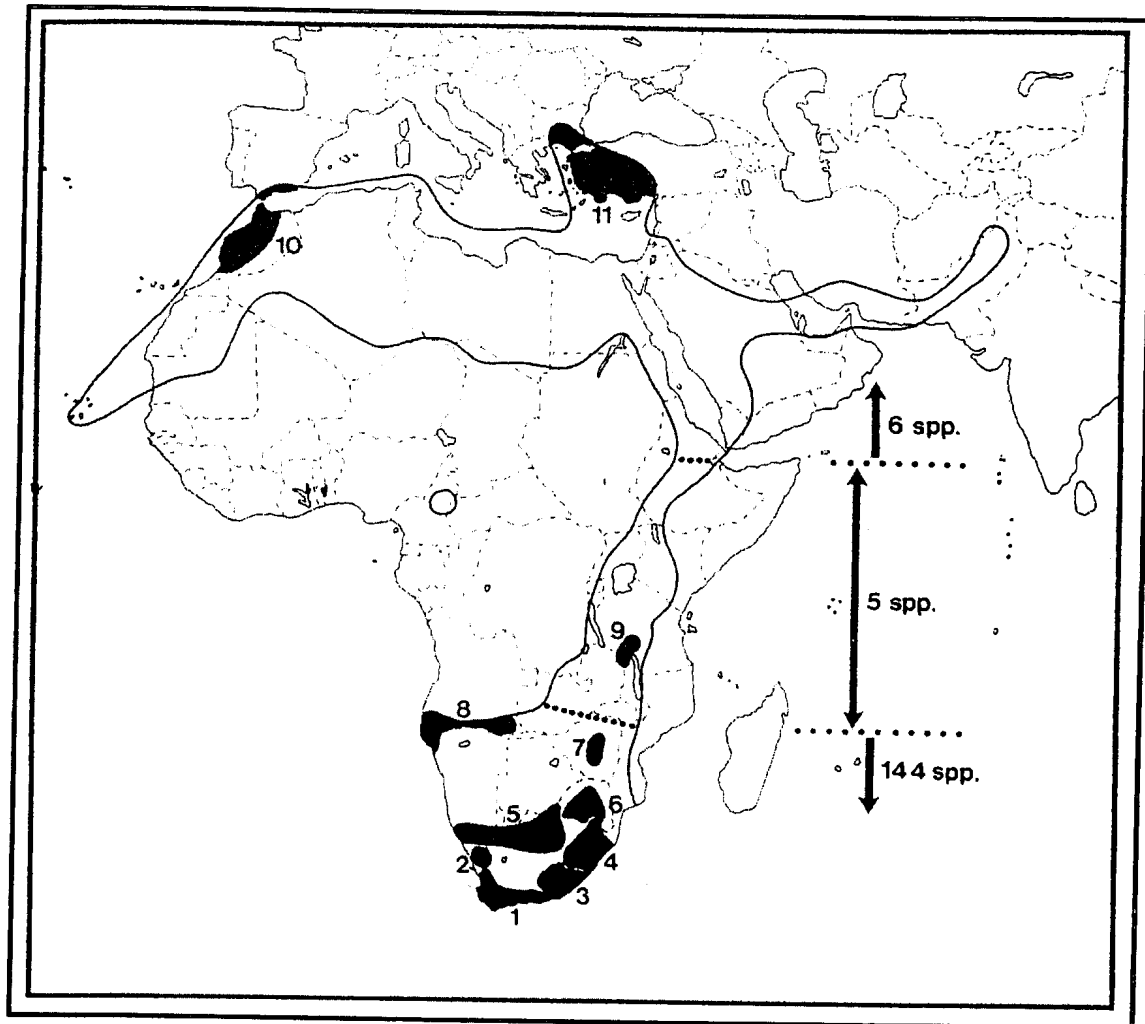
Figure 1.3 The approximate geographical distribution of the genus *Lotononis*. Endemic centres are numbered 1 to 11. 1, Cape region; 2, Namaqualand; 3, eastern Cape; 4, Natal-Drakensberg area; 5, southern Namibia and Griqualand-West; 6, Transvaal; 7, central Zimbabwe; 8, north-western Namibia and southern Angola; 9, Nyika Plateau; 10, Morocco and southern Spain; 11, Turkey and south-eastern Bulgaria . (van Wyk, 1991)

2.6.3   Taxonomic history of the genus *Lotononis*

The early taxonomic history of the species presently included in the genus *Lotononis* reflects the uncertainty that existed about generic concepts. Species have been described under various generic names, involving several genera of the tribe *Crotalarieae* and even genera from other tribes (van Wyk, 1991). Marked similarities exist between the genera *Lebeckia, Buchenroedera, Crotalaria, Pearsonia* and *Lotononis*. The critical question was whether these similarities indicate evolutionary

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relationship or merely reflect similar adaptations in unrelated groups (van Wyk, 1986). The classification and nomenclature reached stability only when Bentham (1843) combined a number of smaller genera into a much-enlarged generic concept. In 1991 van Wyk reviewed the taxonomical status of the genus making use of all relevant generic and infrageneric relationships. Parameters such as vegetative morphology, reproductive morphology, chromosome cytology and chemical characters were taken into account. Subsequently it led to the arrangement of all 150 species into 15 sections and 22 subsections (Table 1.2.) [Appendix A1].

2.6.4 Relevance of the genus *Lotononis* towards biological nitrogen fixation and agriculture

Scientists in the pasture and agricultural sciences are increasingly aiming at addressing the lack of suitable pasture legumes which will maintain itself in association with grasses under moderate to heavy grazing conditions in the tropics. *Lotononis* spp. have been studied and evaluated over the past 50 years as an alternative leguminous component for tropical grazing feeds. One species, *Lotononis bainesii* showed to be a particularly promising species for commercial use. Cameron (1985) described *Lotononis bainesii* (Lotononis) as an "enigmatic legume", since its contribution to the pasture sward fluctuated widely (Fujita and Humphreys, 1992). In addition to *L. bainesii*, other species of *Lotononis* such as *L. diviricata*, *L. tenella* and *L. laxa* also have potential value as grazing plants due to their well adapted nature to arid regions (Shearing, 1994). Members of the genus are tolerant to acidic soils and heavy grazing and show a high tolerance towards cold temperatures and frost. The high crude protein content (18-25%) of the genus makes it in particular an interesting alternative for tropical grazing feeds (Thro, 1987).

Table 1.2.   **(continued)**

Section	Number of species/species characters[*]	Geographical habitat
14. Krebsia	12, Perennial shrubs or shrublets, procumbent or erect woody branches, trifoliated leaves, flowers blue	Coastal and mountainous
15. Buchenroedera	11, Perennial shrubs or shrublets, procumbent or erect woody branches or suffrutescent shrubs emerging from woody base, 2- to several-flowered, flowers small and blue	Coastal and mountainous

[*] Species characters as described by van Wyk, 1991.

CHAPTER 3

ABSTRACT:

Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of whole cell protein extracts was used to investigate the taxonomic relationships among 102 isolates obtained from 32 different species of *Lotononis*.

The isolates and reference strains investigated were grouped into three major sections that could be further divided into 25 clusters. Most of these clusters were related to representatives of the rhizobial genera. Furthermore, in most cases, isolates obtained from the same plant species, grouped into various clusters. It is reasonable to conclude that host specificity in the symbiotic interaction is less applicable for some species of *Lotononis*. When considering the geographical origins of the isolates, their diverse nature was clearly illustrated. Most of the 25 clusters showed that isolates from similar geographical regions were evenly distributed throughout the dendrogram. Five prominent clusters, however, contained only isolates obtained from arid areas.

It also became apparent that *Methylobacterium nodulans* and members of the β-subclass of the *Proteobacteria* (*Burkholderia*) are associated with the root nodules of some *Lotononis* species. It was subsequently shown that root-nodule bacteria associated with *Lotononis* species are not restricted to a particular rhizobial genus, but that diversity is evident.

Keywords: *Lotononis*, Rhizobia, *Burkholderia*, *Methylobacterium*, SDS-PAGE

CHAPTER 3

CHARACTERIZATION OF INDIGENOUS RHIZOBIAL ISOLATES ASSOCIATED WITH *LOTONONIS* SPECIES IN SOUTH AFRICA AS DETERMINED BY SODIUM DODECYL-SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

3.1 INTRODUCTION

The first systematic investigation into the identity and taxonomy of root nodule bacteria associated with legumes in South Africa was done by Dagutat (1995). Recently it became apparent, that bacteria other than those belonging to the rhizobial family, the *Rhizobiaceae*, are capable of nodulating leguminous plants. Unlike rhizobia, belonging to the α-subclass of the *Proteobacteria*, bacterial genera belonging to the β-subclass such as *Burkholderia* and *Ralstonia* have been identified as root nodule-associated bacteria capable of forming successful symbiosis with their leguminous hosts (Moulin *et al.*, 2001; Chen *et al.*, 2001). The isolation of a methylotrophic bacterium associated with the root nodules of a *Crotalaria* sp., subsequently led to the description of *Methylobacterium nodulans*, a novel symbiotic nitrogen fixer (Sy *et al.*, 2001).

The genus *Lotononis* occurs throughout South Africa and shows high taxonomical relatedness to the genus *Crotalaria*, belonging to the same tribe, *Crotalarieae*, within the family *Fabaceae*. The diverse habitat and climatological conditions of *Lotononis* species might possibly be reflected in the diversity of the bacteria associated with their root nodules. The aim of this study was to investigate the diversity and taxonomy of root nodule-associated bacteria of *Lotononis* spp. in South Africa. *Lotononis* plants were collected from various geographical regions in South Africa and bacteria isolated from root nodules. Initial screening of putative isolates was done by SDS-PAGE analysis of their whole cell proteins. Type and/or reference strains of the genera *Agrobacterium*, *Azorhizobium*, *Bradyrhizobium*, *Burkholderia*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium* and *Sinorhizobium* were included in the SDS-PAGE analysis.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of root nodule-bacteria from nodulated *Lotononis* spp. growing in natural environments

Eighty-two specimens of *Lotononis* plants representing thirty-two different species and twelve of the fifteen sections within the genus were collected from their natural environments in South Africa. Where possible at least two specimens were collected at each locality. In some instances other genera were mistaken for *Lotononis* and collected, these were nevertheless included in the analysis. Plant specimens were donated to the H.G.W.J. Scheickerdt Herbarium (PRU) at the University of Pretoria, Pretoria, South Africa. Figure 3.1 indicates the range of geographical regions from which root nodule-bacteria were collected. Table 3.1 lists all the isolates investigated in this study.

Root nodules were excised from each specimen and surface sterilized with 50 % (v/v) NaOCl:H_2O for 7 min. and washed repeatedly with sterilized distilled water. Root nodules were squashed individually in 300 µl sterile distilled water. Loopsful of these suspensions were streaked out on yeast extract mannitol (YM) agar plates, containing 1% (m/v) mannitol, 0.5% (m/v) K_2HPO_4, 0.02% (m/v) $MgSO_4.7H_2O$, 0.01% (m/v) NaCl, 0.04% (m/v) yeast extract and 1.5% (m/v) bacteriological agar supplemented with Congo red (Allen, 1959). Plates were incubated for 5-7 days at 28 °C and examined for growth. Cultures were purified by at least three consecutive streakings of single colonies on the same medium. To confirm colony purity, microscopy was done on living and Gram-stained cells.

3.2.2. Maintenance and preservation of cultures

Purified cultures were maintained on YM agar slants at 4°C and long-term storage was done in glycerol. Cultures were allowed to grow in yeast extract mannitol broth (YMB) for 5-7 d at 28 °C with agitation. The resultant suspensions were mixed 1:1 with 50% (v/v) glycerol in sterile cryotubes and copies stored at –20°C and –70°C.

Table 3.1   **Isolate numbers, *Lotononis* host species and their geographical origin**

Isolate	Host plant	Geographical origin	Herbarium reference no[*]
D 1	*Lotononis mucronata*	Natal; Cathederal Peak district, 28° 29° CC	PRU 091486
D 3	*Lotononis mucronata*	Natal; Cathederal Peak district, 28° 29° CC	PRU 091486
D 4	*Lotononis mucronata*	Natal; Cathederal Peak district, 28° 29° CC	PRU 091486
M 2	*Lotononis eriantha*	Mpumulanga; Hendrina, 26° 29° BA	PRU 091488
M 3.3	*Lotononis calycina*	Mpumulanga; Hendrina, 26° 29° BA	PRU 091489
M 4	*Lotononis lanceolata*	Mpumulanga; Badplaas, 25° 30° DC	PRU 091490
M4.1	*Lotononis lanceolata*	Mpumulanga; Badplaas, 25° 30° DC	PRU 091490
M5	*Lotononis carinata*	Mpumulanga, Hendrina district	NA
M6	*Lotononis carinata*	Mpumalanga, Hendrina, 26° 29° BA	NA
M7	*Lotononis carinata*	Mpumalanga, Hendrina district	NA
M7.1	*Lotononis carinata*	Mpumalanga, Hendrina district	NA
M7.2	*Lotononis carinata*	Mpumalanga, Hendrina district	NA
M7.3	*Lotononis carinata*	Mpumalanga, Hendrina district	NA
M8	*Lotononis carinata*	Mpumalanga, Hendrina district	NA
NK 2	*Lotononis platycarpa*	Northern Cape, Springbok, Namaqualand	NA
NK 3	*Lotononis falcata*	Northern Cape, Springbok, Namaqualand	NA
NK4.1	*Lotononis crumaniana*	Northern Cape; Lykso district, 27° 24° AC	PRU 091459
NK 4.2	*Lotononis crumaniana*	Northern Cape; Lykso district, 27° 24° AC	PRU 091459
NK 6[#]	*Melolobium adenodis*	Northern Cape; Bladgrond district, 28° 19° DD	PRU 091462
NK 8[#]	*Melolobium adenodis*	Northern Cape; Naip Suid Namaqualand, 29° 18° AD	PRU 091457
NK 9	*Lotononis falcata*	Northern Cape; Naip Suid Namaqualand, 29° 18° AD	PRU 091458
NK9.1	*Lotononis falcata*	Northern Cape; Naip Suid Namaqualand, 29° 18° AD	PRU 091458
NK 10	*Lotononis sparsiflora*	Northern Cape; Naip Suid Namaqualand, 29° 18° AD	PRU 091453
NK 11.2	*Lotononis platycarpa*	Northern Cape; Naip Suid Namaqualand, 29° 18° AD	PRU 091454
NK 12	*Lotononis platycarpa*	Northern Cape; Naip Suid Namaqualand, 29° 18° AD	PRU 091454
NK 15.1	*Lotononis falcata*	Northern Cape; Richtersveld, 29° 17° BC	PRU 091451
NK 16	*Lotononis leptoloba*	Northern Cape; Richtersveld, 29° 17° BC	PRU 091452
NK 17	*Lotononis leptoloba*	Northern Cape; Richtersveld, 29° 17° BC	PRU 091461
NK 20	*Lotononis leptoloba*	Northern Cape; Springbok district, 29° 17° BD	PRU 091447
NK 22[#]	*Lebeckia serucea*	Northen Cape, Kamiesberg	NA
NK 23	*Lotononis magnifica*	Northern Cape; Kamieskroon district, 30° 18° BD	PRU 091449

Table 3.1 (continued)

Isolate	Host plant	Geographical origin	Herbarium reference no[a]
NK 25.3[a]	Crotalaria humilis	Northern Cape; Kamieskroon district, 30° 18° AC	PRU 091444
NK 27[a]	Lotononis polycephala	Northern Cape; Kamieskroon district, 30° 18° AC	PRU 091441
NK 27.1	Lotononis polycephala	Northern Cape; Kamieskroon district, 30° 18° AC	PRU 091441
NK 28	Lotononis carnea	Northern Cape; Kamieskroon district, 30° 18° AC	PRU 091442
NK 29	Lotononis quinata	Northern Cape; Kamieskroon district, 30° 18° AC	PRU 091439
NK 30	Lotononis mollis	Northern Cape; Kamieskroon district, 30° 17° BB	PRU 091440
NK 32	Lotononis falcata	Northern Cape; Soebatsfontein Namaqualand, 30° 17° BA	PRU 091438
NK 33[a]	Melolobium humile	Northern Cape; Soebatsfontein Namaqualand, 30° 17° BA	PRU 091437
NK34	Lotononis falcata	Northern Cape; Naip Suid, Namaqualand, 29° 18° AD	PRU 091432
NK 37.3.1	Lotononis digitata	Northern Cape; Garies district, 30° 17° BD	PRU 091434
NK 37.3.2	Lotononis digitata	Northern Cape; Garies district, 30° 17° BD	PRU 091434
NW 1[a]	Melolobium microphyllum	North West; Setlagole district, 26° 25° CB	PRU 091485
T 7.1	Lotononis listii	Northern Province; Vaalwater district, 24° 28° AC	PRU 091482
T 9.2	Lotononis calycina	Gauteng; Johannesburg district, 26° 28° AA	PRU 091483
T 10.1	Lotononis calycina	Gauteng; Johannesburg district, 26° 28° AA	PRU 091483
T 13	Lotononis mucronata	Gauteng; Johannesburg district, 26° 28° AA	PRU 091484
T15	Lotononis calycina	Gauteng; Johannesburg district, 26° 28° AA	NA
T17.1	Lotononis calycina	Gauteng; Johannesburg district, 26° 28° AA	NA
T17.2	Lotononis calycina	Gauteng; Johannesburg district, 26° 28° AA	NA
V 1	Lotononis decumbens ssp. decumbens	Free State; Lindley district, 27° 27° DD	PRU 091429
V 2	Lotononis decumbens ssp. decumbens	Free State; Lindley district, 27° 27° DD	PRU 091429
V 4	Lotononis decumbens ssp. decumbens	Free State; Lindley district, 27° 27° DD	PRU 091430
V4.3	Lotononis decumbens ssp. decumbens	Free State; Lindley district, 27° 27° DD	PRU 091430
V 5	Lotononis decumbens ssp. decumbens	Free State; Lindley district, 27° 27° DD	PRU 091430
V 6	Lotononis laxa	Free state; Lindley district, 27° 27° DD	PRU 091431
V 7	Lotononis calycina	Free state; Lindley district, 27° 27° DD	PRU 091425
V 8	Lotononis listii	Free state; Lindley district, 27° 27° DD	PRU 091426
V9	Lotononis listii	Free state; Lindley district, 27° 27° DD	PRU 091426

Table 3.1   (continued)

Isolate	Host plant	Geographical origin	Herbarium reference no[#]
V 9.3	*Lotononis listii*	Free state; Lindley district, 27° 27° DD	PRU 091426
V 10	*Lotononis listii*	Free state; Lindley district, 27° 27° DD	PRU 091427
V 11.1	*Lotononis listii*	Free state; Lindley district, 27° 27° DD	PRU 091427
V 11.2	*Lotononis listii*	Free state; Lindley district, 27° 27° DD	PRU 091427
V 12[#]	*Crotalaria sp.*	Free State; Fouriesburg district, 28° 28° CA	NA
V 12.2[#]	*Crotalaria sp.*	Free State; Fouriesburg district, 28° 28° CA	NA
V 13[#]	*Crotalaria sp.*	Free State; Fouriesburg district, 28° 28° CA	NA
V 14	*Lotononis decumbens ssp. decumbens*	Free State; Fouriesburg district, 28° 28° CA	PRU 091428
V 15.1	*Lotononis adpressa ssp. adpressa*	Free State; Fouriesburg district, 28 ° 28° CA	PRU 091422
V 15.2	*Lotononis adpressa ssp. adpressa*	Free State; Fouriesburg district, 28 ° 28° CA	PRU 091422
V 17.2	*Lotononis sericophylla*	Free State, Golden Gate National Park, 28° 28° DA	NA
V 18	*Lotononis laxa*	Free State; Golden Gate National Park, 28° 28° DA	NA
V 19	*Lotononis laxa*	Free State; Golden Gate National Park, 28° 28° DA	PRU 091424
V 23	*Lotononis lotononoides*	Free State, Golden Gate National Park, 28° 28° DA	NA
WK 1	*Lotononis falcata*	Western Cape; Vanrhynsdorp district, 31° 18° BD	PRU 091463
WK 2	*Lotononis maximiliana*	Western Cape; Vanrhynsdorp district, 31° 18° BD	PRU 091464
WK 3	*Lotononis lenticula*	Northern Cape; Soetwater district, 31° 19° AD	PRU 091465
WK 4	*Lotononis leptoloba*	Northern Cape; Soetwater district, 31° 19° AD	PRU 091466
WK 5	*Lotononis parviflora*	Western Cape; Klawer, 31° 18° DC	PRU 091467
WK 6	*Lotononis falcata*	Western Cape; Klawer, 31° 18° DC	PRU 091468
W 7[#]	*Aspalathus ssp.*	Western Cape, Graafwater district	NA
WK 8	*Lotononis oxyptera*	Western Cape; Citrusdal district, 32° 19° CA	PRU 091469
WK 10.2	*Lotononis fastigiata*	Western Cape; Simon's Town district, 34° 18° AB	PRU 091471
WK 11	*Lotononis involcrata*	Western Cape; Worcestor district, 33° 19° DC	PRU 091472
WK 12.2	*Lotononis tenella*	Western Cape; Barrydale district, 33° 20° DC	PRU 091473
WK 13.1	*Lotononis tenella*	Western Cape; Ladismith district, 33° 21° CA	PRU 091474
WK 14	*Lotononis tenella*	Western Cape; Laingsburg district, 33° 20° BB	PRU 091475
WK 15	*Lotononis pungens*	Western Cape; Kromrivier, 31° 23° CC	PRU 091476
WK 16	*Lotononis rabenaviana*	Western Cape; Kromrivier, 31° 23° CC	PRU 091477
WK 17.1	*Lotononis tenella*	Western Cape; Prince Albert district, 33° 22° AA	PRU 091478
WK 19	*Lotononis tenella*	Northern Cape; Colesberg, 30° 25° CA	PRU 091480

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Table 3.1   (continued)

Isolate	Host plant	Geographical origin	Herbarium reference no#
WK19.1	*Lotononis tenella*	Northern Cape; Colesberg, 30° 25° CA	PRU 091480
WK 20#	*Dichilus gracilis*	Northern Cape; Colesberg, 30° 25° CA	PRU 091481
XCT7	*Lotononis bainesii*	Mozambique, Lourenco Marques	NA
XCT8	*Lotononis bainesii*	Mozambique, Lourenco Marques	NA
XCT9	*Lotononis bainesii*	Zimbabwe	NA
XCT10	*Lotononis bainesii*	East London	NA
XCT12	*Lotononis bainesii*	Gauteng, Rietondale research station.	NA
XCT13	*Lotononis bainesii*	Natal	NA
XCT14	*Lotononis bainesii*	Natal	NA
XCT16	*Lotononis bainesii*	Gauteng, Buffelspoort	NA
XCT17	*Lotononis bainesii*	Gauteng, Rietondale research station.	NA

.   Herbarium reference number of plant specimens at the H.G.W.J. Scheickerdt Herbarium (PRU).

Isolates that were obtained from genera that were mistaken for *Lotononis*

NA   Not available or not deposited

Isolate numbers starting with XCT were obtained from an existing culture collection at the Agricultural Research Council, Pretoria, South Africa

3.2.3. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins

(i)   Strains used

Thirty-one reference strains (Table 3.2), obtained from the Laboratorium voor Microbiologie Gent Culture Collection (State University Gent, Belgium) and the United States Department of Agriculture-ARS National Rhizobium Culture Collection (USA) were used in the SDS-PAGE analysis. These reference strains represented various genera within the *Rhizobiaceae*, and the type strains for *Methylobacterium nodulans* and *Burkholderia* sp. STM815*. A total of 102 putative root nodule-associated strains isolated in this study and ten isolates obtained from the Agricultural Research Council (ARC) were included in the SDS-PAGE analysis. The strains received from the ARC were isolated as described in section 3.2.1.

* Type strains for *Burkholderia* and *Methylobacterium* were donated by C. Boivin-Masson, LSTM, UMR 113 IRD/INRA/AGRO-M/CIRAD, 34398 Montpellier Cedex 5, France.
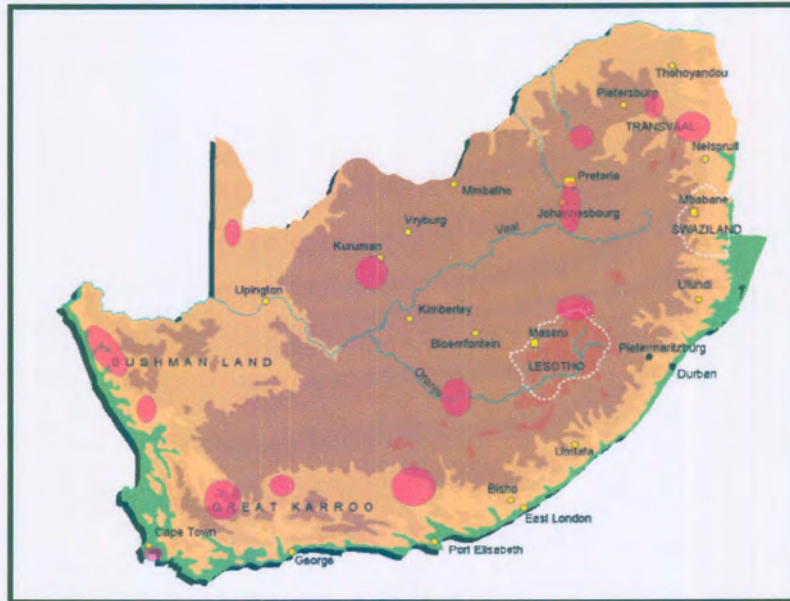
Figure 3.1 **Map of South Africa where the sites from which specimens were collected in this study are indicated by pink dots.**

(ii)   Preparation of whole cell protein extracts

Isolates were incubated in YMB at 28 °C for 5 d with agitation and used to inoculate 50 ml of sterile YMB. These cultures were incubated and grown under the same conditions as described in section 3.2.2. Cells were harvested by centrifugation at 12 000 x g for 10 min. The resultant pellets were washed twice with 0.2 M sodium phosphate buffer (pH 6.88).

Cell pellets were finally resuspended in 1 ml of 0.2 M sodium phosphate buffer (pH 6.88), transferred to Eppendorf tubes and centrifuged (12 000 x g for 10 min). The supernatants were discarded and the pellet sizes determined. According to the pellet size, between 30-400 µl sample treatment buffer (STB) [0.5 M Tris-HCl pH 6.8, 5 % (v/v) 2-β-mercaptoethanol, 10 % (v/v) glycerol], was added and 10 % SDS was added according to the amount of 10% of STB added. The samples were heated to 95 °C and cells disrupted using a Cole-Palmer ultrasonic homogenizer (Series 4710) at 50 % maximum output (40 Watt) for 15 s. Equal volumes of STB and 10% SDS were added, mixed and centrifuged at 12 000 x g for 10 min. The supernatants were transferred to clean Eppendorf tubes and frozen overnight at –20 °C. Samples, containing excess slime was heated to 95 °C for 30 min, cooled to room temperature and centrifuged at 12 000 x g for 10 min. The supernatants were transferred to clean Eppendorf tubes and stored at –20 °C until further use.

(iii)   Polyacrylamide gel electrophoresis

The method of Laemmli (1970), as modified by Kiredjian *et al.* (1986), was used. Gels (1.5 mm thick) consisting of a 5 % stacking gel (0.5 M Tris-HCl pH 6.68, conductivity 31.0 mS/cm) and a 12 % separation gel (1.5 M Tris-HCl pH 8.87, conductivity 16.02 mS/cm) were prepared. Electrophoresis was done in a BioRad Protean II gel apparatus with a constant current of 5 Watt through the stacking gel and 10 Watt through the separation gel. After the completion of electrophoresis Coomassie Brilliant Blue solution [15 % (v/v) of a 2 % (m/v) Coomassie Brilliant Blue R-250 stock solution, 50 % (v/v) methanol] was used to stain gels for 1 h 10 min at room temperature. Gels were destained overnight in a solution containing 25 % (v/v) methanol and 10 % (v/v) acetic acid.

(iv)   Analysis of gels

A Hoefer GS300 Transmittance/Reflectance scanning densitometer (Hoefer Scientific Instruments, San Francisco) was used to scan the electrophoretic patterns of the proteins.

Table 3.2 Reference strains used in the SDS-PAGE analysis

Species	Strain no.[a]	Host plant	Source[*]
Agrobacterium tumefaciens (*Agrobacterium biovar 1*)	LMG 187^T	*Lycopersicon lycopersicum*	Belgium
Azorhizobium caulinodans	LMG 6465^T	*Sesbania rostrata*	Belgium
Bradyrhizobium elkanii	USDA 76^T	*Glycine max*	USA
Bradyrhizobium japonicum	LMG 6138^T	*Glycine max*	Belgium
Burkholderia spp.	STM 815	*Aspalathus* spp.	
Mesorhizobium amorphae	USDA 10001^T	NS	USA
Mesorhizobium ciceri	USDA 3383^T	NS	USA
Mesorhizobium loti	LMG 6125^T	*Lotus corniculatus*	Belgium
	USDA 3471^T	NS	USA
Mesorhizobium mediterraneum	USDA 3392^T	*Cicer arietinum*	USA
Mesorhizobium plurifarium	USDA 3707^T	NS	USA
	LMG 11892	NS	Belgium
Mesorhizobium tianshanense	LMG 18976^T	*Glycyrrhiza pallidiflora*	Belgium
	USDA 3592^T	*Glycyrrhiza pallidiflora*	USA
Methylobacterium nodulans	ORS 2060	*Crotalaria* spp.	
Rhizobium etli bv *phaseoli*	LMG 17827^T	*Phaseolus vulgaris*	Belgium
Rhizobium huautlense	USDA 4900^T	*Sesbania herbacea*	USA
	LMG 18254^T	*Sesbania herbacea*	Belgium
R. leguminosarum bv *trifolii*	LMG 8820^T	*Trifolium pratens*	Belgium
Rhizobium mongolenes	LMG 19141^T	*Medicago ruthenica*	Belgium
	USDA 1844^T	*Medicago ruthenica*	USA
Rhizobium tropici group B	USDA 9030	*Phaseolus vulgaris*	USA
Sinorhizobium arboris	LMG 14919^T	*Prosopis chilensis*	Belgium
Sinorhizobium fredii	LMG 6217^T	*Glycine max*	Belgium
Sinorhizobium kostiense	LMG 19227^T	*Acacia senegal*	Belgium
	USDA 4905	*Acacia senegal*	USA
Sinorhizobium medicae	LMG 18864	*Medicago truncatula*	Belgium
Sinorhizobium meliloti	LMG 6133^T	*Medicago sativa*	Belgium
Sinorhizobium saheli	USDA 4893^T	NS	USA
	LMG 7834	NS	Belgium
Sinorhizobium terangae	USDA 4894^T	NS	USA
Sinorhizobium xinjiangense	LMG 17930	NS	Belguim

[a] LMG, Laboratorium voor Microbiologie Gent Culture Collection; USDA, United States Department of Agriculture-ARS National Rhizobium Culture Collection

[*] Refer to text for detailed description of source   T   Type strain   NS   Not stated

Analysis and comparison of patterns were done using the GelCompare 4.0 computer program (Applied Maths, Kortrijk, Belgium). Densitometric traces were normalized and grouped. The Pearson product moment correlation coefficient (r) between strains was calculated and the unweighted pair group method of arithmetic averages (UPGMA) was used to cluster strains. Whole cell protein extracts of *Psychrobacter immobilis* LMG 1125 was included in each gel (six tracks per gel) to ensure reproducibility. One *P. immobilis* selected in the program, acted as standard and was used to compare the reproducibility of references on successive gels where a correlation (r) of 94% (where r = r x 100%) was considered as reproducible.

(v)   Statistical analysis of SDS-PAGE data

Two dimensional frequency sheets were constructed, revealing the correlation between the sections of *Lotononis* and its distribution throughout the dendrogram as well as the distribution of geographical regions throughout the dendrogram. These results were illustrated graphically.

3.3   RESULTS

3.3.1   Isolation of putative rhizobial strains from *Lotononis* species

In this study more than 100 strains were isolated from the root nodules of thirty-two different *Lotononis* species, representing twelve of the fifteen sections within the *Lotononis* genus. In several instances more than one strain was isolated from the same nodule, for example M7, M7.1 and M7.3 (from a root nodule of *L. carinata*).

3.3.2 SDS-PAGE of whole cell proteins

In this study SDS-PAGE was used for the characterization of a large group of isolates by including reference strains of the various genera within the *Rhizobiaceae* as well representatives from the genera *Burkholderia* and *Methylobacterium*.

The isolates and reference strains grouped into three major sections (arbitrarily defined) that could be further divided into 25 clusters and 5 single isolates (Figure 3.2). The separation into these three sections does not necessarily reflect a closer relationship between the isolates in each section, but simplify the discussion of the results obtained. Section I comprises 21 isolates and 11 reference strains representing the genera: *Mesorhizobium, Sinorhizobium, Rhizobium* and *Bradyrhizobium*. Section II contained reference strains from the genera *Rhizobium, Mesorhizobium, Sinorhizobium* and 38 isolates. Section III grouped three reference strains from the *Sinorhizobium* genus together with *Azorhizobium caulinodans, Agrobacterium tumefaciens, Mesorhizobium mediterraneum, Methylobacterium nodulans* and a *Burkholderia* sp. Section III included 43 of the isolates under investigation.

Section I

A total of 21 isolates grouped in this section. A few isolates grouped with various reference strains from the genera *Mesorhizobium, Rhizobium* and *Sinorhizobium*, while most isolates grouped with the bradyrhizobial reference strains, *B. japonicum* and *B. elkanii*. This section was further subdivided into four clusters (1-4), cluster 2 not containing any reference strain.

Cluster 1

Cluster 1 consisted of six faster-growing isolates and the reference strains: *Mesorhizobium ciceri, M. plurifarium, Rhizobium tropici, R. mongolense, Sinorhizobium*

terangae, S. meliloti, S. arboris and *S. fredii*, with an overall similarity of 81.3 %. Two distinct subgroups (1a and 1b) were distinguished, joining at a similarity value of 81.3 %.

Subcluster 1a contained four isolates and the four reference strains *M. ciceri, M. plurifarium, S. meliloti* and *S. arboris*. Isolates V6 (*L. laxa*), V18 (*L. laxa*) and V9.3 (*L. listii*) grouped closely together with an overall similarity of 88.7 %, showing the highest similarity to both *M. ciceri* and *S. meliloti* (86.8 %,). The *L. laxa* isolate V19 showed the highest similarity (82.5 %) to *M. plurifarium*.

Subcluster 1b contained two isolates: D3 (*L. mucronata*) and M4 (*L. lanceolata*), both showing the highest similarity to reference strains from the genus *Rhizobium*. D3 joined *R. tropici* at a similarity of 92.1 % whilst M4 showed 88.9 % similarity to *R. mongolense*.

Cluster 2

Cluster 2 consisted of six isolates and no reference strain and had an overall similarity of 82.7 % and could be divided into two different subgroups.

Subcluster 2a. The highest similarity value within the cluster (97.3 %) was obtained between the two *L. calycina* isolates (T9.2 and T15). These two isolates grouped at a similarity value of 84.1 % with NK10 (*L. sparsiflora*).

Subcluster 2b joined subcluster 2a at a similarity value of 82.7 % containing the closely related (89.1 %) isolates: NK28 (*L. carnea*), M6 (*L. carinata*) and WK5 (*L. parviflora*).

Cluster 3

Cluster 3 grouped six slower-growing bradyrhizobial isolates corresponding to the *B. japonicum* reference strain LMG6138. Cluster 3 consisted of distinct subgroups having an overall similarity of 80.1 %.

Subcluster 3a group WK1 (*L. falcata*) showed the highest similarity to *B. japonicum* at 90.1 %, followed by V5 (*L. decumbens*) with a value of 88.3 %. V5 was joined by NK8 (*Mellolobium* spp.) at a similarity value of 84.0 %.
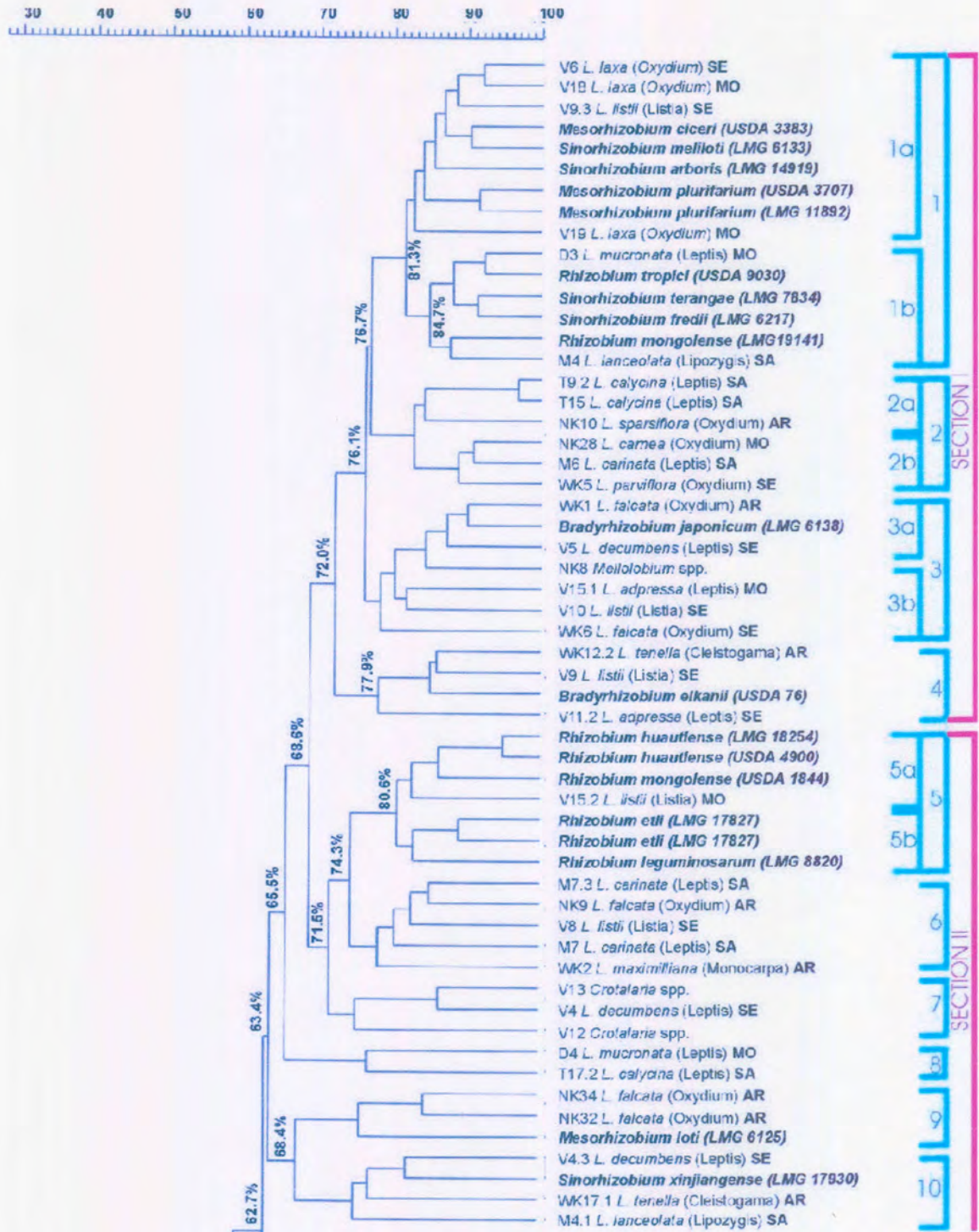
Figure 3.2
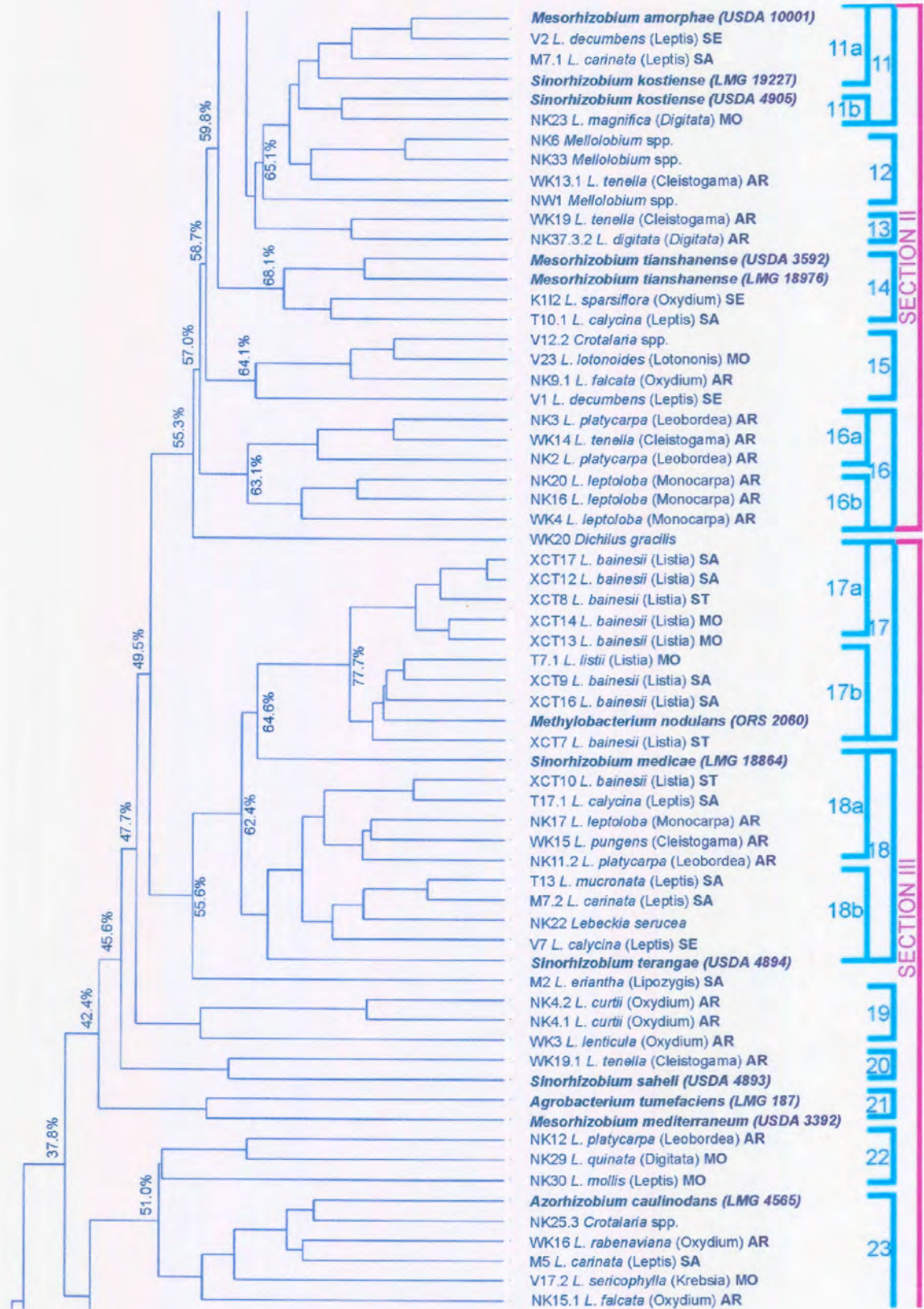
Figure 3.2 (continued)

Figure 3.2 **Detailed dendrogram based on UPGMA analysis of the correlation coefficients (*r*) between protein profiles of *Lotononis* isolates and reference strains of the genera *Agrobacterium, Azorhizobium, Bradyrhizobium, Burkholderia, Mesorhizobium, Methylobacterium, Rhizobium* and *Sinorhizobium*. Correlation (r), where r (%) = *r* x 100, is represented on the x-axis. Reference strains are indicated in bold and italic font. *Lotononis* isolates are indicated by isolate number followed by host plant species name. The representative section within the *Lotononis* genus is indicated in parenthesis followed by the geographical habitat of the host plant. The latter is indicated in bold font where: AR = Arid; MO = Mountainous; SA = Savanna; SE = Semi arid and ST = Strandveld.**

Subcluster 3b V15.1 (*L. adpressa*); V10 (*L. listii*) and WK6 (*L. falcata*) joined subcluster 3a at a similarity value of 78.1 %. WK 6 showed the lowest overall similarity (78.4 %) to any of the isolates within the cluster.

Cluster 4

Cluster 4 grouped three slow-growing bradyrhizobial isolates showing high similarity to *B. elkanii*. WK12.2 (*L. tenella*) and V9 (*L. listii*) (86.2 %) showed the highest relatedness to *B. elkanii* at a similarity value of 85 %. The last isolate within this cluster, V11.2 (*L. adpressa*) showed 77.9 % similarity to *B. elkanii*.

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Section II

Twelve clusters (5-16) comprised this section which contained *Rhizobium, Mesorhizobium* and *Sinorhizobium* reference strains. None of these clusters showed a similarity higher than 74.3 %. Seven of the clusters contained no reference strain.

Cluster 5

Despite only containing 1 isolate (V15.2) and four *Rhizobium* reference strains this cluster could be subdivided into two groups.

Subcluster 5a contained the reference strains *R. huautlense* and *R. mongolense* with the only isolate, V15.2 (*L. listii*), showing the highest relatedness to *R. mongolense* at 82.1 %.

Subcluster 5b grouped the reference strains *R. etli* and *R. leguminosarum* joining subcluster 5a at a similarity value of 80.6 %.

Cluster 6

Cluster 6 contained five isolates and no reference strains. The two isolates M7.3 (*L. carinata*) and NK9 (*L. falcata*) showed the highest similarity (85.2 %) within this cluster. These two isolates were joined by V8 (*L. listii*) at a value of 82.3 %. M7 (*L. carinata*) and WK 2 (*L. maximilliana*) joined V8 at 79.0 % and 77.4 % respectively. The overall similarity value obtained for the cluster was 77.4 %.

Cluster 7

Cluster 7 contained the two *Crotalaria* spp. isolates V12 and V13, having a 76.2 % similarity. However, the *Lotononis decumbens* isolate, V4 showed higher similarity (88.2 %) to V13 than V12.

Cluster 8

This cluster consisted of only two isolates, D4 (*L. mucronata*) and T17.2 (*L. calycina*) having 75.8 % similarity.

Cluster 9

Cluster 9 grouped the two *L. falcata* isolates NK32 and NK34 clustering at 84.2 %. These two isolates showed 75.8 % similarity to *M. loti* (LMG 6125).

Cluster 10

Cluster 10 contained the reference strain *S. xinjiangense* (LMG 17930) with its closest neighbour being V4.3 (*L. decumbens*) at 82.0 % similarity. The other two isolates contained within this cluster, WK17.1 (*L. tenella*) and M4.1 (*L. lanceolata*), respectively displayed similarity values of 77.0 % and 74.3 % to the former isolates.

Cluster 11

Cluster 11 consisted of three reference strains representing the genera *Sinorhizobium* and *Mesorhizobium* and three isolates. Two subgroups could be distinguished within this cluster.

Subcluster 11a constituted the reference strains *M. amorphae* (USDA 10001) and *S. kostiense* (LMG19227) together with the two isolates V2 (*L. decumbens*) and M7.1 (*L. carinata*). V2 and M7.1 showed a higher similarity to *M. amorphae*, with 85.3 % and 77.6 % similarity respectively.

Subcluster 11b contained the reference strain *S. kostiense* (USDA 4905) and the isolate NK23 (*L. magnifica*), sharing 75. 0% similarity.

Cluster 12

Cluster 12 consisted of three *Mellolobium* spp. isolates NK6, NK33 and NW1 grouping with the *Lotononis tenella* isolate WK13.1. Isolates NK33 and NK6 showed the highest similarity of 85.2 % within the cluster. WK13.1 joined NK33 and NK6 at 72.2 %, whilst the *Mellolobium* spp. isolate NW1 were loosely associated with WK13.1 at a similarity value of 65.1 %.

Cluster 13

Cluster 13 contained only two isolates, WK19 (*L. tenella*) and NK37.2.3 (*L. digitata*). These two isolates shared 77.5 % similarity.

Cluster 14

Cluster 14 consisted of the reference strain *M. tianshanense* (LMG 18976 and USDA 3592) together with two isolates. K1I2 (*L. sparsiflora*) and T10.1 (*L. calycina*) clustered at 74.1 % with both these isolates showing a similarity value of 68.1 % to the *M. tianshanense*.

Cluster 15

Cluster 15 was composed of four isolates and no reference strains. The three isolates: V12.2 (*Crotalaria* spp.), V23 (*L. lotonoides*) and NK9.1 (*L. falcata*) showed an overall similarity of 78.1 %. The *Lotononis decumbens* isolate V1, only shared 64.1 % similarity with the former two.

Cluster 16

Cluster 16 contained six isolates and no reference strains sharing an overall similarity of 63.1 %. Two subgroups could be distinguished within this cluster.

Subcluster 16a had an overall similarity of 73.0 % encompassing the two *Lotononis platycarpa* isolates NK2 and NK3, together with WK14 (*L. tenella*). NK3 and WK14 clustered at 82.9 % similarity with NK2 joining the latter two at a similarity value of 71.8 %.

Subcluster 16b All three isolates in this subcluster were isolated from *Lotononis leptoloba* sharing an overall similarity of 71.3 %. Isolates NK20 and NK16 displayed a similarity of 78.0 %, while WK4 is related to them at a similarity value of 69.5 %.

Section III

Section III could be subdivided into nine clusters (17-25) none of which showed a similarity higher then 62.4%. The similarity between section II and section III was 49.5%. Reference strains from the genera *Methylobacterium, Sinorhizobium,*

Agrobacterium, Mesorhizobium, Azorhizobium and *Burkholderia* were included here. A total of 43 isolates were included in this section.

Cluster 17

Except for one isolate, T7.1 (*L. listii*), all the isolates contained in this cluster were obtained from *L. bainesii*. These isolates grouped with the reference strain *Methylobacterium nodulans* (ORS 2060). Two distinct subgroups having 77.7 % similarity could be distinguished.

Subcluster 17a consisted of isolates from the same plant species, *Lotononis bainesii*. The three isolates XCT17, XCT12 and XCT8 shared a similarity value of 91.5 %. These three isolates clustered with XCT14 and XCT13 at 87.0 %.

Subcluster 17b contained the reference strain *Methylobacterium nodulans*, three *L. bainesii* isolates (XCT9, XCT16 and XCT7) and the *L. listii* isolate T7.1. *Methylobacterium nodulans* showed the highest similarity to T7.1, XCT9 and XCT16 (82.0 %). T7.1 clustered with XCT9 at a similarity value of 85.7 % with XCT16 joining these two isolates at 82.9 %. XCT7 clustered with *Methylobacterium nodulans* at a similarity of 80.6 %. Subcluster 17b was loosely joined by the reference strain *Sinorhizobium medicae* (LMG 18864) at a similarity value of 64.6 %.

Cluster 18

Cluster 18 consisted of the reference strain, *S. terangae* (USDA 4894) and nine isolates. The cluster could further be subdivided into two distinct subgroups sharing an overall similarity of 71.1 %.

Subcluster 18a contained no reference strains and five isolates. The highest similarity was obtained between XCT10 (*L. bainesii*) and T17.1 (*L. calycina*) at a similarity value of 88.0 %. NK17 (*L. leptoloba*), WK15 (*L. pungens*) and NK11.2 (*L. polycephala*) joined these two isolates at a similarity of 74.2 %. The overall similarity among NK17, WK15 and NK11.2 was 77.4 %.

Subcluster 18b contained four isolates with an overall similarity of 75.4%. T13 (*L. mucronata*) and M7.2 (*L. carinata*) showed the highest similarity at 87.5 %

followed by NK22 (*Lebeckia serucea*) joining them at 80.0 %. V7 (*L. calycina*) joined the latter three isolates with a similarity value of 75.4 %. The reference strain *S. terangae* (USDA4894) showed 65.5 % similarity to all the isolates contained within this cluster.

Cluster 19

Cluster 19 contained no reference strains. The three isolates contained within this cluster were associated as follows: NK4.2 (*L. curtii*) and NK4.1 (*L. curtii*) at 80.1 %; NK4.1 and WK3 (*L. lenticula*) at 57.0 %.

Cluster 20

Cluster 20 contained two loosely associated strains of which one was the reference strain *Sinorhizobium saheli* (USDA 4893). USDA 4893 clustered with the *Lotononis tenella* isolate WK19.1 at a similarity of 59.8 %.

Cluster 21

Cluster 21 consisted of two reference strains, *Agrobacterium tumefaciens* (LMG 187) and *Mesorhizobium mediterraneum*, clustering at a similarity of 57.0 %.

Cluster 22

Cluster 22 contained three isolates but no reference strains. The highest similarity (61.2 %) was shown between NK12 (*L. platycarpa*) and NK29 (*L. quinata*). These two isolates were joined by NK30 (*L. mollis*) at a similarity of 50.1 %.

Cluster 23

This cluster consisted of five isolates grouping with the *Azorhizobium caulinodans* (LMG 4565) reference strain. Cluster 23 had an overall similarity of 56 %. The highest similarity within the cluster (72.3 %) was between NK25.3 (*Crotalaria* spp.) and *Azorhizobium caulinodans* (LMG 4565). WK16 (*L. rabenaviana*) and M5 (*L. carinata*) clustered at a similarity of 71.0 %. WK16 and M5 joined LMG 4565 and NK25.3 at a similarity value of 67.2 %. These four strains were joined by V17.2 (*L. sericophylla*)

showing a similarity of 62.3 %, followed by NK15.1 (*L. falcata*) at a similarity value of 57.5 %.

Cluster 24

Cluster 24 contained six isolates and no reference strains. The highest similarity (77.2 %) was shown between NK37.3.1 (*L. digitata*) and D1 (*L. mucronata*). The *Lotononis decumbens* isolate V14 grouped together with these two isolates at a similarity value of 70.3 %. M3.3 (*L. calycina*), WK8 (*L. oxyptera*) and M8 (*L. carinata*) joined this group with an overall similarity of 57.9 %. M3.3 joined WK 8 at a similarity value of 68.1 % followed by M8 joining these two isolates, showing 62.5 % similarity.

Cluster 25

Cluster 25 contained one reference strain, *Burkholderia* spp. (STM 815) and four isolates. The highest similarity was shown between NK27 (*L. polycephala*) and WK10.2 (*L. fastigiata*) at a value of 85.2 %. These two isolates were joined by the *Aspalathus* sp. isolate WK7 at a similarity value of 69.8 %. WK7 was joined by WK11 (*L. involucrata*) at a similarity value of 66.0 %. All of the isolates within this cluster grouped with STM 815 with an overall similarity of 55.5 %.

The five loosely associated isolates

Branching points consisting of only one isolate having a similarity of 55.6 % or less to any of the other branching points within the dendrogram were considered as loosely associated.

The single isolate WK20 (*Dichilus gracilis*) was loosely associated with all the isolates contained within section II and section III. WK20 showed the highest similarity to section II (55.3 %). Cluster 18 was loosely joined by the single isolate M2 (*L. eriantha*) at a similarity value of 55.6 %. The two isolates V11.1 (*L. listii*) and NK27.1 (*L. polycephala*) were loosely associated with the isolates contained within clusters 24 and 25. V11.1 and NK27.1 shared 30.5 % similarity.

3.3.3   Statistical analysis

Two dimensional frequency sheets allowed the representation of results as graphical illustrations. These graphs indicate the dispersal of the various sections of *Lotononis* within different clusters (Figure 3.3) and throughout the dendrogram (Figure 3.4). The distribution of geographical regions within the different clusters are shown (Figure 3.5) and throughout the dendrogram (Figure 3.6). Some of these data represented on graphs might be misleading when not considering it together with the data from the dendrogram. For example: a cluster might reveal a 100 % graphical constitution of one single *Lotononis* section, but the dendrogram might indicate that only one isolate was contained within the specific cluster.
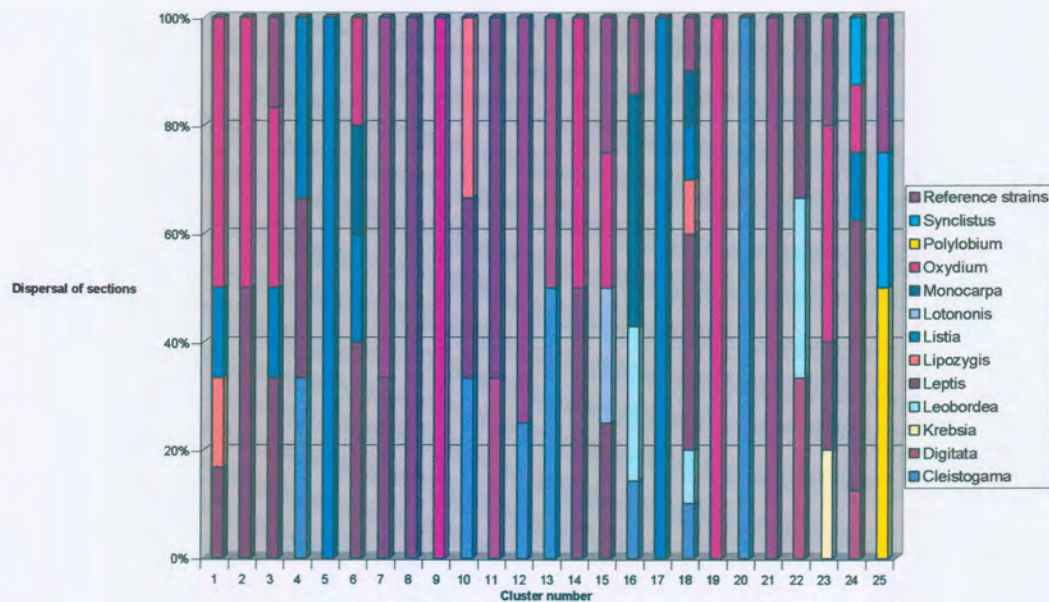
Figure 3.3 Bar chart illustrating the percentage constitution of the various sections within *Lotononis* in each cluster as well as the percentage reference strains in each cluster. The colour code for each section is given in the figure legend.
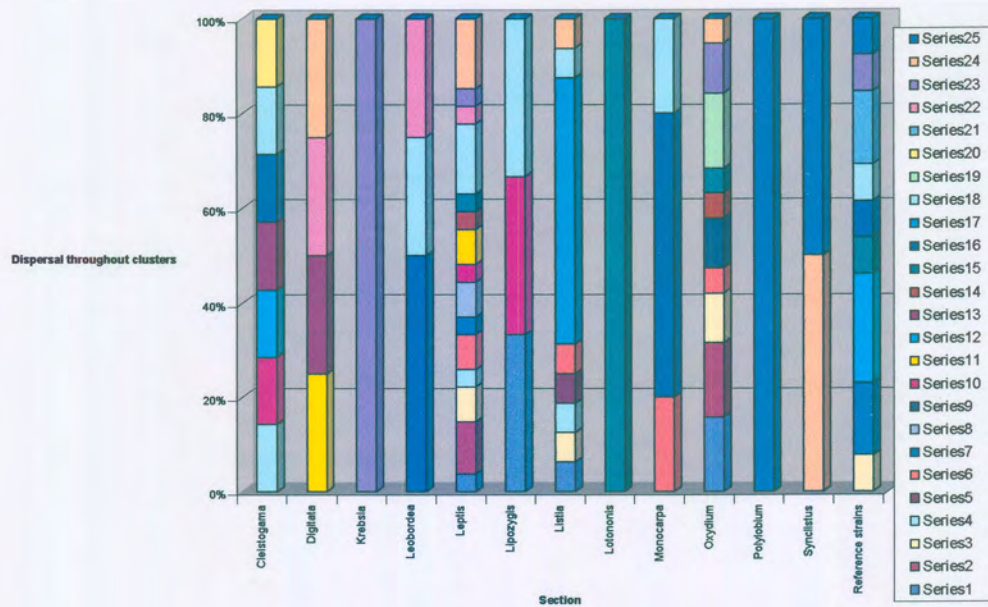
Figure 3.4 Bar chart illustrating the distribution of *Lotononis* section and reference strains throughout the dendrogram. Various clusters are identified by different colours shown in figure legend, where series = cluster.
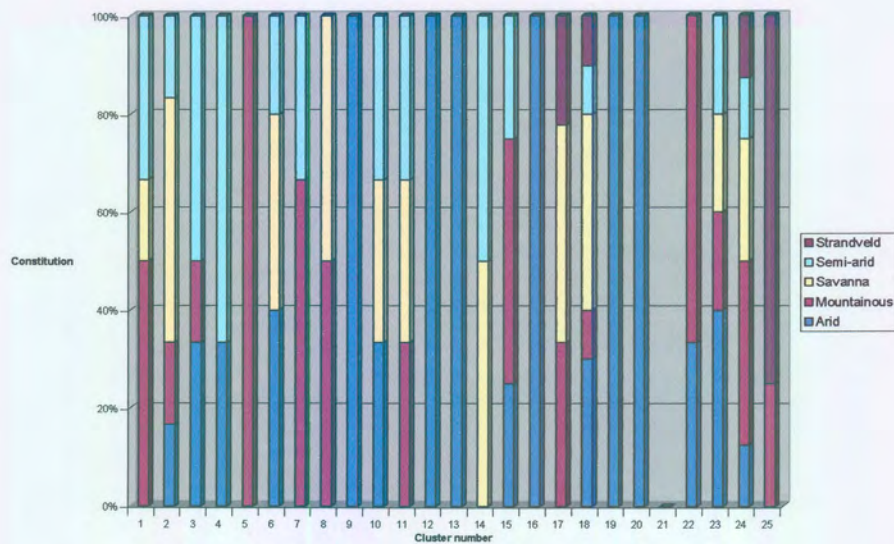
Figure 3.5 (Page 70) Bar chart illustrating the distribution of various geographical regions within different clusters. The colour code for each region is given in figure legend.
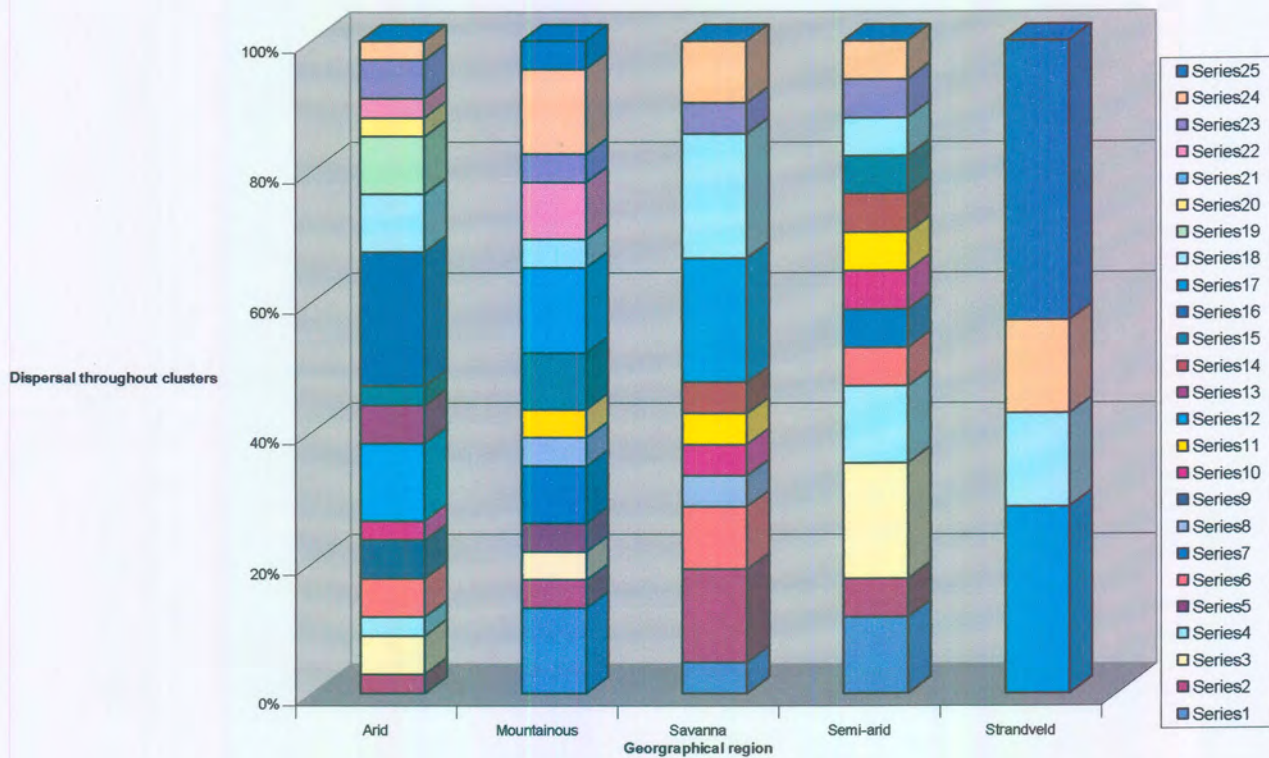
Figure 3.6 Bar chart illustrating the distribution of different geographical regions throughout the dendrogram. The colour code for each cluster is given in the figure legend, where series = cluster.

Figure 3.3 indicated that in most instances different *Lotononis* sections were found throughout the dendrogram. Clusters 5, 8, 17, 19 and 20 grouped only isolates obtained from one specific section within *Lotononis*. When considering figure 3.4 it became apparent that most sections were distributed throughout the dendrogram. The sections

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Krebsia, Lotononis and Polylobium occurred in very low frequencies and thus appear to group with definite specificity. All geographical regions seemed to group throughout the dendrogram (Figure 3.5). Arid regions seemed to be the only geographical environment to group exclusively in some clusters (clusters 9, 12, 13, 16, 19 and 20). Furthermore all the geographical environments were found throughout the dendrogram (Figure 3.6).

3.4 DISCUSSION

Introduction

The diversity of rhizobial isolates associated with *Lotononis* species occurring in South Africa was determined by the isolation of putative root nodule-bacteria from thirty-two different *Lotononis* species. To our knowledge this is the first extensive study to investigate the diversity of the rhizobial symbionts associated with *Lotononis* species. Jaftha *et al.* (2002) characterized the rhizobial symbionts of the single species, *Lotononis bainesii*, subsequently proving these isolates to be related to *Methylobacterium nodulans*. From the SDS-PAGE analysis it became apparent that the members of the *Lotononis* genus are being nodulated by various genera within and outside the *Rhizobiaceae* family. Clusters showed close similarity to the genera: *Mesorhizobium* (clusters 1a, 9, 11a and 14); *Rhizobium* (Clusters 1b and 5); *Bradyrhizobium* (clusters 3 and 4); *Sinorhizobium* (clusters 10, 11b and 20); *Methylobacterium* (cluster 17); *Azorhizobium* (cluster 23) and *Burkholderia* (cluster 25). Earlier studies on the diversity of rhizobia indigenous to South Africa indicated the absence of isolates corresponding to the genus *Azorhizobium* (Dagutat, 1995; Kruger, 1998 and Joubert, 2002). In a few instances in this study, more than one type of rhizobial isolate was obtained from the same root nodule, corresponding to previous reports (Dreyfus and Dommergues, 1981; Dagutat, 1995; Kruger 1998; Joubert, 2002).

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The majority of the *Lotononis* isolates represented fast- and medium-growing strains clustering with reference strains of the genera *Rhizobium, Sinorhizobium* and *Mesorhizobium*. The slower-growing isolates that showed high relatedness to the genus *Bradyrhizobium* were confined to 14.6 % of the total number of isolates. The methylotrophic bacterium *Methylobacterium nodulans* showed a high degree of similarity to nine isolates representing 8.7 % of all the isolates under investigation. The isolate T7.1 that was capable of utilizing methanol as sole carbon source (results not shown) were also contained within this cluster. The utilization of methanol as sole carbon source is a common feature of the species *M. nodulans*. A small number of isolates (3.8 %) corresponded to the β-subclass *Proteobacteria* genus *Burkholderia*. Closely related groups of isolates such as those grouped in cluster 24 that were not related to any of the reference strains included, could warrant the description of novel entities upon further investigation.

Correlation between geographical environment and electrophoretic groupings

When considering the geographical environment of the isolates it became apparent that in most instances very little correlation exists between the geographical region and the grouping of isolates. Isolates obtained from arid regions seemed to show a higher correlation between geographical environment and electrophoretic groupings than those that were obtained from other geographical environments. Clusters 12, 13, 16 and 19 seemed to group only isolates from arid environments. It might be reasonable to conclude that these extreme environments are selective towards specific root nodule-bacteria.

Correlation between host specificity and electrophoretic groupings

The close relatedness that exists between the different species contained in the same sections of *Lotononis* would make the different sections good indicators of host specificity (Van Wyk, B-E; personal communication). It was subsequently decided to use the different sections within *Lotononis* as the representative entities when considering host specificity.

It was subsequently shown that a high degree of host specificity was evident for the isolates that grouped in clusters 17 as well as subcluster 16b.

Subcluster 16b contained 3 isolates representing the section Monocarpa. Although these isolates were obtained from the same geographical environment, the origins were far apart, with WK4 and NK 20 isolated 300 km apart. Subcluster 16b was also contained within cluster 16, a cluster showing correlation between geographical environment and electrophoretic patterns.

Cluster 17 grouped only isolates from the section Listia. Most isolates were obtained from the species *L. bainesii* showing high degrees of similarity towards each other. One isolate, T7.1, obtained from the closest taxonomical neighbour of *L. bainesii, L. listii*, was also contained within this group. This cluster seems to be restricted to isolates obtained from the section Listia.

Most of the isolates under investigation did not show any significant degree of host specificity, being dispersed throughout the dendrogram. The species *L. falcata*, a member of the section Oxydium, was found throughout the dendrogram, having isolates showing similarity to the genera *Bradyrhizobium* (WK6); *Mesorhizobium* (NK32, NK34), etc.. Other *Lotononis* spp. contained within this section showed equal dispersal throughout the dendrogram. Isolates obtained from the section Leptis was also found in various clusters of the dendrogram, showing similarity to the genera *Rhizobium* (D3), *Bradyrhizobium* (V5), *Mesorhizobium* (V2), etc.. The lower degree of host specificity in some instances was further supported by the dispersal throughout the dendrogram of isolates obtained from the same root nodule. M7, M7.1, M7.2 and M7.3 were isolated from the same root nodule (*L. carinata*). M7 and M7.3 were the only isolates showing a relatively high similarity in cluster 6. M7.1 was contained within subcluster 11a and M7.2 in subcluster 18b.

Dispersal of different isolates and reference strains throughout the dendrogram

Different rhizobial species were well separated below the similarity value of 91.0% in the SDS-PAGE analysis. However, *Sinorhizobium saheli* (LMG 7834) and *Sinorhizobium fredii* (LMG 6217), shared a similarity value of 91.6%. de Lajudie and co-workers (1994) characterized strains nodulating *Sesbania* and *Acacia* species from Senegal (West Africa) and concluded that the species *S. saheli* was related to the *Rhizobium meliloti-Rizobium fredii* (now *S.* meliloti-*S. fredii*) rRNA branch. The high similarity obtained in the SDS-PAGE analysis further supported this close relationship.

Similarity values of 92.0 % or more shared between strains were considered as representing the same species, e.g. V6 and V18 in subcluster 1a. Based on the 94.0 % limit for reproducibility, those strains sharing 94.0 % or more similarity were considered identical. One isolate, D3, in subcluster 1b was considered to belong to the species *Rhizobium tropici*. In all the instances where similarity values of more than 94.0% were obtained, the isolates had the same host plant species. In subcluster 2a the *L. calycina* isolates T9.2 and T15 had a similarity value of 97.3 % whilst the two *L. bainesii* isolates XCT12 and XCT17 in subcluster 17a shared a similarity of 97.5 %.

In the results a branch containing reference strains of the genera *Rhizobium, Mesorhizobium* and *Sinorhizobium* was observed (cluster 1), containing various isolates. Although recovered from the same cluster, the two different type strains of *Mesorhizobium plurifarium* (LMG 11892 and USDA 3707) did not share a similarity of 94.0% or higher. It should be emphasized that it is not uncommon for reference strains of the same species to show low similarity in SDS-PAGE analysis (Joubert, 2002; Kruger, 1998). The definite *Rhizobium* branch (cluster 5) contained various species within the genus and one indigenous isolate (V15.2) that showed similarity to *R. mongolense*. Some of the faster-growing indigenous isolates under investigation (clusters 6, 7 and 8) showed relatively high similarity to this *Rhizobium* cluster. The slower-growing isolates grouped within clusters 3 and 4, containing the bradyrhizobial reference strains *B. japonicum* and *B. elkanii*.

Reference strains of the genus *Sinorhizobium* were dispersed throughout the dendrogram, with none of the isolates showing similarity to the *Medicago truncatula* symbiont *S. medicae* (Rome *et al.*, 1996). The phylogenetic closely related *S. terangae* and *S. saheli* showed low similarity in their electrophoretic patterns. Upon the proposition of these two species, de Lajudie *et al.* (1994) reported that divergence existed in the whole-cell protein profiles between these two species. Some of the indigenous isolates showed relatively low similarity to *S. terangae* and one isolate clustered with *S. saheli*.

Azorhizobium caulinodans is the stem and root symbiont of *Sesbania rostrata* in a very host specific way (Dreyfus *et al.*, 1988). Strains corresponding to *A. caulinodans* have never been found in South African soils (Joubert, 2002; Dagutat, 1995; Kruger, 1998). Our results indicated that cluster 23 containing the species *A. caulinodans* also clustered five indigenous isolates showing some degree of similarity towards *A. caulinodans*. The highest similarity was shown to a slower-growing isolate, NK25.3. The taxonomical status of this isolate should be investigated further in future studies. It should be noted that isolate NK25.3 showed unique colony morphology with single colonies appearing as hard pinkish knobs on agar plates.

The recently described nodulating species *Methylobacterium nodulans* (Sy *et al.*, 2001) clustered distinctly with various *Lotononis* isolates, most of which represented isolates of *Lotononis bainesii*. Jaftha *et al.*, 2002 reported the symbionts of *L. bainesii* to be related to *Methylobacterium nodulans*. The emergence of the nodulating member of the β-subdivision of the *Proteobacteria*, *Burkholderia*, obtained from South Africa (Moulin *et al.*, 2001), led to the inclusion of a *Burkholderia* reference strain in this study. Moulin and co-workers (2001) reported the presence of nodulation genes within these *Burkholderia* isolates. It should be noted that whole cellular protein extracts of the isolates contained within cluster 25 were difficult to normalize due to the production of extracellular polysaccharides causing profiles to appear as smears upon analysis. This would correspond to the relatively low overall similarity (55.5%) obtained between STM 815 and the rest of the isolates contained within this cluster. One isolate showing similarity to *Burkholderia* sp., WK7, was obtained from the plant genus *Aspalathus*,

representing the same genus from which Moulin *et al.* (2001) first isolated *Burkholderia* sp. The *Lotononis* isolates grouping within this cluster represents the two sections Synclistus and Polylobium. Interesting to note is that these two sections are some of the most distantly related sections within the plant genus.

Statistical analysis of SDS-PAGE data

Frequency data concerning geographical regions and the dispersal of different *Lotononis* sections in the dendrogram aided to give a visual understanding and interpretation of the results. These results supported the information obtained from the dendrogram showing that most sections within *Lotononis* were evenly distributed throughout the dendrogram, but that a degree of specificity does exists for some sections. The same observation was made when considering the geographical regions of isolates, with some clusters having a definite preference towards grouping isolates obtained from arid environments (the only geographical region seemingly playing a role in groupings of isolates).

Conclusion

SDS-PAGE analysis of whole cellular protein extracts proved to be a useful tool when screening a large number of isolates of closely related strains. SDS-PAGE data should not, however, be used alone, but should rather form part of a polyphasic approach in order to make useful conclusions regarding the strains investigated.

Our results showed that SDS-PAGE analysis was sufficient to differentiate between the rhizobial symbionts of *Lotononis*, subsequently proving them to be diverse. Most isolates showed affinity towards the faster-growing genera, *Rhizobium, Mesorhizobium* and *Sinorhizobium*. A few isolates also corresponded to *Azorhizobium caulinodans*, a species never reported as indigenous to South African soils. A portion of the isolates also grouped with the slower-growing bradyrhizobial reference strains. Interestingly, two of the known genera outside the *Rhizobiaceae* capable of successful nodulation of leguminous plants were represented in the results. Isolates showing high affinities to both *Methylobacterium nodulans* and *Burkholderia* sp. were isolated from the root nodules of *Lotononis* specimens.

It is thus apparent that the root nodule-associated bacteria of the legume genus *Lotononis* show heterogeneity and that the genus *Lotononis* proved to be an excellent indicator of the diversity of root nodule bacteria indigenous to South African soils.

CHAPTER 4

ABSTRACT:

Based on the results of SDS-PAGE analysis, representative rhizobial isolates from the root nodules of *Lotononis* species were selected and subjected to 16S rDNA sequencing. A phylogenetic tree, including various rhizobial reference strains as well as representatives of the β-subclass of the *Proteobacteria*, was constructed. Isolates obtained from the same root nodule seemed to be dispersed between various genera within the *Rhizobiaceae*. A few of the isolates also showed high sequence homology to the genus *Burkholderia*. Using this approach it was possible to get clarity on the taxonomical status on some of the isolates, not clearly resolved in the SDS-PAGE analysis.

Keywords: *Rhizobiaceae, Burkholderia,* 16S rDNA sequencing

CHAPTER 4

CHARACTERIZATION OF SELECTED INDIGENOUS RHIZOBIAL ISOLATES ASSOCIATED WITH *LOTONONIS* BY 16S rDNA SEQUENCING.

4.1 INTRODUCTION

Eight genera are currently described within the *Rhizobiaceae*: *Rhizobium* (Frank, 1889), *Bradyrhizobium* (Jordan, 1982), *Azorhizobium* (Dreyfus *et al.*, 1988), *Sinorhizobium* (Chen *et al.*, 1988; de Lajudie *et al.*, 1994), *Mesorhizobium* (Jarvis *et al.*, 1997), *Allorhizobium* (de Lajudie *et al.*, 1998), *Agrobacterium* (Conn, 1942) and *Phyllobacterium* (Knösel, 1984). During recent years the classification of rhizobia has been progressively revised as the extent of rhizobial diversity has been increasingly uncovered (Diouf *et al.*, 2000). These revisions and improvements of the classification of the legume root-nodulating bacteria is due to the application of the polyphasic approach which has resulted in a greater understanding of the complex intra- and intergeneric relationships of rhizobial species (de Lajudie *et al.*, 1994).

Initial screening methods such as SDS-PAGE are rapid and clusters are formed from complete similarity matrices. The resolution of the fingerprint, however, is not capable of distinguishing relatedness below the species level, and reproducibility is difficult to ensure (Kersters and de Ley, 1975; Jackman, 1985; Vauterin *et al.*, 1993). Under standardized conditions a degree of variation might still exist when applying SDS-PAGE analysis and techniques such as 16S rDNA sequencing are not liable to such variation.

Ribosomal RNA has structural constraints in ribosomes and at the same time variability in some domains. These features make RNA gene sequences (5S, 16S and 23S) very good choices to compare organisms and to infer phylogenies (Woese, 1987) and to use it as a diagnostic DNA segment (Eardly *et al.*, 1992).

Rhizobia phylogenies based on 16S rDNA sequences are generally in agreement with those derived from other gene sequences such as nodulation gene sequences

(Martínez-Romero and Caballero-Mellado, 1996). Based on 16S rDNA phylogeny the slow-growing *Bradyrhizobium* species are clearly separated from the faster-growing *Rhizobium* species, showing closer phylogenetic relatedness to *Rhodopseudomonas palustris* than to the other genera contained within the *Rhizobiaceae* (Terefework *et al.*, 1998). All *Rhizobium* species tend to intermingle with members of the genus *Agrobacterium*, where it was previously reported that *R. tropici* and *A. rhizogenes* could not be distinguished based on 16S rDNA characters (Laguerre *et al.*, 1994). Based on these findings, Young *et al.* (2001) proposed the emendation of the genera *Agrobacterium*, *Rhizobium* and *Allorhizobium* into the single genus *Rhizobium*. Together with the close phylogenetic relationship between *Rhizobium* and *Agrobacterium*, *Allorhizobium undicola* proved to be an outlying branch of the *Agrobacterium-Rhizobium* cluster based on 16S rDNA phylogeny (de Lajudie *et al.*, 1998b). Young and co-workers (2001) argued that no consistency existed in phylogenetic differentiation between these three genera and that they should encompass the single genus *Rhizobium*. Within the genus *Sinorhizobium*, 16S rDNA phylogeny is sufficient to distinguish between *S. fredii* and *S. meliloti* (Jarvis *et al.*, 1992). However, *S. fredii* and *S. xinjiangensis* share 100 % 16S rDNA homology. Yanagi and Kazuhide (1993) proposed the revision of the *Rhizobiaceae* based on 16S rDNA phylogeny, arguing that biological behavior should be excluded as criteria. This would implicate that *Bradyrhizobium*, although capable nodulation and biological nitrogen fixation should form a distinct phylogenetic group separate from the *Rhizobiaceae*. Sequencing of 16S rDNA genes aided as a supplementation technique for the results obtained from SDS-PAGE analysis.

4.2 MATERIALS AND METHODS

4.2.1 Strains used

From the initial screening results (SDS-PAGE) eight isolates were selected and subjected to 16S rDNA sequencing (Table 4.1). Isolates showing relatedness to *Burkholderia* spp. and different isolates from the same root nodule were selected. Corresponding sequences of root-nodule bacteria, isolated from South African soils,

showing close relatedness to the genus *Burkholderia* were also included in the analysis. The 16S rDNA sequences of relevant rhizobial reference strains and other members of the α- and β-*Proteobacteria* were obtained from GenBank database of the National Centre for Biotechnology (NCBI) [*www.ncbi.nlm.nih.gov/Genbank/*].

4.2.2 Extraction of genomic DNA

Isolates were incubated on tryptone yeast extract medium (TY) slants [0.5 % (m/v) tryptone, 0.3 % (m/v) yeast extract, 0.065 % (m/v) $CaCl_2$ and 1.5% (m/v) bacteriological agar] at 28°C for 5 d. Cells were harvested by adding sterile distilled water and gentle shaking until cells were suspended. Genomic DNA was extracted as previously described by Lemanceau *et al.* (1995). Cell suspensions were transferred to sterile plastic tubes. The optical density (OD) of cell suspensions was measured at 620 nm using water as a blank. To calculate the number of cells needed for further use the following formula was used: V (μl) = $^{0.2}/_{OD260}$ x 1000. The appropriate volume of cells was transferred to a clean Eppendorf tube and centrifuged at 12 000 x g for 5 min., the supernatant discarded and the pellet blotted dry. The cell pellet was resuspended in 100 μl of ultra-high quality (UHQ) water, 100 μl Tris-HCl (10 mM, pH 8.2) and 10 μl proteinase K (15.6 mg/ml) and incubated overnight at 55 °C. Proteinase K was inactivated by heating the suspension of 10 min. at 96 °C. The cell lysates were stored at –20°C until further use.

4.2.3 PCR amplification of the 16S rDNA gene

The primers fD1SHRT and rP2SHRT were used to amplify the 16S rDNA gene of selected isolates (Table 4.2). These primers are derived from the universal primers fD1 and rP2 (Weisburg *et al.*, 1991), lacking the linker sequences containing restriction enzyme recognition sites present in the original primers. PCR amplification was carried out in 50 μl reaction volumes instigated as followed: 5 μl of the supernatant of Proteinase K treated cells, 5 μl 10 x PCR buffer, 1.5 mM $MgCl_2$, 250 μM of each dNTP, 12.5 pmol of both fD1SHRT and rP2SHRT and 0.5 U Super-

Table 4.1   **List of putative root nodule-associated bacteria subjected to 16S rDNA sequencing**

Isolate number	*Lotononis* host species
M7	*L. carinata*
M7.1	*L. carinata*
M7.2	*L. carinata*
T17.1	*L. calycina*
WK6	*L. falcata*
WK7	*Aspalathus* sp.
NK27	*L. polycephala*
V15.2	*L. listii*

Therm Taq DNA polymerase (Southern Cross Biotechnologies). Amplification was carried out in a Perkin Elmer GeneAmp System 2400 thermocycler. The following thermal profile was used: an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 3 min. (Laguerre *et al.*, 1994). The size, concentration and purity of PCR products were examined by horizontal gel electrophoresis using 1 % (m/v) agarose gels containing 3 μl ethidium bromide (10 mg/ml) in 1 x TAE buffer (40 mM Tris-HCl, 20 mM NaOAc and 1 mM EDTA, pH 8.5).

4.2.4 Purification of the 16S rDNA PCR products

The QIAquick PCR Purification Kit (QIAGEN) was used to purify the PCR products.

4.2.5 Sequencing of the 3' end (approximately 600 bp) of the 16S rDNA gene

The primers rP2SHRT (Weisburg *et al.*, 1991) and 16SRNAII-S (Kuhnert *et al.*, 1996) was used to sequence the 3' end of the 16S rDNA gene (Table 4.2). Sequencing was carried out in 5 μl reaction volumes instigated as followed: 100 ng purified 16S

Table 4.2   **Primers used for PCR amplification and partial sequencing of 16S rDNA**

Primer	Sequence (5' to 3')	Reference
fD1SHRT	AGAGTTTGATCCTGGCTCAG	Weisburg *et al.*, 1991
rP2SHRT	ACGGCTACCTTGTTACGACTT	Weisburg *et al.*, 1991
16SRNAII-S	GTGTAGCGGTGAAATGCGTAG	Kuhnert *et al.*, 1996

rDNA template, 2 μl BigDye™ Terminator RR mix (PE Applied Biosystems), 10 pmol of 16SRNAII-S or rP2SHRT. Amplification was carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler using the following thermal profile: 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s and extension at 60 °C for 4 min. Sequencing reaction products were precipitated with 100 % ethanol and 3 M NaOAc on ice for 10 min. The precipitate was centrifuged at 12 000 x *g* for 30 min and the supernatant discarded, followed by 2 successive wash steps with 70 % (v/v) ethanol. The precipitate was vacuum dried and stored at –20 °C until further use. Prior to electrophoresis, the purified products were resuspended in 3.5 μl Blue dextran/EDTA loading buffer, prepared by combining deionized formamide and 25 mM EDTA (pH 8.0) containing 50 mg/ml Blue dextran in a ratio of 5:1 formamide to EDTA/Blue dextran. Sequencing samples were run overnight on an ABI 377 Automated Sequencer at the sequencing facility at the University of Pretoria, South Africa.

4.2.6 Analysis of sequence data

Sequences were analyzed with the ABI Prism Sequencing Analysis 3.1 and the ABI Prism Sequencing Navigator 1.0.1 computer programms (PE Applied Biosystems). Phylogenetic relationships were determined using the Neighbour-joining algorithm (Saitou and Nei, 1987) in ClustalX (Thompson *et al.*, 1997). Confidence levels of phylogenies were estimated by using the bootstrap method (Felsenstein, 1985). The NJPLOT computer programme was used to display the phylogenetic trees.

The partial 16S rRNA gene sequences of eight of the indigenous isolates under investigation were compared with those of members of the rhizobial genera *Agrobacterium, Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium*, as well as the genera *Burkholderia, Ensifer, Methylobacterium* and *Ralstonia* available from the GenBank database. A phylogenetic tree (Figure 4.1) was constructed by the neighbour-joining algorithm (Saitou and Nei, 1987) using the ClustalX computer programme.

The tree was composed of two lineages representing members of the α- and β-subclasses within the *Proteobacteria* respectively.

WK 6 (*Lotononis falcata*) was related to *S. meliloti, S. fredii* and *S. xinjiangensis*. Isolates M7, M7.1 and M7.2 were all obtained from the same specimen (*L. carinata*) and were related to three different rhizobial genera. M7 was related to the *Rhizobium* branch of the tree showing the highest relatedness to *R. indigoferae*. M7.1 showed relatedness to the *Agrobacterium rhizogenes-Rhizobium tropici* branch whilst M7.2 and T17.1 showed a high relationship to *Bradyrhizobium elkanii*.

Within the α-subclass of the *Proteobacteria* the genus *Bradyrhizobium* was clearly differentiated from the faster-growing rhizobial genera showing only an overall relationship of 95.4 % to these members of the *Rhizobiaceae*. A higher relationship of 96.7 % was obtained between *Bradyrhizobium* and the *Methylobacterium-Azorhizobium* branch.

The β-subclass of the *Proteobacteria* was represented by various reference strains from the genera *Burkholderia* and *Ralstonia*.

2 %

67 % *Sinorhizobium xinjiangensis* (D12796)
Sinorhizobium fredii (X67231)
Sinorhizobium meliloti (X67222)
WK6 (Lotononis falcata)
Sinorhizobium medicae (L39882)
68.4 % *Sinorhizobium kummerowiae* (AY034028)
Sinorhizobium arboris (Z78204)
Sinorhizobium kostiense (Z78203)
Sinorhizobium saheli (X68390)
Sinorhizobium morelense (AY024335)
Ensifer adhaerens (AY040360)
Sinorhizobium terangae (X68387)
Rhizobium giardinii (U86344)
63.3 % *Mesorhizobium loti* (X67229)
Mesorhizobium chacoense (AJ278249)
Mesorhizobium amorphae (AF041442)
Mesorhizobium tianshanense (Y71079)
Mesorhizobium huakuii (D12797)
70.6 % *Mesorhizobium ciceri* (U07934)
62.4 % *Mesorhizobium plurifarium* (Y14158)
Mesorhizobium mediterraneum (L38825)
Agrobacterium tumefaciens (X67223)
Agrobacterium radiobacter (AJ389904)
95 % *Agrobacterium rubi* (D14503)
Agrobacterium larrymoorei (Z30542)
99.5 % *Agrobacterium vitis* (D14502)
Allorhizobium undicola (Y17047)
M7.1 (Lotononis carinata)
100 % *Agrobacterium rhizogenes* (X67224)
Rhizobium tropici (U89832)
95.1 % *Rhizobium hainanensis* (U71078)
Rhizobium leguminosarum (X67227)
99 % *Rhizobium indigoferae* (AY034027)
M7 (Lotononis carinata)
Rhizobium gallicum (U86343)
Rhizobium mongolense (U89817)
Rhizobium etli (U28916)
Rhizobium galegae (AF025853)
86.7 % *Rhizobium huautiense* (AF025852)
V15.2 (Lotononis listii)
Rhizobium yanglingense (AF19031)
M7.2 (Lotononis carinata)
84.8 % *Bradyrhizobium elkanii* (U35000)
99.7 % **T17.1 (Lotononis calycina)**
Bradyrhizobium japonicum (X66024)
100 % *Bradyrhizobium liaoningense* (AJ250813)
65.9 % *Methylobacterium nodulans* (AF220763)
100 % *Azorhizobium caulinodans* (X67221)

Burkholderia ubonensis (AB030584)
85.5 % *Burkholderia vietnamensis* (AF097534)
Burkholderia multivorans (Y18703)
Burkholderia gladioli (U96934)
Burkholderia andropogonis (X67037)
Burkholderia plantarii (U96933)
Burkholderia glumae (U96931)
Burkholderia anthina (AJ420880)
Burkholderia ambifaria (AY028444)
Burkholderia cepacia (AF265235)
Burkholderia pseudomallei (AF093052)
49.4 % *Burkholderia thailandensis* (U91838)
Burkholderia caryophylli (AB021423)
Burkholderia mallei (AF110187)
97.5 % *Burkholderia pyrrocinia* (U96930)
Burkholderia stabilis (AF148554)
95.1 % *Burkholderia phenazinium* (U96936)
Burkholderia sp. CS 2 (AY178065)
98.5 % *Burkholderia* sp. CD 9 (AY178076)
100 % *Burkholderia sordicola* (AF512827)
Burkholderia glathei (U96935)
Burkholderia sp. CS 6 (AY178066)
Burkholderia sp. CM 1 (AY178058)
58.8 % *Burkholderia fungorum* (AF215705)
Burkholderia sp. UCT 71 (AY178064)
Burkholderia caledonica (AF215704)
92.1 % **WK 7 (Aspalathus spp.)**
NK 27 (Lotononis polycephala)
Burkholderia graminis (U96941)
53.8 % *Burkholderia* sp. UCT 29 (AY178062)
73 % *Burkholderia* sp. Cses 4 (AY178063)
Burkholderia tropicalis (AJ420332)
Burkholderia sp. STM678 (AJ302311)
Burkholderia sp. UCT 70 (AY178075)
Burkholderia sp. CI 3 (AY178072)
Burkholderia sp. UCT 30 (AY178067)
100 % *Burkholderia brasilensis* (AJ238360)
Burkholderia kururiensis (AB024310)
76.7 % *Burkholderia sacchari* (AF263278)
Burkholderia sp. STM815 (AJ302312)
Burkholderia caribiensis (Y17009)
97.5 % *Burkholderia norimbergensis* (Y09879)
100 % *Ralstonia taiwanensis* (AF300324)
Ralstonia picketti (AB004790)

100 %

Alpha subdivision of the *Proteobacteria*

Beta subdivision of the *Proteobacteria*

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Figure 4.1 (Page 86) Phylogenetic relationships between members of the α- and β-subdivisions of the *Proteobacteria* compared to selected isolates from this study. The tree was constructed by the neighbour-joining method from partial 16S rDNA sequences. Bootstrap values are indicated at the branching points. The scale bar represents 2 % nucleotide difference. Isolates from this study are indicated in blue and their respective host species in parenthesis. Corresponding sequences of root-nodule bacteria, isolated from South African soils, showing close relatedness to the genus *Burkholderia* were also included in the analysis (green). Accession numbers are indicated in parenthesis.

4.4 DISCUSSION

Although SDS-PAGE analysis gives high discriminatory results the reproducibility depends highly on the standardization of all procedures that might influence the protein composition of the bacterial cell. Furthermore, SDS-PAGE analysis proves very little value when comparing distantly related bacteria. Small subunit rDNA sequencing would thus clarify or support the taxonomical positions of isolates as obtained from SDS-PAGE analysis.

The comparison of SDS-PAGE and 16S rDNA sequencing data confirmed that identification below the species level is not possible based on the SDS-PAGE data alone. This was clearly reflected for the isolate M7.2 (*L. carinata*) that grouped within cluster 18b in the SDS-PAGE analysis, together with the reference strain *S. terangae* (USDA 4894). However, partial 16S rDNA sequencing, revealed this isolate to show a high degree of sequence relatedness to *Bradyrhizobium elkanii*. In a similar way, Dupuy *et al.* (1994) found that putative *Bradyrhizobium* strains grouped in clusters only containing fast growing species. Slower-growing rhizobia strains tend to produce lower quality (less distinct and sharp) protein profiles than the faster-growing strains that might influence the fingerprint being produced by SDS-PAGE analysis (Moreira *et al.*, 1993).

The other *L. carinata* isolate (M7.1) obtained from the same specimen than M7.2 showed high sequence homology to the *Agrobacterium rhizogenes-Rhizobium tropici* branch. A previous report based upon ARDRA analysis of the 16S rRNA genes of the genus *Rhizobium* and other related genera indicated that variation between 16S rDNA sequences was sufficient to permit the identification of individual species, except for *R. tropici* and *A. rhizogenes* (Laguerre *et al.*, 1994). These results correspond to phylogenetic data based on 16S rDNA gene sequencing, showing that *R. tropici* group A, *R. tropici* group B and *A. rhizogenes* are intermixed in a tight cluster (Sawada *et al.*, 1993; de Lajudie *et al.*, 1994; Dupuy *et al.*, 1994; Amarger *et al.*, 1997; Chen *et al.*, 1997; ; van Berkum *et al.*, 1998a; Wang *et al.*, 1998; Young *et al.*, 2001).The last *L. carinata* isolate (M7) grouped with the reference strains from the genus *Rhizobium* (*R. leguminosarum; R. huautlense; R. etli* and *R. mongolense*) [cluster 5 and 6] in the SDS-PAGE analysis. These results were consistent with the phylogenetic analysis, with M7 showing high sequence homology to the *R. leguminosarum-R. indigoferae-R. gallicum-R. mongolense-R. etli* branch.

SDS-PAGE analysis grouped the *L. listii* isolate (V15.2) [cluster 5a] with the *Rhizobium* reference strains *R. mongolense* and *R. huautlense*. These results were supported when phylogenetic analysis showed V15.2 to share high sequence homology with *R. huautlense*.

Electrophoretic groupings of whole cellular protein profiles grouped the two isolates WK7 (*Aspalathus* sp.) and NK27 (*L. polycephala*) within cluster 25 containing the *Burkholderia* sp. reference strain, STM 815. This reference strain was described by Moulin *et al.* (2001) as the β-subclass *Proteobacterium* nodulating *Aspalathus* sp. The authors subsequently showed, based on 16S rDNA sequence homology, that isolate STM 815 is phylogenetically closest related to *Burkholderia graminis*. WK7 was isolated from the root nodules of an *Aspalathus* sp. due to misinterpretation that this host plant might represent a *Lotononis* sp. This isolate revealed close sequence homology to *B. graminis*, supporting SDS-PAGE results and being consistent with results indicating that *Aspalathus* sp. can also be nodulated by species closely related to *Burkholderia*. NK27 (*L. polycephala*), showed an even higher phylogenetic relationship to *B. graminis*, again consistent with the results obtained from SDS-

PAGE analysis. It was decided to include most members of *Burkholderia* genus to get a clear picture of the identity of strains from this study. Species like *B. cepacia* and *B. vietnamensis* showing relatively distant relatedness to our strains has previously been described as symbionts capable of successful nitrogen fixation (Baldani *et al.,* 2000; Santos *et al.,* 2001; Cruz *et al.,* 2001; etc.).

To conclude, it was shown that isolates obtained from the same root nodule (M7, M7.1, M7.2) constituted three different genera within the *Rhizobiaceae*. This is in corroboration with previous reports indicating that diverse rhizobia were isolated from the same leguminous host and even the same root nodule (Dreyfus and Dommergues, 1981, Dagutat, 1995, Kruger, 1998; Joubert, 2001). Furthermore it was shown that rhizobial symbionts corresponding to the β-subclass *Proteobacterial* genus, *Burkholderia*, seem to be capable of nodulating *Aspalathus* and *Lotononis* species.

CHAPTER 5

ABSTRACT:

The occurrence of rhizosphere bacteria capable of promoting growth of their associative host plants is a well-known phenomenon. *Bacillus circulans* species has been known to produce lipochitooligosaccharides (LCO's) that cause root hair deformation on soybean roots. Using various techniques such as 16S rDNA sequencing, HPLC analysis of extracellular polysaccharides, SDS-PAGE analysis and SEM/TEM electron microscopy we identified and characterized three novel filamentous *Bacillus* isolates as the rhizosphere inhabitants of leguminous shrubs. HPLC purification of a plant-growth promoting LCO proved to be similar to that reported for *Bacillus circulans*. LCO activity was tested by inducing root hair deformation on soybean roots.

Keywords: *Bacillus*, filamentous, lipochitooligosaccharide, root hair deformation

CHAPTER 5

NOVEL FILAMENTOUS *BACILLUS* ISOLATES CAPABLE OF INDUCING ROOT HAIR DEFORMATION BY THE PRODUCTION OF AN EXTRACELLULAR LIPOCHITOOLIGOSACCHARIDE (LCO).

5.1 INTRODUCTION

The emergence of rhizosphere inhabitants eliciting plant-growth promotion or stimulation other than the conventional rhizobia-legume symbionts, is well documented (Santos *et al.,* 2001). Different free-living rhizobacteria such as *Azospirillum* ssp. (Dobbelaere *et al.,* 2001, Kloos *et al.,* 2001), *Bacillus* ssp. (Lian *et al.,* 2001), *Klebsiella* spp. (Riggs *et al.,* 2001) and *Paenibacillus* spp. (Seldin *et al.,* 1998) and *Burkholderia* spp. (Santos *et al.,* 2001) have already been identified and their positive attributes towards different plants described. Most of these organisms make use of the same molecular strategies as those of known symbionts to accomplish a beneficial association with their plant partner. Examples of these strategies include the production of signal molecules similar to that produced by rhizobia and leaching of roots allowing more effective ion transport. The production of lipochitooligosaccharides (LCO's) is an example whereby organisms mimic the so-called Nod-factors produced by rhizobia. LCO's are host-specific rhizobial-to-plant signals and its interaction with legume root hairs has been studied in various legumes (Relic *et al.,* 1993; Heidstra *et al.,* 1994). These oligosaccharides have various effects on plant morphogenesis (Spainck, 1992). LCO's cause root hair deformation by targeting hydrolytic enzymes on root hair tips or reinitiating root hair growth on existing root hairs (Heidstra *et al,* 1994). These molecules can also depolarize root hair membrane potential (Ehrhardt *et al.,* 1992) and influence ion fluxes, particularly Ca^{2+} (Felle *et al.,* 1999).

Recently, Lian *et al.* (2001) reported the production of a compound by the silicate bacterium *Bacillus circulans* which corresponds to the lipochitooligosaccharide, Nod *Bj* V (C18:1 MeFuc) produced by the rhizobial soybean symbiont *Bradyrhizobium*

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japonicum. The authors subsequently proved that a purified fraction of this compound was capable of inducing root hair deformation on soybean. This was the first time such activity by a LCO was reported for bacteria other then those belonging to the *Rhizobiaceae.*

During the isolation of root nodule bacteria associated with *Lotononis* spp. (Chapter 3, section 3.2.1) highly filamentous, endospore-forming, Gram positive strains were repeatedly obtained. The repeated occurrence of these morphologically unique strains as possible contaminants prompted further characterization. These isolates were specifically characterized and investigated for plant-growth promoting factors. We subsequently report that the isolates showed very high phylogenetic relatedness to the newly described *B. funiculus* species and reveal a unique morphological nature. The isolates were also capable of synthesizing a LCO compound in the presence of genistein, similar to that previously reported for *B. circulans.* Purified fractions of this compound were shown to cause root hair deformation on soybean roots.

5.2 MATERIALS AND METHODS

5.2.1 Strains used and growth conditions

Strains NK1.2, NK20.3 and NK37.3 were isolated whilst attempting to isolate root nodule bacteria from surface-sterilized root nodules obtained from the legume genus *Lotononis.* Root nodules were surface-sterilized for 7 min using 50% NaOCl and washed repeatedly with sterilized distilled water. Cultures were grown on yeast extract mannitol (YM) agar (Vincent, 1970), supplemented with 0.5% (w/v) peptone, 0.5% (w/v) glucose and 20% (v/v) soil extract. One hundred g of soil, from which the plants were originally collected, was mixed in 1 L of water, autoclaved and filtered to prepare the soil extract. Favourable growth was obtained after incubating plates for 4 to 5 d at 28 °C. The ability of strains to fix atmospheric nitrogen was tested by growth on nitrogen-free medium, *Azotobacter* medium (ATCC Medium 240) [Appendix A2]. Reference strains used in SDS-PAGE analysis are listed in Table 5.1.

Table 5.1 Reference strains used in this study

Strain	Species	Origin	Reference
Btl1	*B. thuringiensis*	South Africa	Gift from P. L. Steyn[*]
ATCC 10702	*B. cereus*	South Africa	Lindsay *et al.*, 2002
B.aiz	*B. thurigiensis* bv. aizawai	South Africa	Gift from P. L. Steyn
B.kur	*B. thuringiensis* bv. kurstaki	South Africa	Gift from P. L. Steyn
Bta	*B. thurigiensis*	South Africa	Gift from P. L. Steyn
NAF001[T]	*B. funiculus*	Japan	Ajithkumar *et al.*, 2002
NAF002	*B. funiculus*	Japan	Ajithkumar *et al.*, 2002
B-Rus	*B. subtilus*	South Africa	Gift from P. L. Steyn
DL5	*B. cereus*	South Africa	Lindsay *et al.*, 2002
Strain 168	*B. subtilus*	NS	Gift
NK1.2	*B. funiculus*	South Africa	This study
NK20.3	*B. funiculus*	South Africa	This study
NK37.3	*B. funiculus*	South Africa	This study

[*] P. L. Steyn is a professor emeritus in the department for Microbiology and Plant Pathology at the University of Pretoria, Pretoria, South Africa.

NS   Not stated

5.2.2   Electron and phase microscopy

Strains NK1.2, NK20.3 and NK37.3 were observed by scanning (SEM) and transmission (TEM) electron microscopy. Cell suspensions were diluted with 0.1% glutaraldehyde solution (0.075 M phosphate buffer, pH 7.2) and centrifuged at 1280 x g for 10 min. The supernatant was discarded and the pellet resuspended in 2.5 % glutaraldehyde (0,075 M phosphate buffer, pH 7.2) and fixed for 1 h before pelleting at 12 000 x g for 10 min. The pellets were washed twice with 0.075 M phosphate buffer (15 min) after which it was fixed in 1 % osmium tetroxide and washed twice in 0.075 M phosphate buffer (15 min). The samples were then dehydrated sequentially with 30 %, 50 %, 70 % and 100 % ethanol, each step repeated three times. For SEM, a fraction of the prepared samples was then critical-point-dried in CO_2 and sputter-coated with gold prior to SEM analysis. Samples were analyzed on a JOEL 840 SEM using 5 kV and a magnification of 5, 000x. For TEM analysis, the remaining samples were sequentially infiltrated with 30 %, 60 %, 90 % and 100 % Quetol resin (ref), each step repeated three times. Samples were then embedded in 100 % Quetol resin and polymerized at 60 °C for 48 h. Sections (90 nm) were prepared with an

ultramicrotome and mounted on copper grids, contrasted with uranyl acetate (5 min) and lead citrate (2 min). Samples were studied using a Phillips 301 TEM. Phase contrast microscopy was done by photographing the cell suspension with a Nikon Digital Camera DXM1200, using a Nikon Optiphot microscope and a magnification of 1000x.

5.2.3 PHBA staining

The presence of polymeric beta-hydroxybutyric acid (PHBA) granules was determined by staining cells with Sudan Black B, 0.3 % (w/v) in 60 % ethanol for 10 min. After rinsing with water, contrast staining was performed using Safranin O, 0.5 % w/v aqueous. Light microscopy was used to detect the presence of PHBA granules.

5.2.4 DNA extraction, amplification and sequencing of the 16S rRNA gene

Cultures were grown in YM broth supplemented with 0.5 % (w/v) peptone, 0.5 % (w/v) glucose and 20 % (v/v) soil extract (as previously described) for four to six d. Genomic DNA was extracted as previously described by Lemanceau *et al.* (1995). Depending on the optical density at 620 nm, between 200 and 1500 μl of each suspension was pelleted. Total cellular DNA was extracted by the incubating cell pellets in 100 μl distilled water with the addition of 100 μl Tris-HCl (10 mM) and 10 μl Proteinase K (15.6mg/ml) overnight at 55 °C. The activity of proteinase K was inactivated by heating the samples to 96 °C for 10 min. Cell debris were removed by centrifugation at 12 000 x *g* for 8 min and the DNA-containing supernatant stored at – 20 °C until needed.

The 16S rDNA genes of strains NK1.2, NK20.3 and NK 37.3 were amplified using the primers fD1SHRT and rP2SHRT. These primers are derived from the universal primers fD1 and rP2 (Weisburg *et al.*, 1991), lacking the linker sequences containing restriction enzyme recognition sites present in the original primers. Amplification was performed in a Perkin Elmer GeneAmp PCR System 2400 apparatus with a temperature profile as described previously (Laguerre *et al.*, 1994). The resulting PCR products were purified using a Qiagen purification kit according to the

manufacture's instructions. For sequencing two internal primers: 16SRNAII-S and 16SRNAVII-S (Kuhnert *et al.*, 1996) was used to obtain nearly full-length 16S rDNA sequence data. The purified PCR products were sequenced directly using an ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems). The resultant reaction products were run on an ABI Prism model 377 DNA sequencer. The sequences were edited and overlapped using the ABI Prism Sequencing Navigator 1.0.1 computer programme. Phylogenetic relationships were determined by comparing the sequencing data with published sequences of relevant members within the *Bacillaceae*, obtained from the GenBank sequence database, (*http://www.ncbi.nlm.nih.gov/GenBank/*). The phylogenetic tree was constructed from distance matrix using the neighbour-joining method of Saitou and Nei (1987). The bootstrap method (Felsenstein 1985) was used in combination with the neighbour-joining method to estimate confidence levels of the phylogenies. The phylogenetic tree was displayed using Njplot (Perrière and Gouy, 1996).

5.2.5   SDS-PAGE analysis of whole cellular protein extracts

Strains (Table 5.1) were allowed to grow in YM broth supplemented with 0.5% (w/v) peptone, 0.5 % (w/v) glucose and 20 % (v/v) soil extract (as described in section 5.2.1) for five d at 28 °C. Whole-cell proteins were prepared by disrupting cells using a Cole-Palmer ultrasonic homogenizer (Series 4710) at 50 % maximum output (40 Watt) for 15 s. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970), as modified by Kiredjian *et al.*, (1986) using a Biorad Protean II system. Electrophoretic patterns of the proteins were scanned with a Hoefer GS300 densitometer (Hoefer Scientific Instruments, San Francisco). Data were analyzed with the GelCompar 3.0 Program (Applied Maths, Kortrijk, Belgium). Normalized densitometric traces were grouped and the similarities calculated between all organisms using the Pearson product moment correlation coefficient (r), converted to a percentage. The cluster analysis was performed using the unweighted pair group method of arithmetic averages (UPGMA).

5.2.6 Production and isolation of LCO-like compound from filamentous *Bacillus* strain NK37.3.

The production and isolation of a LCO-like compound was done as previously described (Lian *et al.*, 2001). A culture of strain NK37.3 was grown in YM broth supplemented with 0.5 % (w/v) peptone, 0.5 % (w/v) glucose and 20 % (v/v) soil extract (as described in section 5.2.1) per liter of medium. The culture was incubated with agitation at 28°C until an OD_{620} of between 0.4 and 0.6 was reached (3-4 d). Twenty milliliters of this culture was used as inoculum per 1000 ml of media to prepare 2 l of bacterial subculture. This subculture was incubated with agitation for 5 d at 28°C. At this stage genistein (5 µM) was added as a possible inducer of LCO or LCO-like compound production and the subculture was further incubated for an additional 2 d. Phase partitioning of the bacterial subculture was done by adding 400 ml of HPLC-grade 1-butanol per liter of subculture and incubation overnight. The upper butanol layer was transferred to a one to one evaporation flask and concentrated to 2 to 3 ml of light brown, viscose material with a rotary evaporator operated at 80 °C (Büchi RE120, Germany). The resultant extract was resuspended in 5 ml of 18 % acetonitrile and stored in the dark at 4 °C until needed.

HPLC analysis (Jupiter 5µ C5 300 Å, 4.6 mm x 25 cm, Phenomenex, USA, flowspeed: 1ml/min) was conducted with a Nova-Pak C18 reverse-phase column (Waters Millipore). The detector was set at 214 nm. As a baseline, 18% acetonitrile (AcN/H_2O; w/w) was run through the system for at least 40 min prior to injection. The sample was loaded and isocratic elution was conducted for 90 min with 18-82% AcN. The LCO-like compound was eluted at 90 to 94 min of HPLC run time.

5.2.7 Root hair deformation by purified LCO-like compound

The ability of the putative LCO-like compound to induce root hair deformation was tested as previously described (Prithiviraj *et al.*, 2000). Germinated soybean (*Glycine max*) seeds were used to obtain root hairs. These seeds were surface sterilized in 2 % sodium hypochlorite for 2 min, followed by three successive washing steps with sterile distilled water. These surface sterilized seeds were allowed to germinate for 6

d at 25 °C in Petri dishes containing 1.5 % water agar. The lateral roots emerging from the geminating seeds were excised with a sharp scalpel blade and placed on microscopic slides containing 50 µl of the test solution produced in section 5.2.6. These slides were incubated at 25 °C in moist chambers for another 24 h. The roots were stained with methylene blue stain containing 0.02 % (w/v) methylene blue, 20 % (v/v) glycerol and 10 % (w/v) phenol and observed under a UV light microscope for root hair deformation.

5.3 RESULTS

Using the dilution method, isolates obtained from surface-sterilized root nodules were streaked out on YMA plates. Colonies appeared after 5 d of incubation at 28 °C. It became apparent that extremely filamentous, endospore-forming isolates could be distinguished, initially thought to be contaminants able of surviving the sterilization step. The same type of colonies was however seen for two other isolates, which led to the investigation of these isolates. On YMA these strains seem to have circular colony shape with a cream colour and distinct odour. Furthermore, these strains were capable of growth on nitrogen-free media indicating their capability to fix atmospheric nitrogen.

5.3.1 Microscopy

Scanning electron microscopy (SEM) revealed that the three putative *Bacillus* isolates had an extremely filamentous nature and were morphologically unique compared to most other members of the *Bacillus* genus (Figure 5.1). The filaments appeared to be branched and surrounded by extracellular slime with the vegetative cells contained within these structures (Figure 5.1). Transmission electron microscopy (TEM) showed numerous intracellular granules within individual cells. These were subsequently identified as polymeric beta-hydroxybutyric acid (PHBA) upon staining with Sudan Black (Figure 5.2). The TEM results corresponded to those previously reported for the filamentous *Bacillus* sp., *B. funiculus* (Ajithkumar *et al.*, 2001). An

'intermediate filamentous' form of growth was observed when the isolates were repeatedly streaked out. The isolates did not completely lose their filamentous
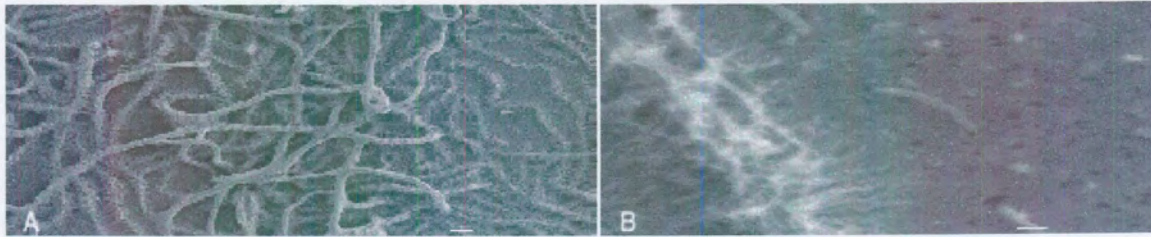
Figure 5.1 Scanning electron microscopy micrographs of strain NK37.3. (A) Extreme filamentous structures formed by *Bacillus* isolates, showing numerous branching, Bar = 10 μm. (B) Higher resolution micrograph showing filaments surrounded by extracellular slime/polysaccharides, Bar = 1 μm.

Figure 5.2 A transmission electron microscopy micrograph of strain NK37.3 indicating two cells contained within a filament. Note the abundance of intracellular PHBA.
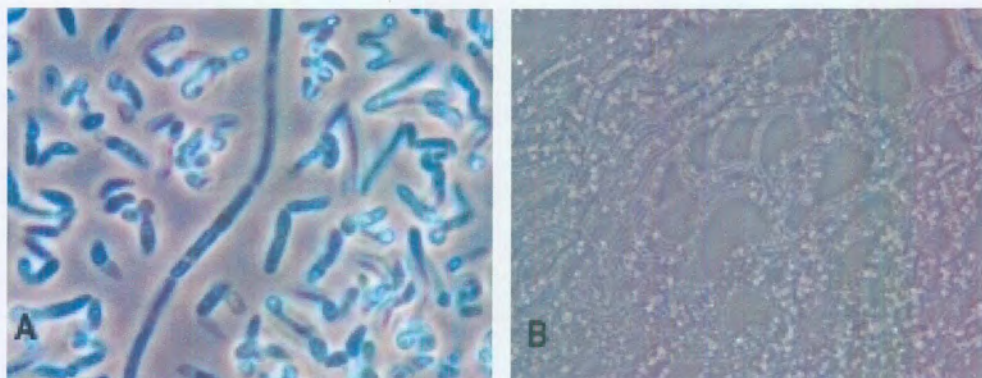
Figure 5.3 (from previous page) Phase contrast microscope micrographs indicating (A) the 'intermediate filamentous' form of growth and (B) the highly filamentous form as observed for strain NK 1.2.

structures, but tended to form shorter filament structures. This is shown in Figure 5.3 as observed by phase microscopy.

5.3.2 Phylogenetic analysis based on 16S rDNA sequence homology

Nearly full-length 16S rDNA sequences (1270 bp) of all three isolates (NK1.2, NK20.3 and NK37.3) were determined. These isolates had identical 16S rDNA sequences and were related to members of the *Bacillaceae* (Figure 5.4). The isolates formed a distinct cluster with *B. funiculus*, showing an overall sequence homology of 99.99 %. Related sequence homology values for this cluster were *B. cohnii* (95.1 %); *B. cereus, B. megaterium, B. halmapalus* and *B. simplex* (94.9 %). *B. mycoides* and *B. weihenstephanensis* showed 94.7 % relatedness. The silicated *Bacillus* species, *B. circulans*, showed 91.9 % similarity to the cluster. Filamentous members of the *Bacillaceae* (*B. stearothermophilus, B. caldovelox* and *Geobacillus stearothermophilus*) included in the analysis showed no significant relationship (86 %) to the cluster.

5.3.3 SDS-PAGE analysis of whole cellular protein extracts

SDS-PAGE analysis grouped the isolates and reference strains under investigation in three clusters and 1 outgroup (4) (Figure 5.5).

Cluster 1

Cluster 1 encompassed four different strains of *B. thurigiensis*, the *B. cereus* reference strain ATCC 10702, and strain NAF002 representing the newly described filamentous species, *B. funiculus*. Strain NAF002 showed the highest similarity to *B. thuringiensis* strain Bta with a similarity value of 72.7 %.
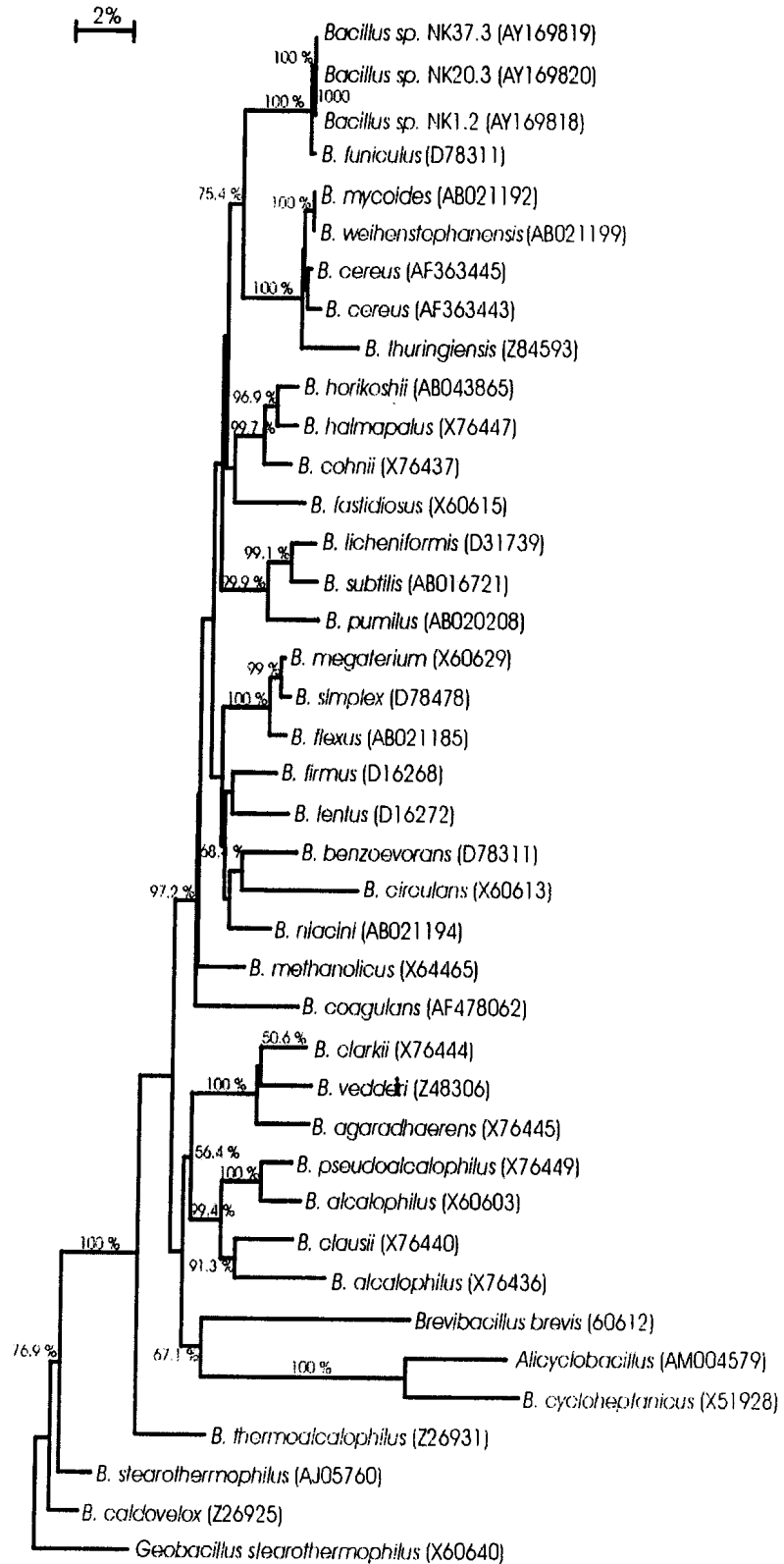
Figure 5.4 Phylogenetic analysis of 16S rRNA gene sequences showing the relationship among the three filamentous isolates obtained from this study and selected members of the *Bacillaceae*. The significance of branches is indicated by bootstrap values. The accession numbers of all the strains are shown in parenthesis. Bar = estimated substitutions per nucleotide position.

Cluster 2

Cluster 2 grouped all three filamentous *Bacillus* strains , NK1.2, NK20.3 and NK37.3 and the *B. subtilus* strain B-Rus. The 'intermediate filamentous' form of NK1.2 was also included in order to determine if this feature influenced whole cellular protein content. The profile of the 'intermediate filamentous' form joined the highly filamentous NK1.2 and NK20.3 at a similarity of 89.9%. NK37.3 had the highest similarity to the 'intermediate form' of isolate NK1.2 at 75.8%.

Cluster 3

Cluster 3 grouped the type strain described for *B. funiculus* (NAF001) joining the *B. cereus* strain DL5 at a similarity value of 65%.

Outgroup (4)

The outgroup consisted of the one strain (*B. subtilus* 168).
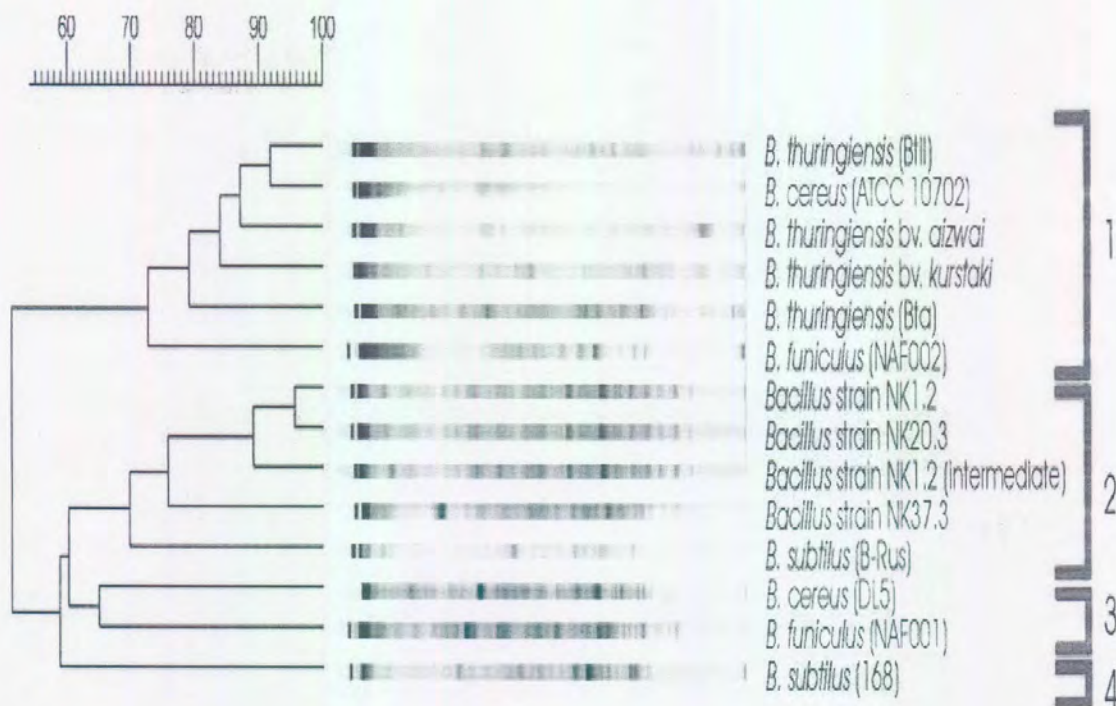
Figure 5.5 A dendrogram based on UPGMA analysis of the correlation coefficients (r) between protein profiles of the three isolates obtained in this study and other selected *Bacillus* species.

5.3.4 Production and isolation of a LCO-like compound

The LCO-like compound was isolated from genistein-induced cultures of the filamentous *Bacillus* isolate NK37.3. Fractionation of the butanol extract by HPLC led to the identification of a peak that had a retention time of between 90 to 94 min as previously described for LCO-like compounds (Lian *et al.*, 2001; Prithiviraj *et al.*, 2000) [Figure 5.6]. This fraction was collected for biological activity study.
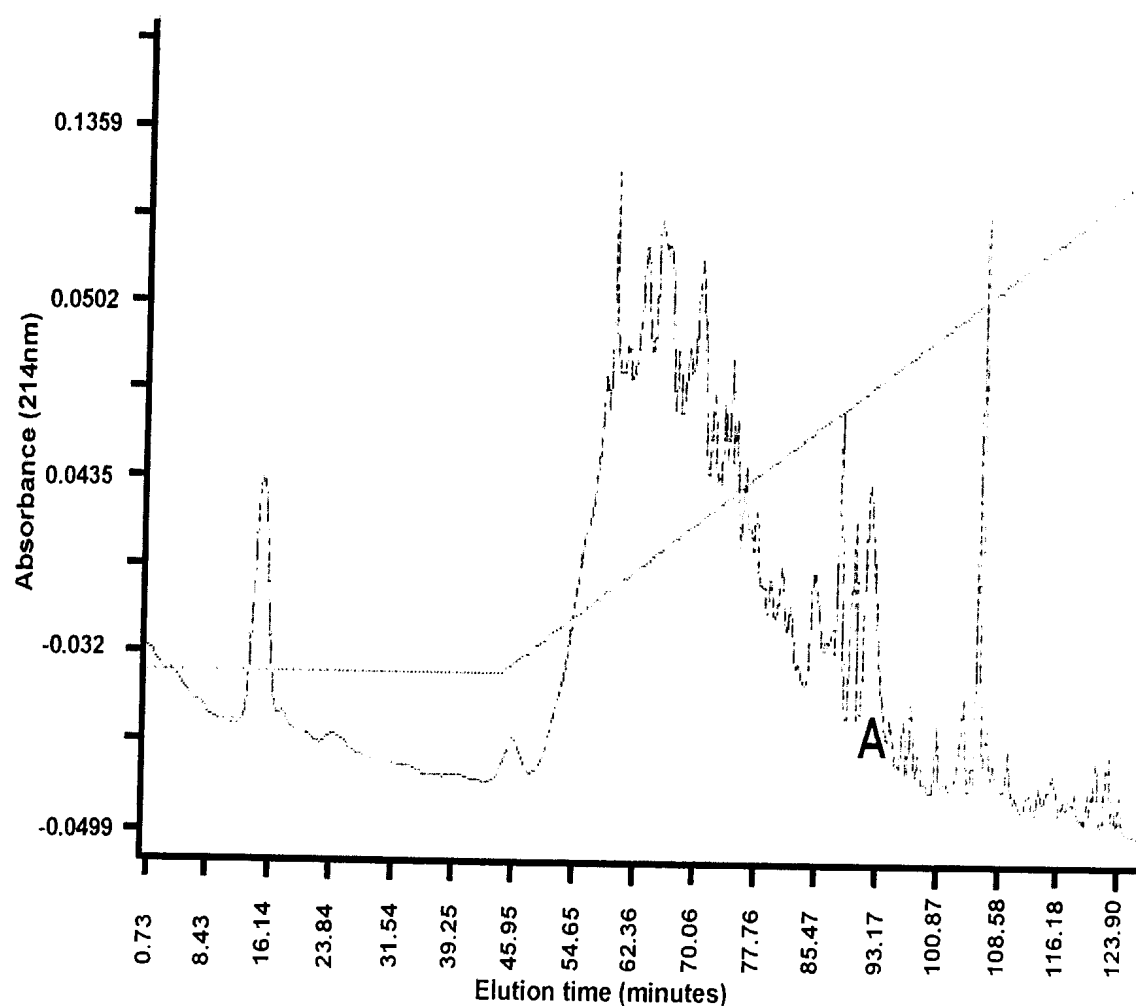
Figure 5.6 A graph illustrating the fractionation of extracellular polysaccharide extract. The LCO-like compound was eluted at 90 to 94 minutes (A).

5.3.5 Root hair deformation assay

The biological activity of the LCO-like compound was assayed making use of the soybean root hair deformation assay (Lian *et al.*, 2001). A morphological change similar to that previously observed for LCO's was obtained. The LCO-like compound isolated in this study seems to cause bulging at the root hair base (Figure 5.7).
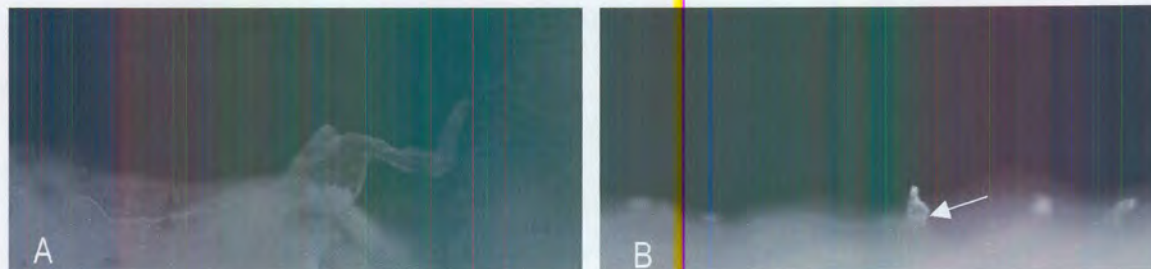
Figure 5.7 Root hair deformation induced by the isolated LCO-like compound produced by filamentous isolates obtained in this study. The control showing a normal root hair (A) and the treated root hairs (B) showing bulging at the base of the root hair indicated with an arrow.

5.4 DISCUSSION

NK1.2, NK20.3 and NK37.3 were found to be natural soil bacteria associated with plant rhizospheres. To our knowledge, extensive filamentous structures as reported here, have not been described for *Bacillus* species occurring naturally in soil. Filamentous structures seem to be less dense and evident after repeated streaking of the isolates on the same media. This 'intermediate filamentous' form might be the result of the rich media on which the isolates were grown. Filamentous structures are normally a more evident characteristic when organisms experience nutrient deficiencies (Eikelboom, 1975). Small subunit RNA gene sequencing proved that the

'intermediate filamentous' form represented the same organisms as the highly filamentous form (results not shown).

Phylogenetically the isolates showed the highest similarity to the recently described *B. funiculus* (Adjithkumar et al., 2002). *B. funiculus* was also described as a highly filamentous species isolated from activated sludge. In contrast our isolates formed branching filaments and did not produce spore-like resting cells (SLRC's) as reported for *B. funiculus*, but rather endospores. *B. funiculus* also showed no phases of intermediate growth or loss of filamentous structures (Ajithkumar *et al.*, 2001).

Different electrophoretic groupings for the same species within the *Bacillaceae* are normally the result of intraspecific variation (Heyndrickx *et al.*, 1996), and as a result same species might show low similarity values. The divergence that was observed in the electrophoretic grouping of the two *B. cereus* strains ATCC 10702 and DL5 supports these findings. Strains NAF001 and NAF002 (*B. funiculus*) proved only distantly related towards each other and towards our strains as determined by their whole cellular protein content. Ajithkumar *et al.* (2002) reported that these two strains showed particular preferences towards growth media, indicating the existence of physiological divergence between them. Results from SDS-PAGE data emphasized the high degree of relatedness between strains NK1.2, NK20.3 and NK37.3. The high similarity value that the 'intermediate filamentous' form of NK1.2 showed towards the other strains indicates that this phase of growth slightly alters the intracellular protein content and merely reflects a physiological adaptation. The high similarity values might indicate that the three isolates NK1.2, NK20.3 and NK37.3 represent a more homogenous genotypic group than NAF001 and NAF002.

The fact that the described three strains were isolated from the rhizospheres of three different plants suggests that they might have a significant function coupled with this preferred habitat. The biological activity of the LCO-like compound identified by HPLC provides circumstantial evidence that it might resembles (at least in terms of function) a LCO similar to those previously described (Lian *et al.*, 2001; Prithiviraj *et al.*, 2000).

CHAPTER 6

CHAPTER 6
CONCLUDING REMARKS

The taxonomy of the family *Rhizobiaceae* experienced important advances in recent years. Numerous studies to clarify the taxonomy and phylogeny of members of this family are now being carried out resulting in many proposals for new species. This has been stimulated both by the isolation from new sources and by the recent advances in molecular methods applied in taxonomy. Subsequently, rhizobia are currently encompassing a more diverse group of organisms than anticipated previously.

Despite the diversity of organisms identified as nodulating symbionts of leguminous plants, it is evident that in some instances a high degree of specificity exists for this association in order to be successful. Various molecular signals for both the bacterial and the plant symbiont have been identified as essential for nodulation and nitrogen fixation to occur. It would thus be reasonable to assume that the rhizobial symbionts associated with one specific legume genus might give a better reflection of the various aspects involved in the symbiotic process than those obtained from various unrelated legume genera.

SDS-PAGE analysis of whole cellular protein extracts is a useful classification tool for determining the relationships among large groups of closely related strains having potential species status and provides differentiation at intraspecies level. SDS-PAGE data should, however, be supplemented with additional techniques such as genotypic data to get clarity on the exact taxonomical status of clusters above the species level.

The diverse nature of rhizobia associated with *Lotononis* was clearly illustrated by the different rhizobial genera that were in some instances isolated from the same root nodule. Furthermore, the genus *Lotononis* represents host species capable of forming symbiosis with various genera within the *Rhizobiaceae*, which include both fast- and slow-growing genera. This would emphasize the broader host range characteristics of the genus *Lotononis*.

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It was also shown that members of the genus *Lotononis* are nodulated by members outside the *Rhizobiaceae* family. Some *Lotononis* species showed to be nodulated by methylotrophic bacteria showing close relatedness to the species *Methylobacterium nodulans* (Sy *et al.*, 2001). A few *Lotononis* species were also shown as hosts of members of the β-subclass *Proteobacterium* genus, *Burkholderia* (Moulin *et al.*, 2001).

Results from this study revealed isolates showing some degree of similarity to *A. caulinodans*. No indigenous strains of *A. caulinodans* have previously been reported from South African soils. The exact taxonomical status of these isolates should be determined in future.

Loosely associated isolates in SDS-PAGE data not showing particular affinity towards any of the reference strains should be genotypically investigated to clarify their taxonomical status.

Three novel filamentous *Bacillus* isolates were characterized in this study. It was subsequently shown that these isolates were capable of altering root hair morphology by the production of a lipochitooligosaccharide, a molecule similar to that being produced by rhizobia. These isolates might prove as plant growth-promoting towards agricultural important crops and their applicability should be assessed in future.

The bacterial symbionts of a large number of indigenous legumes have not been studied systematically. The increasing isolation of such symbionts is a strategy that will improve our knowledge concerning the diversity of indigenous rhizobia. The supplementation of classical taxonomical approaches applied in rhizobial taxonomy with both phylogenetic and phenotypic traits would ensure a systematic system reflecting a better understanding as to the diversity of indigenous rhizobia. Many legume root nodule bacteria lacking typical rhizobial characters have in the past been discarded as contamination during isolation. Caution should be taken for repetition of such mistakes, especially when dealing with unfamiliar tropical legume species. This predicts that several new groups of legume symbiotic bacteria (genera and species) may emerge in the future.

CHAPTER 7

CHAPTER 7

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APPENDIX

APPENDIX

Table A1 The corresponding sections and subsections in which *Lotononis* species are classified.

Section	Subsection	Species
1. Listia	1A. Macrocarpa	*L. macrocarpa*
	1B. Listia	*L. solitudinis*
		L. subulata
		L. marlothii
		L. angolensis
		L. minima
		L. listii
		L. bainesii
2. Digitata		*L. plicata*
		L. digitata
		L. quinata
		L. longiflora
		L. benthamiana
		L. magnifica
3. Lipozygis	3A. Bracteolata	*L. procumbens*
		L. difformis
	3B. Lipozygis	*L. sutherlandii*
		L. eriantha
		L. grandis
		L. corymbosa

Table A1 (Continued)

Section	Subsection	Species
		L. pulchra
		L. lanceolata
		L. foliosa
		L. spicata
4. Leptis		*L. tapetiformis*
		L. mirabilis
		L. mollis
		L. arida
		L. pusilla
		L. prolifera
		L. mucronata
		L. pariflora
		L. stolzii
		L. decumbens
		L. carinata
		L. wilmsii
		L. adpressa
		L.calycina
		L. acuticarpa
		L. lupinifolia
		L. genistoides
		L. maroccana
		L. bullonii
5. Leobordea		*L. platycarpa*
		L. newtonii

Table A1 (Continued)

Section	Subsection	Species
		L. bracteosa
		L. stipulosa
		L. schoenfelderi
		L. furcata
6. Synclistus		*L. oligocephala*
		L. laticeps
		L. globulosa
		L. longicephala
		L. pentaphylla
		L. rosea
		L. bolusii
		L. anthyllopsis
7. Oxydium	7A. Distans	*L. pallens*
	7B. Delicata	*L. delicata*
		L. pseudodelicata
	7C. Tenuis	*L. tenuis*
	7D. Falcata	*L. strigillosa*
		L. falcata
		L. parviflora
		L. fruticoides
		L. sabulosa
		L. linearifolia
		L. pachycarpa
	7E. Striata	*L. schreiberi*
	7F. Rosea	*L. pallidirosea*
	7G. Brachyantha	*L. curtii*

Table A1 (Continued)

Section	Subsection	Species
		L. brachyantha
		L. crumanina
		L. burchellii
		L. maculata
	7H. Laxa	*L. macrosepala*
		L. laxa
		L. serpentinicola
	7I. Oxydium	*L. glabra*
		L. monophylla
	7J. Pumila	*L. micrantha*
		L. pumila
	7K. Quinata	*L. acutiflora*
	7L. Rostrata	*L. rostrata*
		L. arenicola
		L. oxyptera
		L. perplexa
		L. stenophylla
		L. carnea
	7M. Fragilis	*L. rabenaviana*
		L. sparsiflora
	7N. Oppositiflora	*L. lenticula*
8. Monocarpa		*L. venosa*
		L. leptoloba
		L. maximiliana
9. Cleistogama		*L. pungens*

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Table A1 (Continued)

Section	Subsection	Species
		L. tenella
10. Euchlora		*L. hirsuta*
11. Polylobium		*L. racemiflora*
		L. fastigiata
		L. involucrata
		L. brevicaulis
12. Lotononis		*L. lamprifolia*
		L. acuminata
		L. gracilifolia
		L. argentea
		L.prostrata
		L. villosa
		L. varia
		L. complanata
		L. elongata
		L. azurea
		L. filiformes
13. Aulacinthus		*L. dissitinodis*
		L. dahlgrenii
		L. rigida
		L. densa
		L. viborgioides

Table A1 (Continued)

Section	Subsection	Species
		L. comptonii
		L. acocksii
		L. nutans
		L. exstipulata
		L. azureoides
		L. umbellata
		L. purpurescens
14. Krebsia	14A. Krebsia	*L. eriocarpa*
		L. stricta
		L. sericophylla
		L. divaricata
		L. jacottetii
		L. galpinii
		L. minor
		L. caerulescens
	14B. Glabrifolia	*L. dichiloides*
		L. bachmanniana
		L. carnosa
		L. pottiae
15. Buchenroedera	15A. Racemosa	*L. lotonoides*
		L. amajubica
		L. glabrescens
		L. virgata
		L. viminea

Table A1 (Continued)

Section	Subsection	Species
	15B. Buchenroedera	*L. pulchella*
		L. trichodes
		L. alpina
		L. meyeri
		L. holosericea
		L. harveyi

A2

Azotobacter Medium (ATCC Medium 240)

Composition per liter:

Agar	15.0 g
$MgSO_4.7H_2O$	0.2 g
KH_2PO_4	0.15 g
K_2HPO_4	0.05 g
$CaCl_2$	0.02 g
$Na_2MoO_4.2H_2O$	2.0 mg
$FeCl_3$	1.0 mg