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GENERIC IDENTITY OF PUTATIVE RHIZOBIAL ISOLATES AS
DETERMINED BY PCR-RFLP OF 16S rDNA AND
SELECTEDPHENOTYPIC PROPERTIES

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GENERIC IDENTITY OF PUTATIVE RHIZOBIAL ISOLATES AS DETERMINED BY PCR-RFLP OF 16S rDNA AND SELECTED PHENOTYPIC PROPERTIES

by

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I certify that the thesis hereby submitted to the University of Pretoria for the degree M. Sc. (Agric) Microbiology has not previously been submitted by me in respect of a degree at any other university.

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DEPARTMENT: Microbiology and Plant Pathology

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SUMMARY

The taxonomy of root nodule bacteria is in a state of transition, primarily as a result of the increased isolation of rhizobia from legumes not investigated before and the advances made in molecular methods for classification of bacteria (Young, 1996). In order to ensure a stable taxonomy, it is important to include representative South African isolates. The first comprehensive study of indigenous rhizobia was done by Dagutat (1995). More than 300 putative rhizobia were isolated from diverse leguminous hosts and diverse geographic regions, and characterized with sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins. Although a large group of these isolates showed similarity to previously described genera, possible new taxa were recognized as many isolates did not show any relatedness to authentic rhizobial strains included. The question arose whether these isolates were rhizobia capable of symbiotic nitrogen fixation or merely opportunistic endosymbionts.

The aim of this study was to further characterize putative indigenous rhizobia at a genetic level with restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA, and at a phenotypic level with the Biolog system (i.e. oxidation of 95 substrates), and to evaluate these methods as rapid, reliable tools for characterization of new rhizobial isolates. The collection of indigenous rhizobia was also expanded with the isolation of rhizobia from diverse leguminous hosts.

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Ninety six indigenous isolates and 35 reference strains were first characterized with SDS-PAGE of whole cell proteins, and representative strains were selected for further analysis with PCR-RFLP and the Biolog system. Although most of the indigenous isolates investigated were slow-growers belonging to the genus *Bradyrhizobium*, some isolates did show close relationship to species of the genera *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*. Characterization of isolates with 16S rDNA PCR-RFLP analysis and the Biolog system showed good agreement with results obtained with SDS-PAGE. Close relationships between strains could be distinguished with SDS-PAGE, whereas PCR-RFLP and Biolog analysis were limited in the distinction of closely related strains. PCR-RFLP and Biolog proved to be valuable tools for the differentiation of rhizobia at species and higher level.

GENERIESE IDENTITEIT VAN SOGENAAMDE RHIZOBIUM ISOLATE SOOS BEPAAL DEUR PKR-RFLP VAN 16S rDNS EN GESELEKTEERDE FENOTIPIESE EIENSKAPPE

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OPSOMMING

Die taksonomie van wortel-nodulerende bakterieë verander voortdurend, hoofsaaklik as gevolg van 'n toename in die isolasie van rhizobia uit peulplante wat nie voorheen ondersoek is nie, asook die vooruitgang in molekulêre metodes vir klassifikasie van bakterieë (Young, 1996). Om 'n stabiele taksonomie te verseker, is dit nodig om Suid-Afrikaanse isolate in te sluit. Die eerste volledige ondersoek na Suid-Afrikaanse rhizobia is gedoen deur Dagutat (1995). Meer as 300 rhizobia is geïsoleer uit 'n verkeidenheid peulplant-gashere oor 'n wye geografiese gebied en gekarakteriseer met natrium dodesiel sulfaat poli-akrielamied gel elektroforese (SDS-PAGE) van heelselproteïene. Alhoewel 'n groot groep van die isolate verwantskap getoon het met reeds bestaande genera, het baie isolate geen assosiasie getoon met outentieke rhizobium rasse nie en kon moontlike nuwe taksa onderskei word. Die vraag het ontstaan of hierdie isolate inderdaad rhizobia in staat tot simbiotiese stikstofbinding was, of bloot net opportunistiese endosimbionte.

Die doel van hierdie studie was om sogenaamde inheemse rhizobia verder te karakteriseer op genotipiese vlak met analise van restriksiefragment lengte-polimorfismes (RFLP) van 16S rDNS geamplifiseer deur die polimerase ketting reaksie (PKR) en ook op fenotipiese vlak met die

Biolog sisteem (d.i. oksidasie van 95 substrate). Hierdie tegnieke is geevalueer as vinnige, betroubare metodes vir die karakterisering van nuwe isolate. Die versameling inheemse rhizobia is ook uitgebrei met isolasie uit verskeie genoduleerde peulplante.

Ses en negentig inheemse isolate en 35 verwysingstamme is eerstens gekarakteriseer met SDS-PAGE van heelselproteïene. Verteenwoordigende isolate is hieruit gekies vir verdere Biolog- en PKR-RFLP analise. Alhoewel meeste van die isolate wat ondersoek is, stadige-groeiers is en aan die genus *Bradyrhizobium* behoort, toon sekere isolate wel verwantskap met *Rhizobium, Sinorhizobium* en *Mesorhizobium* spesies. Resultate van PKR-RFLP en Biolog- analise het goed ooreen-gestem met dié van SDS-PAGE. Na-verwante isolate kon onderskei word met SDS-PAGE, terwyl PKR-RFLP en Biolog analise beperk was in die vermoë om hierdie isolate te onderskei. PKR-RFLP en Biolog is waardevolle tegnieke vir die onderskeiding van rhizobium isolate op spesie- en hoër vlakke.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BNF	biological nitrogen fixation
bp	base pair
bv	biovar
C	carbon
°C	degrees Celsius
cm	centimeter
DNA	deoxyribonucleic acid
e^-	electrons
EDTA	ethylenediamine-tetra-acetic acid
ERIC-PCR	enterobacterial repetitive intergenic consensus PCR
g^{-1}	per gram
GN	Gram negative
h	hour
ha	hectare
HCl	hydrogen chloride
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulphide
IAA	indole acetic acid
IGS	intergenic spacer region
kg	kilogram
LMG	Laboratorium voor Microbiologie Gent Culture Collection
M	molar
MLEE	multilocus enzyme electrophoresis
mg	milligram
ml	milliliter
mm	millimeter
m/v	mass per volume
N	nitrogen
N_2	dinitrogen

NaCl	sodium chloride
PCR	polymerase chain reaction
PGPR	plant growth promoting rhizobacteria
RAPD	randomly amplified polymorphic DNA
rDNA	ribosomal DNA
REP-PCR	repetitive extragenic palindromic PCR
rRNA	ribosomal RNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl-sulphate polyacrylamide gel electrophoresis
sp.	species
ssp.	subspecies
S_{SM}	simple matching coefficient
STB	sample treatment buffer
TY	tryptone yeast medium
U	units
UPGMA	unweighted pair group method using arithmetic averages
USDA	United States Department of Agriculture - ARS National Rhizobium Culture Collection
US$	USA dollar
µl	microliter
µm	micrometer
V	volts
v/v	volume per volume
YEB	yeast extract beef medium
YMA	yeast mannitol agar
YMB	yeast mannitol broth

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CHAPTER 1

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INTRODUCTION

The rapidly growing human population is marked by an escalation in the demand for fixed nitrogen as a nutrient for crop and pasture production. With the high costs of nitrogen fertilizers, increased application of biological nitrogen fixation (BNF) is required (Bohlool *et al.*, 1992). The assimilation of atmospheric nitrogen in root nodules of legumes was first described in 1888 by Hellriegel and Wilfarth. These bacteria were subsequently isolated and classified as *Rhizobium* by Frank in 1889 (Elkan, 1992). The development of rhizobial inoculants for crop and pasture legumes has been the main practical impact of BNF and as agriculture extends into new regions, more diverse and competitive rhizobia will be needed (Brockwell *et al.*, 1982).

In 1984, two genera, *Rhizobium* and *Bradyrhizobium*, were recognized representing the fast- and slow-growing rhizobia respectively (Jordan, 1984). Improved methods for identification and classification with regard to molecular techniques and increase in the range of leguminous hosts investigated for nodulation, led to significant changes in the taxonomy of these bacteria (Young, 1996). Bacteria able to infect leguminous plants and establish a nitrogen-fixing symbiosis are currently assigned in five genera: *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium* and *Mesorhizobium* in the alpha subdivision of the Proteobacteria.

In addition to *Rhizobium* and *Bradyrhizobium*, the genus *Azorhizobium* was described for rhizobia nodulating the stems of *Sesbania rostrata*. Only one species, *A. caulinodans*, is recognized thus far (Dreyfus *et al.*, 1988). The genus *Bradyrhizobium*, initially created for slow-growing soybean rhizobia, currently contains two species, *B. japonicum* (Jordan, 1984) and *B. elkanii* (Kuykendall *et al.*, 1992). A third species, *B. liaoningense* (Xu *et al.*, 1995) has been described but not validated.

Within the genus *Rhizobium*, three species were recognized in 1984: *R. leguminosarum* with three biovars: trifolii (*Trifolium*), phaseoli (*Phaseolus*) and viciae (*Vicia, Pisum, Lathyrus, Lens*),

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R. meliloti and *R. loti* (Jordan, 1984). Several new species have since been described : *R. galegae* for *Galega* spp. symbionts (Lindström, 1989), *R. tropici* for biovar phaseoli type II strains (Martínez-Romero *et al.*, 1991), *R. etli* for biovar phaseoli type I strains (Segovia *et al.*, 1993), *R. hainanensis* (Gao *et al.*, 1994), *R. gallicum* and *R. giardinii* for *Phaseolus* strains (Amarger *et al.*, 1997) and *R. mongolense* from *Medicago* (Van Berkum *et al.*, 1998). The latter four species have been proposed, but not recognized as *bona fide* species.

In 1984, the species *R. fredii* was described for species nodulating soybean (Scholla and Elkan, 1984), but was subsequently transferred, together with *R. meliloti* (nodulates *Medicago, Melilotus* and *Trigonella*) to the new genus *Sinorhizobium* (Chen *et al.*, 1988; De Lajudie *et al.*, 1994). In addition this genus also contains the species *S. xinjiangensis*, fast-growing soybean symbionts (Chen *et al.*, 1988), *S. saheli* and *S. teranga* from *Sesbania* and *Acacia* (De Lajudie *et al.*, 1994) and *S. medicae* from annual *Medicago* spp. (Rome *et al.*, 1996b).

Several species related to *R. loti* (isolated from *Lotus* spp.) have been described and these bacteria form a monophyletic group within the fast-growing rhizobia and has subsequently been transferred to the genus *Mesorhizobium* (Jarvis *et al.*, 1997). Species described in this genus are *M. loti, M. huakuii* for symbionts of *Astragalus sinicus* (Chen *et al.*, 1991), *M. ciceri* (Nour *et al.*, 1994) and *M. mediterraneum* (Nour *et al.*, 1995) for chickpea symbionts, *M. tianshanense* (Chen *et al.*, 1995) and *M. plurifarium* (De Lajudie *et al.*, 1998).

The first comprehensive study of the taxonomy of South African rhizobia was done by Dagutat (1995). More than 300 putative rhizobia were isolated from 147 diverse leguminous hosts and a widely diverse geographic region and characterized with sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole cell proteins. The exclusive use of symbiotic performance for the differentiation of taxa was once again questioned with the isolation of diverse strains from the same species and even the same nodule. The need to examine a wider range of legumes was emphasized with the isolation of rhizobia from three genera, *Cassia, Bauhinia* and *Schizolobium*, previously described as non-nodulated legumes. Although a large group of the indigenous isolates showed similarity to previously described genera, possibly new taxa were recognized as many isolates did not show any relatedness to authentic reference rhizobial strains.

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The question arose whether these isolates were rhizobia capable of symbiotic nitrogen fixation, or merely opportunistic endosymbionts.

The purpose of this study was to characterize indigenous rhizobial strains at both phenotypic level (SDS-PAGE analysis of whole cell proteins and the Biolog system) and genetic level (restriction fragment length polymorphism (RFLP)- analysis of PCR amplified 16S rDNA), and to assess the value of PCR-RFLP and the Biolog system for rapid and reliable characterization of new isolates.

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CHAPTER 2

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LITERATURE REVIEW

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1. INTRODUCTION

Nitrogen is an essential element needed by plants for the production of proteins, enzymes and nucleic acids, but it is the nutrient most commonly deficient that leads to substantial reductions in agricultural yields. Almost no nitrogen in mineral form is present in nature and yet, above every hectare of soil at sea level there is 78 million kilogram of inert nitrogen gas (Elkan, 1992). Atmospheric dinitrogen is converted into a biologically available substrate, ammonia, through biological nitrogen fixation or chemical reduction.

Non-biological nitrogen fixation occurs through the effect of lightning (10 million metric tons per year) and through the 80 million tons of ammonia produced annually through the Haber-Bosch process, 75% of which is available for fertilizer (Elkan, 1992). Currently, the annual cost of nitrogen fertilizer exceeds 20 billion US$ (National Research Council, 1994). Large quantities of hydrogen, usually obtained from (limited) natural gas, are needed for the industrial manufacturing of nitrogen fertilizers. A significant amount of energy is also needed to achieve the high temperature and pressure conditions required for the process (Bohlool *et al.*, 1992). Thus, with regard to environmental and economic aspects, increase in biological nitrogen fixation is mandatory (Bohlool *et al.*, 1992; Burton, 1979).

Biological nitrogen fixation is sustainable, less polluting and cheaper compared to industrial nitrogen fixation. Rhizobium-legume plant associations show the greatest promise of all systems for increasing crop yield and improving soil fertility. Nitrogen fixed in this manner, is transformed into leguminous proteins and these proteins can be consumed directly by animals and the excess returned to the soil via animal wastes, or nitrogen may be returned directly to the soil as organic mulches (Gutteridge and Shelton, 1994). Estimates of nitrogen fixation by food legumes range

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from 0 to 360 kg N/ha/year and accounts for approximately 175 million tons of fixed nitrogen per year in total (Bohlool *et al.*, 1992).

Legumes and BNF are of marked importance in developing countries where the high human population growth rate necessitates increased food production through improving yields (Elkan, 1992). Depletion of forests that leads to the scarcity of fuel wood, fodder and timber, coupled with increasing wasteland formation is a major concern of these countries. Leguminous trees and shrubs show great potential as a renewable source of fuel and wood products. Leguminous mulches are widely used as a source of nutrient-rich organic matter and as nitrogen for crops. Furthermore, it is important as high quality forages for livestock, both in cultivated pastures and in naturally occurring associations (Lal and Khanna, 1993).

Inoculation of legumes with root-nodule bacteria is one of Man's successful exploitation of micro-organisms for agricultural purposes. As more legumes come into cultivation and are agronomically improved, more diverse and better rhizobia will be needed (Brockwell *et al.*, 1982). In the past, emphasis was placed on the isolation of rhizobia from agriculturally important plants and hence there was an inadequate exploration of legumes. It is necessary to isolate rhizobia from a wide range of leguminous hosts and investigate the rhizobial symbionts for competitiveness and effectiveness (Dagutat, 1995).

The classification of the root- and stem-nodulating bacteria is changing continually, with numerous proposals for new species being made. This can be attributed to the fact that symbionts are being isolated from leguminous plants not investigated previously, as well as by the advances in molecular methods, especially DNA sequencing and DNA-DNA hybridization (Young, 1996). Proper and simpler methods for classification of the root- and stem-nodulating bacteria need to be developed. As knowledge is increased and new isolates are studied, new species are described and former genera and/or species split (Martínez-Romero and Caballero-Mellado, 1996).

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2. THE RHIZOBIUM-LEGUME ASSOCIATION

Symbiosis is the close association of two dissimilar organisms (symbionts) for their mutual benefit (Shurtleff and Averre, 1997). In the case of rhizobium-legume symbiosis, the host plant (or macrosymbiont) supplies the bacteria (microsymbiont) with photosynthates. This in turn leads to the improved availability of important mineral nutrients of which the supply would otherwise be limiting to plant growth (Pawlowski and Bisseling, 1996).

The symbiotic association is a multistep process initiated by preinfection events. Specific signals are exchanged between the plant and bacterium. Establishing symbiosis involve colonization of the rhizosphere, entrance of the root hairs resulting in the formation of an infection thread, multiplication of the bacteria in the membrane envelopes of the nodule cells, conversion of bacteria to bacteroids, and establishment and continuance of a shared metabolism between plant and bacterium (Trinick, 1982).

2.1. PLANT SYMBIONT : LEGUMES

Of all the plants used by man, only the grasses are more important than legumes. Legumes however, show the greatest promise for future development and exploitation (Gutteridge and Shelton, 1994).

2.1.1. The Family Fabaceae

The family Fabaceae consists of approximately 750 genera, containing 16 000 - 19 000 species of which few are economically exploited and grown over large areas. Up until 1981, only about 15% of the legume species had been examined for their ability to be nodulated by rhizobia (Allen and Allen, 1981).

The family is divided into three subfamilies mainly on the basis of floral differences and include trees, shrubs, annual and perennial herbs and woody vines. The subfamily **Caesalpinioideae**

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contains 177 genera, most of which are trees and shrubs of tropical savannahs and forests of Africa, South America and Asia. **Mimosoideae** (66 genera) are predominantly small trees and shrubs of semi-arid tropical regions of Africa, Australia and the Americas. *Acacia* species are the best known example. **Papilionoideae** (505 genera), comprises mainly of herbs and small shrubs which are distributed world-wide and includes well-known grain legumes such as beans and peas (Allen and Allen, 1981; Gutteridge and Shelton, 1994).

It is estimated that about 94 - 98% of the species of the Papilionoideae and 92 - 96% of the species of the Mimosoideae form root nodules. In contrast, only 30 - 34% of the species of Caesalpinioideae is believed to do so (Corby *et al.*, 1983).

2.1.2. The importance of legumes

Legume seeds are two to three times richer in protein than cereal grain and accounts for approximately 20% of food production. The biggest consumers include the former Soviet Union, South and Central America, Mexico, India, Turkey and Greece (National Research Council, 1994).

Species of the Mimosoideae and Caesalpinioideae are used for timber, dyes, tannins, resins, gums, insecticides, medicines and fibres. Members of the Papilionoideae are important as nutritional crops for humans and animals. The consumption of legumes is quantitatively in the following order: dry bean (*Phaseolus vulgaris*), dry pea (*Pisum sativum*), chickpea (*Cicer arietinum*), broad bean (*Vicia faba*), pigeon pea (*Cajanus cajan*), cowpea (*Vigna unguiculata*) and lentil (*Lens culinaris*). Peanut (*Arachis hypogaea*) and soybean (*Glycine max*) are important sources of cooking oil and are also a major source of protein for human consumption (National Research Council, 1994).

Forage legumes such as alfalfa (*Medicago sativa*), clovers (*Trifolium* spp.), *Stylosanthes* spp., and *Desmodium* spp. are extensively grown. These are either directly grazed by animals or fed as hay or silage. Silage not only provide roughage and protein needed, but also vitamins, minerals and other nutrients (National Research Council, 1994).

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Woody perennial legumes are used to prevent erosion and provide fodder, shade, mulch and green manure. The high wood densities and low moisture content of genera such as *Acacia, Leucaena, Prosopis* and *Acrocarpus* make them excellent trees for the production of fuelwood (National Research Council, 1994). In addition, *Acacia* species are also popular for use in land rehabilitation because of their fast growth rate, even on marginal lands (Lal and Khanna, 1993).

2.2.   MICROBIAL SYMBIONT : RHIZOBIA

Rhizobia are aerobic, motile, rod shaped bacteria that are abundant in soil and capable of forming a symbiotic association with members of the Fabaceae. In this association specialized structures, the nodules are formed on the roots and/or stems in which the conversion of atmospheric nitrogen takes place.

2.2.1. Taxonomy of the rhizobia

2.2.1.1. Historical overview

In 1888, Hellriegel and Wilfarth established that atmospheric nitrogen was assimilated through the root nodules of legumes. Beijerinck isolated these bacteria and proposed the name *Bacillus radicicola*. These organisms were renamed as *Rhizobium* by Frank in 1889 (Elkan, 1992).

In 1929, Baldwin and Fred proposed taxonomic characterization of the rhizobia based on bacteria-plant cross inoculation groups. This was based on extensive cross testing of only a few legume hosts which included *Pisum, Vicia, Lens, Lathyrus, Trifolium* and *Medicago*. Studies showed that isolates from one genus would not necessarily nodulate plants in another genus, and that the legumes formed groups based on the ability of their associated rhizobia to be mutually interchangeable. Rhizobia nodulating each group were named according to one of the hosts of that group (Elkan, 1992). However, it soon

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became evident that these groups were not discrete and many reports of boundary jumping are found in literature (for details see Trinick, 1982).

The division of root-nodulating bacteria into two groups, fast- and slow-growing on laboratory media, was first recognized by Fred and his co-workers in 1932, who also showed that some of the fast-growing rhizobia were closely related to *Agrobacterium* (Elkan, 1992). Norris (1965), suggested the tendency of slow-growing, non acid-producing rhizobia to be associated with tropical legumes and fast-growing, acid-producing rhizobia with temperate hosts. Although numerous authors supported this view, contradictory evidence was found. Slow-growing symbionts were isolated from the temperate legumes *Lupinus* and *Coronilla*, and fast-growing rhizobia from the tropical legumes *Acacia*, *Leucaena* and *Sesbania* (De Lajudie *et al.*, 1994, George *et al.*, 1994). Dagutat (1995) obtained isolates from different *Acacia* species that were closely related to fast-growing *R. leguminosarum* bv. viciae.

The initial view that a nodule is occupied only by a single rhizobial strain has also been proven wrong. Several reports exist of one legume species having more than one species of rhizobium symbiont. Diverse rhizobial types, i.e. fast- and slow-growing, have been isolated from the same species, e.g. *Glycine max* (Dagutat, 1995; Scholla and Elkan, 1984) and even from the same plant, e.g. *Acacia* (Dreyfus and Dommergues, 1981) and *Prosopis* (Jenkins *et al.*, 1987). In addition, Dagutat (1995) showed that up to four rhizobial strains can occupy the same nodule. Therefore, symbiotic performance that has been used mostly for differentiation between species, has become less applicable and the need to use a polyphasic approach is apparent (Dagutat, 1995; Laguerre *et al.*, 1994).

In the ninth edition of *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt, 1984), the rhizobia were divided into two genera, *Rhizobium* and *Bradyrhizobium*, representing the fast- and slow-growing strains respectively. At the time, the genus *Rhizobium* was divided into three species: *R. leguminosarum* (with three biovars: trifolii, phaseoli and viceae), *R. meliloti* and *R. loti*, whereas *Bradyrhizobium* contained only one species, *B. japonicum* (Jordan, 1984). Extensive changes in the taxonomy of the rhizobia

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have taken place since 1984 with the description of three new genera *Azorhizobium,* *Sinorhizobium* and *Mesorhizobium* and numerous new species.

In spite of the wealth of indigenous legume species in South Africa, a comprehensive study of South African rhizobia was sorely lacking. Although Grobbelaar *et al.* (1964, 1967, 1972, 1974, 1975, 1979, 1983) reported on the nodulation status of indigenous legumes, rhizobial symbionts were not isolated. The first in depth investigation of South African rhizobia was done by Dagutat (1995). She isolated bacteria from nodules of a wide variety of legumes including trees, perennials, annuals, shrubs and characterized the isolates by sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the cellular proteins. Many of the isolates were members of existing rhizobial genera, but some could not be assigned to previously described species. In her study, rhizobia were isolated from three genera, *Cassia, Schizolobium* and *Bauhinia,* previously reported as non-nodulating. This once again emphasized that it is essential to investigate a wide range of legumes from diverse geographic and climatic regions.

2.2.1.2. Current taxonomic status

As can be seen in Figure 1, rhizobia isolated thus far, all represent different rRNA branches in the alpha subclass of the Proteobacteria (Willems and Collins, 1993). Molecular data show that root- and stem-nodulating bacteria are polyphyletic, i.e. no single branch of the evolutionary tree carries all the rhizobia and no other bacteria. Distinct lineages were identified in the genus *Rhizobium.* These included the *R. meliloti -* *R. fredii,* the *R. leguminosarum - R. tropici,* the *R. huakuii - R. loti* and the *R. galegae* lineage. The genera *Agrobacterium, Phyllobacterium, Mycoplana, Brucella* and *Ochrobacterium* are imbedded in these lineages (Sawada *et al.,* 1993; Yanagi and Yamasato, 1993). Because the genus *Rhizobium* is such a heterogeneous group of bacteria, phenetic and phylogenetic differences among the species are obscured (Jarvis *et al.,* 1997; Willems and Collins, 1993; Young, 1996). One solution to this problem has been to recognize each distinct lineage as a separate genus, such as the transfer of two species, *R. meliloti* and *R. fredii* to the new genus *Sinorhizobium* (De Lajudie *et al.,*

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1994) and the proposal of a new genus *Mesorhizobium* for the "meso-growing" rhizobia, or the previously known *R. huakuii - R. loti* lineage (Jarvis *et al.*, 1997).

Bradyrhizobium constitutes a separate lineage and is related to *Rhodopseudomonas palustris*, *Blastobacter denitrificans* and the genera *Afipia* and *Nitrobacter* (Willems and Collins, 1993). *Azorhizobium* represents a separate lineage that is more closely related to *Bradyrhizobium* than to *Rhizobium* (Dreyfus *et al.*, 1988).

Thus, although *Rhizobium* and *Bradyrhizobium* establish a quite similar symbiotic process with legume hosts and are related with regard to the genes involved in nodulation and fixation, these two genera are more distantly related to each other than *Rhizobium* and *Agrobacterium* or *Phyllobacterium* (Jordan, 1984; Jarvis *et al.*, 1996).

As more rhizobia are isolated and described, some will undoubtedly fall outside the existing phylogenetic groups and new genera will be needed which would imply possible further subdivision of the remaining *Rhizobium* species (Jarvis *et al.*, 1997; Young, 1996). The SDS-PAGE work on South African rhizobia points in the same direction (Dagutat, 1995). The current taxonomic status is given in Table 1 and an overview of the species proposed until 1998 follows.

2.2.1.2.1. Rhizobium

Rhizobium leguminosarum

Originally, only symbionts of the tribe Vicieae were described as *R. leguminosarum*, but isolates from clover nodules and *Phaseolus* bean nodules could not be distinguished from *R. leguminosarum* except by their host range. Hence, three biovars were recognized: biovar viciae which nodulates *Pisum, Vicia, Lathyrus,* and *Lens,* biovar trifolii which nodulates *Trifolium* and biovar phaseoli which nodulates *Phaseolus* (Jordan, 1984). This biovar designation is essentially a description of the plasmid, rather than of the chromosomal background of the strain because of the fact that genes encoding nodulation and nitrogen fixation, as well host determinants are carried on plasmids (Young, 1996).

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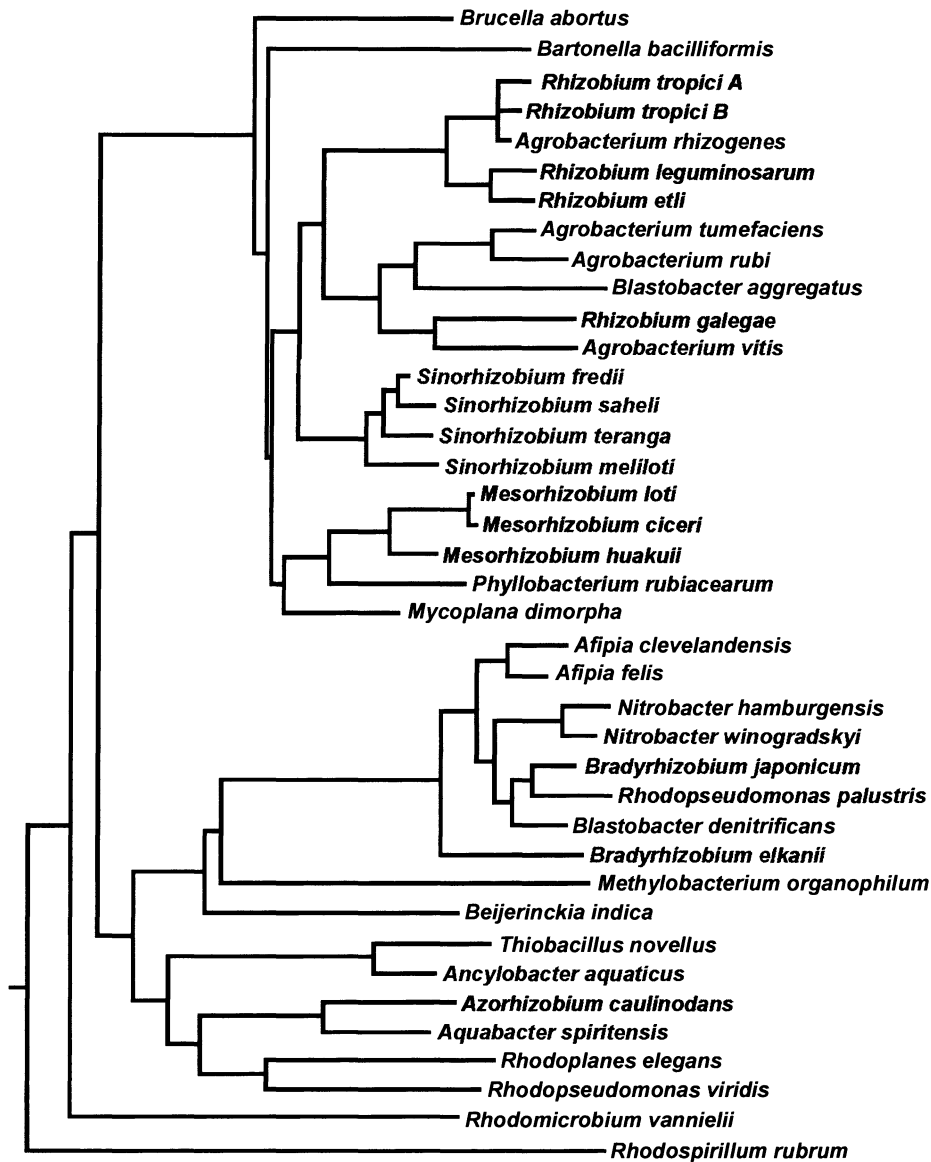
Fig. 1. Phylogenetic tree of members of the alpha subclass of Proteobacteria as determined by 16S rDNAsequence analysis. The tree was constructed by the Neighbor-Joining method from near full-length sequences of preferably type strains obtained from Genbank/EMBL. *Mesorhizobium tianshanense, M. mediterraneum* and *Bradyrhizobium liaoningense* were omitted as full sequences were not available (Young, 1996).

Rhizobium galegae

These fast-growing rhizobia are very host specific and nodulate either *Galega orientalis* or *Galega officinalis*. Strains from one *Galega* species form only ineffective nodules on other host species and no nodules on other legumes. Rhizobia from other species do not infect *Galega* hosts (Kaijalainen and Lindström, 1989; Lindström, 1989; Lipsanen and Lindström, 1989; Young, 1996). Based on DNA and rRNA homology, lipopolysaccharide and protein patterns, phage typing, numerical taxonomy and cross-nodulation studies, it forms a distinct taxonomic group within the genus *Rhizobium* (Lindström, 1989). Based on full-length 16S rDNA sequence analysis, it is on the same phylogenetic branch as the genus *Agrobacterium*, with the closest relative *Agrobacterium vitis* (Fig. 1). The latter appears to be closer related to *R. galegae* than it is to *Agrobacterium tumefaciens* and *A. rubi* (Terefework *et al.*, 1998; Young, 1996). Some controversy exist over this fact as PCR-RFLP analysis of the 16S and 23S rRNA genes and partial sequencing (800bp) of the 16S rDNA showed *R. galegae* is phylogenetically distinct from other rhizobia and agrobacteria. In other studies, partial 16S rDNA sequencing (260bp) and PCR-RFLP analysis of the 16S-intergenic spacer region (IGS) showed *R. galegae* to be closely related to *M. loti* (Nour *et al.*, 1994; Young *et al.*, 1991).

Rhizobium tropici

Symbionts of *Phaseolus* classified as *R. leguminosarum* biovar phaseoli were rather diverse. Genetic studies identified two major groups, Type I and Type II. The latter group has numerous distinctive phenotypic and genetic characteristics, notably only one copy of the nitrogenase reductase gene, *nifH*, and effective nodulation of both *Phaseolus vulgaris* and *Leucaena* species (Martínez-Romero *et al.*, 1991; Young, 1996). On the basis of data from multilocus enzyme electrophoresis (MLEE), DNA-DNA hybridization, 16S rDNA sequencing and phenotypic characteristics, this group was classified as a separate species, *R. tropici* (Martínez-Romero *et al.*, 1991). Two distinct subgroups, IIA and IIB with distinctive phenotypic features, excist within this species which differ sufficiently to warrant description of a separate species (Amarger *et al.*, 1997; Dagutat, 1995; Geniaux *et al.*, 1995; Martínez-Romero *et al.*, 1991; Young, 1996).

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Rhizobium etli

Type I symbionts of *Phaseolus vulgaris* differ from "typical" *R. leguminosarum* in 16S rDNA sequence and have been reclassified as *R. etli*. This group is characterized by the ability to effectively nodulate *Phaseolus vulgaris*, multiple copies of the *nifH* gene on the plasmid, organization of the common nodulation genes into two separate transcriptional units bearing *nodA* and *nodBC*, and the presence of the polysaccharide inhibition gene, *psi*. This species include at least one biovar, *R. etli* biovar phaseoli. Included in this group is genetically related non-symbiotic soil isolates from Mexico (Segovia *et al.*, 1993). According to Laguerre *et al.* (1993a) and Martínez-Romero *et al.* (1988), *R. etli* isolates are restricted to the Americas and only nodulate beans. This was contradicted in the study by Dagutat (1995), who showed that isolates from *Desmodium, Melolobium, Indigofera, Acacia melanoxylon* and *Chamaecrista stricta* exhibited relatedness to *R. etli* and suggested that the host-plant specificity should be expanded.

Rhizobium hainanensis

Rhizobia isolated from various species of leguminous plants in the tropical Hainan Province in China, were characterized by numerical taxonomy and DNA-DNA hybridization and the slow-growing strains were classified as *Bradyrhizobium japonicum*. The fast-growing strains however, were phenotypically and genotypically diverse. Some strains belonged to previously described species, while others formed unique subgroups. One of these groups, subgroup IV, was distinguished from all previously described *Rhizobium* species and was classified as *R. hainanensis*. This group is closely related to *R. tropici* on the basis of 16S rDNA sequence analysis and DNA-DNA homology (Gao *et al.*, 1994). However, recognition of these isolates as a *bona fide* species was discouraged at a meeting held in St. Petersburg until further DNA homology studies with a larger number of strains have been done (Lindström *et al.*, 1995).

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Table 1. Root and stem-nodulating bacteria and their principle host legumes.

Bacterial species	Host legume	Reference
Rhizobium leguminosarum bv. viciae	*Pisum, Vicia, Lathyrus, Lens*	Jordan, 1984
Rhizobium leguminosarum bv. trifolli	*Trifolium*	Jordan, 1984
Rhizobium leguminosarum bv. phaseoli	*Phaseolus vulgaris*	Jordan, 1984
Rhizobium tropici	*Phaseolus vulgaris, Leucaena* spp.	Martínez-Romero *et al.*, 1991
Rhizobium galegae	*Galega officinalis, G. orientalis*	Lindström, 1989
Rhizobium etli	*Phaseolus vulgaris*	Segovia *et al.*, 1993
Rhizobium hainanensis *	*Lotus, Crotalaria, Desmodium, Leucaena*	Gao *et al.*, 1994
Rhizobium gallicum *	*Phaseolus vulgaris*	Amarger *et al.*, 1997
Rhizobium giardini *	*Phaseolus vulgaris*	Amarger *et al.*, 1997
Rhizobium mongolense *	*Medicago ruthenica, Phaseolus vulgaris*	Van Berkum *et al.*, 1998
Bradyrhizobium japonicum	*Glycine max*	Jordan, 1984
Bradyrhizobium elkanii	*Glycine max*	Kuykendall *et al.*, 1992
Bradyrhizobium liaoningense *	*Glycine max, G. soja*	Xu *et al.*, 1995
Azorhizobium caulinodans	*Sesbania rostrata*	Dreyfus *et al.*, 1988
Sinorhizobium fredii	*Glycine max, G. soja* and other	Scholla and Elkan, 1984
Sinorhizobium meliloti	*Medicago, Melilotus, Trigonella*	Eardly *et al.*, 1990
Sinorhizobium teranga	*Acacia, Sesbania*	De Lajudie *et al.*, 1994
Sinorhizobium saheli	*Acacia, Sesbania*	De Lajudie *et al.*, 1994
Sinorhizobium medicae *	*Medicago*	Rome *et al.*, 1996b
Sinorhizobium xinjiangensis *	*Glycine max, G. soja*	Chen *et al.*, 1988
Mesorhizobium huakuii	*Astralagus sinicus*	Chen *et al.*, 1991
Mesorhizobium loti	*Lotus* spp.	Young, 1996
Mesorhizobium ciceri *	*Cicer arietinum*	Nour *et al.*, 1994
Mesorhizobium tianshanense *	*Glycine max, Glycerrhiza uralensis,* and other	Chen *et al.*, 1995
Mesorhizobium mediterraneum *	*Cicer arietinum*	Nour *et al.*, 1995
Mesorhizobium plurifarium *	*Acacia* species, *Leucaena leucocephala*	De Lajudie *et al.*, 1998

* Not yet validated as *bona fide* species

Rhizobium gallicum and *Rhizobium giardinii*

Laguerre *et al.* (1993b), isolated a group of strains from *Phaseolus vulgaris* in France which differed genotypically from *R. leguminosarum*. Strains which belonged to this group were isolated by Geniaux *et al.* (1993) as well. In the latter study a second group of strains which differed from the first, as well as from the three species of bean symbionts, was identified. These two groups were assigned to two new genomic species on the basis of DNA-DNA hybridization and analysis of a partial 16S rRNA sequence (Laguerre *et al.*, 1993a). The new species, *R. gallicum* and *R. giardinii* were proposed for genomic species 1 and 2 respectively, on the basis of phenotypic, genotypic and phylogenetic analyses. Each species is divided into two biovars: *R. gallicum* biovar gallicum and biovar phaseoli, and *R. giardinii* biovars giardinii and phaseoli (Amarger *et al.*, 1997). A study done by Sessitch *et al.* (1997), showed that Austrian isolates are members of *R. gallicum* biovar gallicum.

Rhizobium mongolense

Rhizobial strains isolated from *Medicago ruthenica*, a crop legume from the temperate regions of Inner Mongolia, were phenotypically and genotypically characterized by Van Berkum *et al.* (1998). On the basis of substrate utilization, MLEE, DNA-DNA hybridization and 16S rDNA sequencing it was concluded that these isolates do not represent any previously described rhizobial species, but is closely related to species in the genus *Rhizobium*. A new species, *R. mongolense* which nodulate *Medicago ruthenica* and *Phaseolus vulgaris* was proposed. This is in contrast with symbionts from other *Medicago* species which belong to the genus *Sinorhizobium*.

2.2.1.2.2. Bradyrhizobium

Bradyrhizobium japonicum

This species effectively nodulates soybean (*Glycine max*). Within *B. japonicum* two DNA-homology groups have been recognized on the basis of fatty acid analysis, antibiotic resistance and DNA-DNA hybridization (Graham *et al.*, 1991; Jordan, 1984).

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Bradyrhizobium elkanii

Hollis *et al.* (1981), showed that strains belonging to DNA homology group II of *B. japonicum*, showed maximum 30% homology with the type strain of *B. japonicum* and suggested that these strains represent a distinct species. This group of strains was reclassified as *B. elkanii* on the basis of DNA homology, RFLP analysis, fatty acid and antibiotic resistance profiles, and 16S rRNA sequence data (Kuykendall *et al.*, 1992).

Bradyrhizobium liaoningense

Extra-slowly growing strains from *Glycine soja* and *Glycine max* clustered closely in the genus *Bradyrhizobium*. Colonies are 0.2 - 1 mm in diameter within 7 - 14 days on yeast mannitol agar, compared with 5 - 7 days for other *Bradyrhizobium* species. This group was separated from *B. japonicum* at species level and at genus level from *Rhizobium* and *Agrobacterium* species. Based on results from numerical taxonomy, G+C analysis, DNA-DNA hybridization and partial 16S rRNA sequence analysis, *B. liaoningense* was proposed (Xu *et al.*, 1995).

Bradyrhizobium sp.

Strains which belong to the genus *Bradyrhizobium* but do not nodulate soybean, are known as *Bradyrhizobium* sp., followed by the name of the legume host in parentheses e.g. *Bradyrhizobium* strain (*Arachis*) (Van Rossum *et al.*, 1995; Young, 1996). Strain BTAi1 which nodulates the stem of *Aeschynomene indica* belongs to this group (Young *et al.*, 1991). These isolates are of significance because of their ability to photosynthesize. Similar bacteria have been isolated from stem nodules of several other *Aeschynomene* species (Wong *et al.*, 1994). Phylogenetic analysis of a 260 base pair segment of the 16S rDNA suggested that BTAi1 is closely related to *Bradyrhizobium japonicum* and the photosynthetic species *Rhodopseudomonas palustris* (Wong *et al.*, 1994; Young *et al.*, 1991). Although the name *"Photorhizobium"* has been used to describe these bacteria, it has not been formally proposed (Young, 1996). According to Young *et al.* (1991), the 16S rRNA sequence data do not justify proposal of a new genus. These bacteria can play an important role in the improvement of symbiotic nitrogen fixation for crop production,

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since the demand for chloroplast-generated ATP needed for the nitrogen fixation processes is reduced by bacterial photo-phosphorylation (Wong *et al.*, 1994).

2.2.1.2.3. Azorhizobium

Dreyfus *et al.* (1988), isolated strains nodulating the stems and roots of *Sesbania rostrata* and showed on the basis of DNA-rRNA hybridization that these strains constitute a separate branch, clearly different from *Rhizobium* and *Bradyrhizobium*. A new genus, *Azorhizobium* was described. Only one species, *A. caulinodans* is recognized thus far. The symbiosis between *A. caulinodans* and *Sesbania rostrata* is highly specific and these strains have the ability to fix dinitrogen in the free-living state and to utilize the fixed nitrogen for growth (Dreyfus *et al.*, 1983). Stem nodules are viewed as an adaptation to flooding to ensure adequate oxygenation (Kennedy and Tchan, 1992).

In a study done by Rinaudo *et al.* (1991), two genomic species were identified in the genus *Azorhizobium*. Of the strains analyzed, 92% was members of genomic species 1, which corresponds to *A. caulinodans*. DNA-DNA hybridization data showed that genomic species 2 differed sufficiently from genomic species 1 to constitute a new species. However, this species has to be differentiated by some phenotypic characteristic prior to naming it. In addition, seven unclassified strains were isolated which nodulated the stem and roots of *S. rostrata*, but were quite different from *Azorhizobium* strains. The G+C content was lower and nitrogen was not fixed under free-living conditions. Further investigation is necessary to obtain a better understanding of the diversity among members of this genus (Rinaudo *et al.*, 1991).

2.2.1.2.4. Sinorhizobium

In 1984, a new species nodulating soybean, *Rhizobium fredii* was proposed by Scholla and Elkan. Chen *et al.* (1988), proposed the transfer of this taxon on phenotypic grounds to the new genus *Sinorhizobium* and also described a second species, *S. xinjiangensis*. In 1992, however, Jarvis *et al.*, concluded on the basis of partial 16S rRNA analysis, that the name *Sinorhizobium* is synonymous to the name *Rhizobium*, that all fast-growing soybean-nodulating strains belong to

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the species *R. fredii*, and that additional studies are needed to confirm the status of *Sinorhizobium* as a separate taxon. In 1994, De Lajudie *et al.*, emended the genus *Sinorhizobium*, reclassified *R. meliloti* as *S. meliloti* and proposed two new species. This was accepted in 1996 by the Subcommittee on the Taxonomy of *Agrobacterium* and *Rhizobium* of the International Committee on Systematic Bacteriology (Lindström, 1996).

Sinorhizobium fredii

This species effectively nodulates soybean *(Glycine max, G. soja)*, cowpea (*Vigna unguiculata*) and pigeon pea (*Cajanus cajan*). Two groups within the species are recognized on the basis of DNA-DNA hybridization and serology: chemovars fredii and siensis (Scholla and Elkan, 1984).

Sinorhizobium meliloti

Formerly known as *R. meliloti*, these organisms stimulate the development of root nodules in three related genera *Melilotus, Medicago* and *Trigonella* (Eardly *et al.*, 1990; Young, 1996). This species is differentiated from other *Sinorhizobium* species by its protein profiles, DNA-DNA hybridization data and 16S rRNA sequences (De Lajudie *et al.*, 1994).

Sinorhizobium xinjiangensis

Fast-growing soybean isolates from the suburbs of Xinjiang in the People's Republic of China were described as *S. xinjiangensis* (Chen *et al.*, 1988). This was however, based only on results from numerical analysis of 240 biochemical traits and determination of G+C content, and further analysis of this group is needed to confirm separate species status.

Sinorhizobium saheli and *Sinorhizobium teranga*

In 1994, De Lajudie and his co-workers studied isolates obtained from different *Acacia* and *Sesbania* hosts in the Sahel area in Senegal. On the basis of 16S rRNA sequences, most of the strains clustered on the *S. meliloti* - *S. fredii* branch. These strains have been classified as *S. teranga* with two biovars, i.e. sesbaniae and acaciae for the strains

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nodulating *Sesbania* and *Acacia* respectively (Lortet *et al.*, 1996). Strains isolated from *Sesbania* hosts that are able to nodulate *Sesbania, Acacia seyal* and *Leucaena leucocephala* were classified as *Sinorhizobium saheli* (De Lajudie *et al.*, 1994). In a study done by Boivin *et al.* (1997), it was shown that *S. teranga* bv. sesbaniae and *S. saheli* were also able to nodulate the stems of *Sesbania rostrata* when roots were not inoculated previously. Nodulation was as effective as nodulation by *Azorhizobium*.

Sinorhizobium medicae

Although a thorough study has been made of the symbiotic strains from the perennial species *Medicago sativa*, the taxonomic status of symbionts of annual *Medicago* species is less well understood (Rome *et al.*, 1996b). Eardly *et al.* (1990), demonstrated with MLEE and RFLP-analysis of the 16S rDNA that two genotypic groups exist within the sinorhizobia from *Medicago*. Analysis of DNA polymorphisms of four amplified DNA regions and DNA-DNA hybridization corroborated these results (Rome *et al.*, 1996a). The first genotypic group corresponds to *S. meliloti*, whereas the second group shows low levels of DNA homology with *S. meliloti* strains (Rome *et al.*, 1996b). Nine strains from annual *Medicago* species which were genomically distinct from *S. meliloti*, were compared with reference strains of *S. meliloti* and *S. fredii* by phenotypic tests, 16S rRNA sequencing and symbiotic properties. Results showed that *S. meliloti* and genomic species II have diverged sufficiently to be considered two distinct species and a new species, *S. medicae* was proposed (Rome *et al.*, 1996b).

2.2.1.2.5. Mesorhizobium

Rhizobium loti was first described for strains isolated from *Lotus* species (Jarvis *et al.*, 1982). Results of several analyses indicated that *R. leguminosarum, R. galegae, S. meliloti, S. fredii, Agrobacterium* biovars 1 and 2 were more closely related to each other than to *R. loti* (Jarvis *et al.*, 1997). Subsequently several other species that are related to *R. loti* have been described, namely *R. huakuii, R. ciceri, R. mediterraneum* and *R. tianshanense.* This monophyletic group constitutes the most distant branch within the fast-growing rhizobia and is phenetically and phylogenetically distinct from the other rhizobial genera (Jarvis *et al.*, 1997; Young, 1996). In

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1995, the Subcommittee on the Taxonomy of *Agrobacterium* and *Rhizobium* of the International Committee on Systematic Bacteriology recognized that the phylogenetic branch of *R. loti* - *R. huakuii* merits recognition as a new genus (Lindström, 1996) and *Mesorhizobium* was proposed (Jarvis *et al.*, 1997; Lindström *et al.*, 1995). Many strains in this group grow at rates intermediate between typical fast-and slow-growers (Young, 1996). This group is intermediate between the *Agrobacterium - Rhizobium - Sinorhizobium* complex and the genera *Azorhizobium* and *Bradyrhizobium* in the alpha subdivision of the Proteobacteria (Jarvis *et al.*, 1997). The name *Mesorhizobium* therefore not only implies growth rate but also phylogenetic position.

Mesorhizobium loti

Effective nodules are formed on species of among others, *Lotus, Lupinus* and *Anthyllis*. Nodulation and nitrogen fixation genes are usually encoded on the chromosome rather than on the plasmid as in most fast-growing rhizobia (Young, 1996).

Mesorhizobium huakuii

Chen *et al.* (1991) studied strains isolated from root nodules of *Astragalus sinicus* with gel electrophoresis of whole-cell proteins, G+C content analysis and DNA-DNA hybridization data. These strains constituted a homologous group that was separate from previously described rhizobial species and was designated *M. huakuii*.

Mesorhizobium ciceri

Previously, chickpea (*Cicer arietinum*) symbionts were classified as either *R. loti* or *Bradyrhizobium* sp., depending on their generation time. Nour *et al.* (1994, 1995) showed that all the chickpea strains belong to the genus *Rhizobium* regardless of generation time, that they constitute a separate branch closely related to but clearly different from *R. loti*, and that two phylogenetic lineages (Groups A and B) are present. The homogeneous group B was described as *M. ciceri* (Nour *et al.*, 1994).

Mesorhizobium mediterraneum

In contrast with group B chickpea strains which were identified as *M. ciceri*, great heterogeneity was observed among group A strains (Nour *et al.*, 1994). In a study done in

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1995, these strains grouped into three genomic species. On the basis of genotypic and phenotypic data, it was proposed that genomic species 2 strains should be reclassified as a new species, *M. mediterraneum*, which is differentiated from *M. ciceri* and other mesorhizobia by 16S rRNA sequence, DNA-DNA hybridization and fatty acid analysis (Nour *et al.*, 1995).

Mesorhizobium tianshanense

Moderately and slowly growing isolates from various legumes in the arid saline environment of the Xinjiang Province in China were identified as *M. tianshanense*. Based on the results of partial 16S rRNA sequence analysis, this species is closely related to *M. loti*, *M. huakuii* and *R. galegae* but not to *Bradyrhizobium japonicum* and *Azorhizobium* (Chen *et al.*, 1995). However, the exact taxonomic position of this species was controversial as the partial 16S rRNA sequence was identical to that of *M. ciceri*, and the DNA-DNA relatedness between this species and newly emerged species had not been determined. This uncertainty was cleared up in a study done by Tan *et al.* (1997), that showed that *M. tianshanense* is closely related to the *Mesorhizobium* phylogenetic branch, that it could be distinguished from the other mesorhizobial species and that these bacteria indeed constitute a distinct rhizobial species.

Mesorhizobium plurifarium

In 1994 De Lajudie *et al.*, described a group of strains isolated from *Acacia* species, Brazilian legumes and *Lotus* species. This group, Cluster U, belonged to the genus *Mesorhizobium*, but constituted a protein electrophoretic cluster distinct from the other *Mesorhizobium* species, with the *M. huakuii* group as its nearest neighbour. The taxonomic position of these 32 strains was further investigated (De Lajudie *et al.*, 1998). On the basis of SDS-PAGE of proteins, DNA hybridization and 16S rDNA sequencing, a new species *M. plurifarium* was described. Two subclusters *a* and *b* were distinguished. Most of the strains nodulate *Acacia senegal, A. tortilis* ssp. *raddiana, A. nilotica, A. seyal, Leucaena leucocephala* and *Neptunia oleracea.*

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2.2.1.3. Approaches to systematics of the rhizobia

Classification of rhizobia had been based on symbiotic performance for a long time. A classification system based on a single plasmid-encoded phenotypic characteristic leads to an artificial, ecologically based classification (Eardly *et al.*, 1990). Taxonomy should not only involve the naming of new species and genera, but should also serve an ecological and evolutionary purpose (Lindström *et al.*, 1995).

Bacterial classification should reflect as closely as possible the natural relationship between bacteria, which is the phylogenetic relationship as encoded in the 16S or 23S rDNA. This taxonomic scheme should however, show phenotypic consistency. Therefore phenotypic description at every taxonomic level cannot be ignored (Vandamme *et al.*, 1996). Modern classification systems should stem from extensive databases which integrate data from different levels in which phenotypic, genotypic and phylogenetic information are included. The use of such a polyphasic approach is necessary for delineation of taxa at all levels (Goodfellow and O'Donnell, 1993; Vandamme *et al.*, 1996).

Minimal standards were proposed for the description of new taxa of root- and stem-nodulating bacteria. Traits to be considered in assigning specific status include symbiotic performance with selected hosts, cultural and morphological characteristics, DNA:DNA relatedness, rRNA:DNA hybridization and 16S rRNA analysis, DNA restriction fragment length polymorphisms and MLEE (Graham *et al.*, 1991).

Because of the wealth of new rhizobia being described, it is critical that easy and rapid methods are developed for the identification of new isolates (Laguerre *et al.*, 1994). The most objective method to denote bacterial species remains DNA reassociation. However, due to the technical difficulty and high cost of this method, alternative approaches have to be sought. Sequencing and probing of the rRNA genome fragment are well-established techniques with wide application in bacterial taxonomy. Total genomic restriction analysis followed by hybridization with rDNA probes (ribotyping) is laborious (Vandamme *et al.*, 1996; Vaneechoutte *et al.*, 1992). Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA, SDS-PAGE of cell proteins, RAPD analysis, total cellular fatty

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acids analysis, MLEE and numerical taxonomy should be used as initial screening methods after which DNA relatedness and rDNA sequences should be determined and taxa described with respect to phenotypic traits (Lindström *et al.*, 1995).

2.2.1.3.1. Phenotypic characterization with the Biolog system

Classical phenotypic studies can be labour intensive and difficult to interpret especially when slowly growing bacteria are used (Dupuy *et al.*, 1994). The need for a phenotypic test system allowing many tests to be done in one experiment, has long been realized (Bochner, 1989b; Grimont *et al.*, 1996). One of these systems, Biolog GN MicroPlates (Biolog Inc., Hayward, CA, USA) characterize Gram-negative aerobic bacteria by examining carbon source utilization profiles.

The redox chemistry responds to the process of metabolism rather than to the metabolic by-products such as acids. Utilization of 95 carbon sources is detected as an increase in respiration of the cells in the wells, which lead to the irreversible reduction of a redox dye, tetrazolium to formazan. Purple colour development or turbidity changes in the wells is indicative of a positive reaction. If an isolate is incapable of oxidizing a particular carbon source, no respiration will take place and hence, a coloured product is not formed (Bochner, 1989a). The result is a pattern of purple and colourless wells which represents the organism's "metabolic fingerprint" (Bochner, 1989b) and therefore the development of an extended database is possible.

One of the biggest advantages of this system is its simplicity. Prefilled plates are easily inoculated with a cell suspension, there is no need for the addition of colour-developing reagents or oil overlays and no follow-on tests are needed. The efficiency and speed of characterizing and identifying a large number of strains is greatly increased (Bochner, 1989a). Phenotypic testing and identification of large numbers of isolates are accelerated by at least two orders of magnitude because of its speed, simplicity and efficiency (Bochner, 1989b). However, application of phenotypic fingerprinting systems such as Biolog and inclusion in official descriptions, restrict examination of bacterial phenotype to a minimum, thus also restricting knowledge of the phenotype (Vandamme *et al.*, 1996).

2.2.1.3.2. SDS-PAGE of whole cell proteins

Cellular proteins is the second level of information for determining the relationships between organisms. Electrophoresis of cellular proteins results in a complex banding pattern, or electrophoregram, which is a stable and characteristic fingerprint of the bacterial strain. Sodium dodecyl sulphate (SDS) is an anionic detergent which binds to proteins through hydrophobic interactions. The majority of proteins are solubilized and all the proteins are negatively charged by the excess SDS and loose their spatial conformation. The addition of 2-mercapto-ethanol leads to the disruption of the disulphide bridges that link the polypeptide chains. Proteins are thus reduced to basic polypeptide chains with relatively the same negative charge per mass unit. Patterns obtained through denaturing of proteins in the presence of SDS, detect differences in molecular weight (Vauterin *et al.*, 1993).

Each protein band represents a number of structurally different proteins with the same electrophoretic mobility. All growth conditions such as medium composition, incubation temperature and growth time should be kept as constant as possible. Strains grown under identical conditions produce constant electrophoretic patterns (Vauterin *et al.*, 1993). Strains with highly similar protein patterns, most probably belong to a single species. Small but reproducible differences may provide taxonomic information at intraspecific levels (Vancanneyt *et al.*, 1996).

Analysis of SDS-PAGE gels start with the densitometric measurement of the photometric absorbency after which normalization takes place. Normalization is the standardization of gel length and compensation for discrepancies within and between gels. These are inherent to the system and can be caused by factors such as variation in acrylamide and buffer composition, polymerization and running conditions. Comparison of protein electrophoregrams is best done using the Pearson product-moment correlation coefficient (*r*) because of its insensitivity to experimental differences in the amount of protein applied. The resulting matrix is clustered using the unweighted pair group method using arithmetic averages (UPGMA) and groupings are represented in a dendrogram (Kersters and De Ley, 1975; Vauterin *et al.*, 1993).

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The biggest advantage of SDS-PAGE of whole cell proteins is that large numbers of strains can be compared with a relatively rapid, inexpensive and reliable method. Once electrophoretic groups have been established, the number of strains that needs to be further investigated by genomic methods can be substantially reduced. Electrophoretic traces are stored in a database which can be subsequently used to identify unknown isolates. With this method discrimination can be made at species and subspecies level (Kersters and De Ley, 1975; Vancanneyt *et al.*, 1996; Vandamme *et al.*, 1996; Vauterin *et al.*, 1993).

The drawbacks of this method are that a relatively large amount of cell material (~50 mg wet mass) is needed and that it is more time consuming than other fingerprinting techniques such as gaschromatography of cellular fatty acids (Vauterin *et al.*, 1993).

2.2.1.3.3. 16S rDNA PCR-RFLP analysis

The rRNA molecule is central to the function of the cell which implicates that it cannot be transferred among species and therefore rRNA phylogeny denotes the true phylogeny of the organism. Furthermore this molecule is functionally constant which makes it an accurate molecular chronometer and because it is such a large molecule it contains many functionally defined domains. These are the three main reasons for the use of 16S rRNA in comparative analysis of bacterial species (Woese *et al.*, 1985).

Sequencing of the 16S rRNA genes and DNA-rRNA hybridization is time-consuming and not appropriate for routine identification. Variation in the 16S rRNA genes can also be determined with restriction fragment length polymorphism (RFLP) analysis of enzymatically amplified genes. This is a rapid method that can be used for differentiation at species and subspecies level, and results obtained with PCR-RFLP analysis are in good agreement with those from DNA-DNA homology. (Laguerre *et al.*, 1994; Vandamme *et al.*, 1996; Vaneechoutte *et al.*, 1992).

Advantages of using 16S rDNA PCR-RFLP analysis (or sometimes referred to as ARDRA, amplified rDNA-restriction analysis) are that results are quickly obtained, the

restriction patterns are simple (less than 10 fragments) and therefore are highly reproducible, and differences are studied at a genotypic level. Primers used are complementary to well conserved regions in the 16S rDNA regions of the prokaryotic genome, therefore application of this technique is widespread (Shah and Romick, 1997; Vaneechoutte *et al.*, 1992). Unique patterns obtained can be stored in a database and unknown isolates can be matched to reference strains for confirming suspected species (Shah and Romick, 1997).

This technique has particular application when rapid examination of numerous isolates is needed and to select strains to be studied further by sequencing, DNA:DNA hybridization or other more laborious taxonomic techniques. PCR-RFLP analysis can be used in routine investigations, particularly for taxa which are difficult or laborious to differentiate (Laguerre *et al.*, 1994).

The main drawback of this technique is that a single extra restriction site can cause aberrant patterns (Vaneechoutte *et al.*, 1992). Because of the conserved nature of 16S rDNA sequences, Laguerre *et al.* (1994) found that this method may be limited in differentiation of closely related species as in the case of *Rhizobium tropici* and *Agrobacterium rhizogenes*.

2.2.2. Characteristics of rhizobia

Rhizobia are rod-shaped, Gram-negative, heterotrophic soil bacteria capable of nodulating the roots and/or stems of leguminous plants in temperate and tropical regions. Endospores are not formed. These organisms are aerobic with a respiratory type of metabolism with oxygen as terminal electron acceptor (De Lajudie *et al.*, 1994; Jordan, 1984). The main characteristics of the rhizobia are given in Table 2.

Table 2.　　　**Characteristics of rhizobia.**

	Rhizobium	*Bradyrhizobium*	*Sinorhizobium*	*Azorhizobium*	*Mesorhizobium*
Cell size	0.5 - 0.9 μm x 1.2 - 3.0 μm	0.5 - 0.9 μm x 1.2 - 3.0 μm	0.5 - 1.0 μm x 1.2 - 3.0 μm	0.5 - 0.6 μm x 1.5 - 2.5 μm	---
Cell shape	• pleomorphic under adverse growth conditions • older cells contain granules of poly-ß-hydroxybutyrate	• pleomorphic under adverse growth conditions • older cells contain granules of poly-ß-hydroxybutyrate	• pleomorphic under adverse growth conditions • contain granules of poly-ß-hydroxybutyrate	---	• many strains contain poly-ß-hydroxybutyrate inclusion bodies
Motility	• peritrichous flagella	• one polar or sub-polar flagellum	• one (sub)polar flagellum or 2 - 6 peritrichous flagella	• peritrichous flagella on solid media • one lateral flagellum in liquid medium	• usually one polar or sub-polar flagellum
Colony morphology	• circular, convex, semi-translucent and mucilaginous • diameter of 2 - 4 mm within 3 - 5 days at 25°C • extracellular polysaccharide slime production when grown on carbohydrate media	• circular, opaque, rarely translucent, white, convex • tend to be granular in texture • 1 mm in diameter within 5 - 7 days at 25°C • some strains isolated from *Lotononis bainesii* produce red colonies because of intracellular pigmentation	• circular, convex, semi-translucent, raised and mucilaginous • 2 - 4 mm in diameter within 3 - 5 days at 25°C	• circular with a creamy colour • 2 - 4 mm in diameter within 3 - 7 days at 25°C	• circular, convex, opaque, colorless • 2 - 4 mm in diameter within 3 - 7 days at 25°C
G+C content	59 - 64 mol%	62 - 66 mol%	57 - 66 mol%	66 - 68 mol%	59 - 64 mol%
Optimum pH	pH 6 - 7	pH 6 - 7 (lower for strains from acid soils)	pH 6 - 8	pH 5.5 - 7.8	pH 4.0 - 9.5

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Table 2 (continued).   Characteristics of rhizobia.

	Rhizobium	*Bradyrhizobium*	*Sinorhizobium*	*Azorhizobium*	*Mesorhizobium*
Nutritional requirements	• chemoorganotrophic • utilize a wide range of carbohydrates, with the exception of cellulose and starch • ammonium salts, nitrate, nitrite and most amino acids are utilized as N sources • peptone is poorly utilized	• chemoorganotrophic • utilize a wide range of C sources, with pentoses the preferred source • cellulose and starch are not utilized • ammonium salts, nitrate, nitrite and most amino acids are utilized as N sources • peptone is poorly utilized, except for strains from *Lotononis*	• chemoorganotrophic • utilize a wide range of C sources, with pentoses the preferred source • cellulose and starch are not utilized • ammoniumchloride and nitrates rather than amino acids are preferred as N sources • peptone is poorly utilized	• chemoorganotrophic • among sugars, only glucose is oxidized • organic acids such as lactate or succinate are preferred C sources	• chemoorganotrophic • utilize D-glucose and rhamnose as sole C sources
Other	Produce acidic reaction in mineral salts medium containing mannitol	Produce alkaline reaction in mineral salts medium containing mannitol	• fimbriae occur in few strains • all strains produce acid on YMA	---	Assimilate glucose and rhamnose with acid end products
Symbiotic associations	Invade root hairs of temperate-zone and some tropical-zone leguminous plants	Invade root hairs of tropical-zone and some temperate-zone leguminous plants	Do not exhibit a wide host range. Effectively nodulate *Glycine soja, G. max* and *Vigna unquiculata*	Nodulates roots and stems of *Sesbania rostrata*	Nodulates a restricted range of legumes.
References	Giller and Wilson, 1991 Hirsch and Skinner, 1992 Holt *et al.*, 1994 Jordan, 1984	Giller and Wilson, 1991 Hirsch and Skinner, 1992 Holt *et al.*, 1994 Jordan, 1984	De Lajudie *et al.*, 1994 Holt *et al.*, 1994	Dreyfus *et al.*, 1988 Holt *et al.*, 1994 Graham *et al.*, 1991	Jarvis *et al.*, 1997

Organisms which are most likely to be confused with fast-growing *Rhizobium*, are *Agrobacterium*. Typical of *Agrobacterium* and different from most rhizobia are its ready growth on glucose peptone agar, tolerance to 2% NaCl, precipitate formation with calcium glycerophosphate, reduction of Nile blue, production of H_2S, and the production of 3-ketolactose from lactose (Vincent, 1982).

2.3. SYMBIOSIS

In fast-growing rhizobia, the genes needed for symbiosis, i.e. nodulation (*nod*) and nitrogen fixation (*nif, fix*) genes, are located primarily on the *sym*-plasmid. These genes are chromosomally encoded in slow-growing rhizobia (Roth and Stacey, 1991). Most rhizobia have a narrow host range and specific recognition of the signals exchanged between plant and rhizobia account for much of the specificity (Baron and Zambryski, 1995; Van Rhijn and Vanderleyden, 1995). Rhizobium-legume infection is several successive recognition events involving interactive, complementary bacterial and plant functions (Fig. 2.).

2.3.1. Nodulation of legumes

Rhizobia in the rhizosphere are chemoattracted to compounds such as amino acids, sugars, organic acids, flavonoid compounds, polysaccharides, peptides, enzymes, lectins and other glycoproteins excreted by the legume roots (Roth and Stacey, 1991; Wall and Favelukes, 1991). Invasion of the roots can take place through the root hair, through cracks or through epidermal invasion in the absence of root hair (Davidson and Davidson, 1993).

Initial nodulation events can be characterized as a two-way molecular conversation (Fig. 2). Inducers from the plant, which are phenolics of the flavonoid family of plant secondary metabolites, stimulate expression of the bacterial *nod* genes in conjunction with the NodD protein (Baron and Zambryski, 1995; Fisher and Long, 1992; Roth and Stacey, 1991; Van Rhijn and Vanderleyden, 1995). Nod factors synthesized by the bacteria have a variety of biological activities including inhibition of root growth, induction of plant root hair deformation, induction of cortical cell division, the induction of nodule formation, as well as causing increased exudation

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of flavonoids which in turn induce expression of the *nod* genes (Baron and Zambryski, 1995; Roth and Stacey, 1991).

Nodulation genes are divided into three groups: the regulatory *nodD* gene, which encodes for the NodD protein, a transcriptional regulatory protein that presumably interacts directly with the flavonoids; the so-called "common" nod genes (*nodABCIJ*) which are essential for nodulation to occur, and host-specific nodulation genes which are found only in certain strains and help to determine the host range shown by the bacteria, e.g. *nodE* gene of *Rhizobium leguminosarum* which specifies the nodulation of clover or peas, depending on the biovar source of the gene, i.e. trifolii or viciae respectively (Fisher and Long, 1992; Roth and Stacey, 1991; Spaink, 1994).

After attachment of the rhizobia to the root hair tips, infection is initiated by curling of the root hairs. Rhizobia become entrapped inside the so-called shepherd's crook structures (Spaink, 1995). The cell wall of the host invaginates at the site of infection and new wall material is deposited to form the infection thread (Roth and Stacey, 1991). The bacteria divide and differentiate into pleomorphic nitrogen fixing bacteroids which are characterized by an increase in cell size, changes in surface antigens and morphological change from rod-shaped to branched cells (Baron and Zambryski, 1995; Pawlowski and Bisseling, 1996). The membrane-enclosed bacteroids are released into the plant cytoplasm and develop into nitrogen fixing endosymbionts in mature nodules.

Nodules are lateral outgrowths of the root and/or stem, arising in the cortex following infection of the root-hair by rhizobia. The meristem localizes, either apically or spherically, and thus determines the shape, either oblate or elongate (Corby *et al.*, 1983). Differentiation is made between effective (i.e. nitrogen fixing) and ineffective nodules. Effective nodules are elongate, cylindrical and have apical meristems. These nodules are characterized by two coloured regions: a white region that includes the nodule meristem, cortex and the zone of infection thread invasion, and a pink region with cells containing bacteroids and leghaemoglobin (Corby *et al.*, 1983; Heichel and Vance, 1983). Ineffective nodules appear similar in structure and development to effective nodules with no apparent differences in early symbiotic development. These nodules, however, senesce more rapidly and show a green coloured region over a large portion of the

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nodule. Cells in this region are either empty or contain bacteroids in various stages of deterioration (Heichel and Vance, 1983).
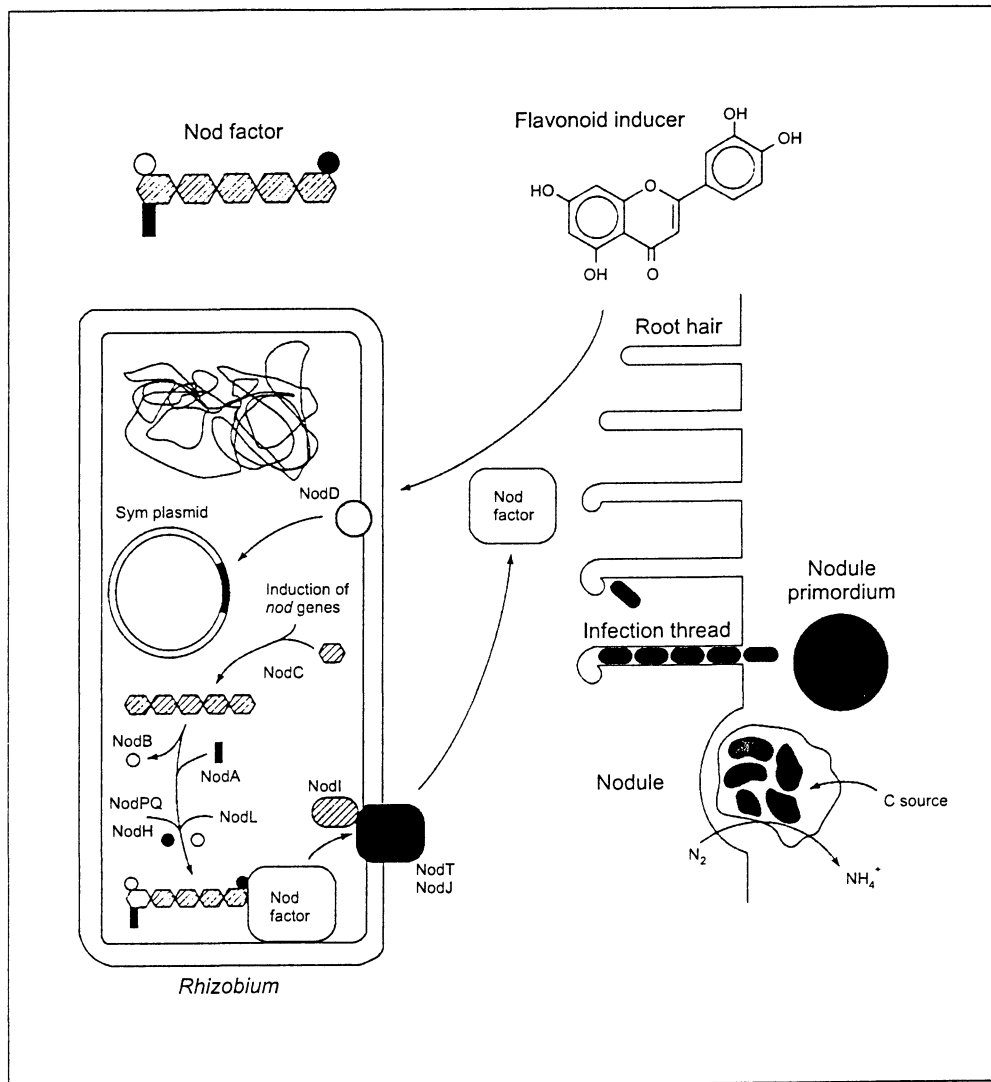
Fig. 2. Nodulation of legumes by rhizobia (Baron and Zambryski, 1995).

Symbols used: ⬢ N-acetylglucosamine, ⬭ glucosamine,
▮ fatty acid (C16:2), ● sulphate, ○ acetyl.

The energy dependent conversion of inert dinitrogen gas (N_2) to ammonia, a substrate which can be utilized by most organisms is catalyzed by nitrogenase, a metalloenzyme which comprises two proteins (Dean *et al.*, 1993). Energy requirements for fixation are met by products from photosynthesis, which include sugars and organic acids used for the generation of ATP, and the reduction of electron donors such as ferredoxin and flavodoxin (Burris, 1997). The net reaction is as follows:

$$N \equiv N + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$$

2.3.2. Nodulation of non-legumes

Nodulation by rhizobia is restricted to the family Fabaceae with only one exception, i.e. nodulation of the non-legume *Parasponia* of the family Ulmaceae (Akkermans *et al.*, 1978). Most wild-type bradyrhizobia isolated from this highly effective symbiosis fail to effectively nodulate legumes. Strain NGR 231 was tested for its ability to nodulate *Parasponia* and several legumes. Effective nodulation of *Parasponia* and four legume species, *Vigna sinensis*, *V. sinensis* ssp. *sesquipedalis*, *Macroptilium artropurpureum* and *M. lathyroides* was observed. Fifteen leguminous species failed to nodulate (Trinick, 1973). *Parasponia* nodules differ structurally from those formed on legumes by the same strain in that it resembles modified roots with a central vascular bundle surrounded by an endophyte-infected zone. Bradyrhizobia are not enclosed in symbiosomes as in legumes, but are retained within the infection threads (De Bruijn, 1997).

3. AGRICULTURAL APPLICATION OF BNF

The main practical impact of BNF has been the development of rhizobial inoculants for crop and pasture legumes. In South Africa, inoculants for the commercial market have been available since 1952 (Strijdom, 1998). As agriculture begins to extend into regions which are marginal because of low and/or erratic rainfall or poor fertility, rhizobia that are able to nodulate a wider range of host plants effectively and survive a wider range of environmental conditions will be needed (Brockwell *et al.*, 1982).

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3.1.   EFFICIENCY OF NITROGEN FIXATION VERSUS FERTILIZATION

Nitrogen fertilizer applied to crops can be temporarily or permanently lost and rarely more than 50% is assimilated. After assimilation efficiency of utilization can range from 20 to 50%, depending on the soil and climatic conditions. A considerable amount of energy is needed for the uptake of nitrogen from the soil through the cell membranes of the roots, and even more energy is needed for the subsequent conversion to a metabolically available substrate. Nitrate can be lost to streams and rivers due to the movement of surface water or leached into groundwater, thereby contaminating wells and threaten human health. Denitrifying bacteria convert nitrate to nitrous oxide (a greenhouse gas) and gaseous nitrogen in wet soils, and therefore the use of nitrogen fertilizer may contribute to global warming (National Research Council, 1997).

In contrast, BNF is very efficient in meeting the high nitrogen requirements of legumes. Conversion of N_2 to NH_3 takes place inside the roots after bacterial penetration of the roots and fixed nitrogen is readily available to be used in the synthesis of proteins. In addition, because nitrogen fixation in the roots is dependent on translocation of carbohydrates from the leaves, the rate of fixation and plant growth is therefore "synchronized" (National Research Council, 1997).

3.2.   INOCULATION WITH RHIZOBIA

Three situations can be identified when inoculation with rhizobia is necessary: in the absence of compatible rhizobia, where the population of compatible rhizobia is too small to give sufficient and rapid nodulation, and where indigenous rhizobia are less effective than the inoculant strain or when ineffective (Giller and Wilson, 1991). Good inoculation responses have been found in situations where cultivated legumes are introduced into a region for the first time, with annual legume crops for which the density of effective soil rhizobia is low and where the level of mineral nitrogen is insufficient for adequate plant growth (Bottomley, 1992; Henzell, 1988). Two kinds of rhizobial inoculants are used: those for application to leguminous seeds and those for direct application to the soil. Seed inoculants are more commonly used (Burton, 1979).

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3.2.1. Selection of inoculant strains

Good inoculant strains are distinguished by their:

- ability to compete with other strains for infection sites on the roots of the host legume,
- ability to fix nitrogen over a wide range of environmental conditions,
- ability to nodulate and fix nitrogen in the presence of soil nitrogen,
- ability to multiply in culture medium and survive in carrier medium,
- persistence in soil and the ability to migrate from the initial site of inoculation,
- ability to survive adverse physical conditions such as desiccation, heat and freezing, and
- stability during storage and growth (Brockwell *et al.*, 1982; Burton, 1979).

3.2.2. Quality of inoculants

The quality of legume inoculants depends on the number of rhizobia it contains, as well as their effectiveness in fixing nitrogen. The standard by which inoculants are ultimately judged is the field performance under different conditions. Because these differ widely, a rigid set of standards cannot be set. Where legumes are to be introduced into a rhizobium-free soil in good condition, 100 rhizobia per seed provide a satisfactory inoculum level. However, when large numbers of ineffective rhizobia are present and/or conditions are adverse for the survival of rhizobia, in excess of 10^6 rhizobia per seed may be needed (Roughley and Pulsford, 1982).

Inoculum quality varies from country to country and no set of international standards exists. Standards for the level of viable rhizobia in inoculants have been established in several countries (Table 3). The highest standards have been set in France and include field testing, an expiry date, 10^6 *Bradyrhizobium japonicum* per seed for soybean and contaminants must be absent (Olsen *et al.*, 1994).

Although the United States of America applies no standards and the quality of the inoculant is left to the manufacturer's own discretion, most USA inoculants exported to Canada contain 1×10^8 rhizobia per gram (Olsen *et al.*, 1994).

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Table 3. **Standards for commercially available inoculants (Olsen *et al.*, 1994; Strijdom and Jansen van Rensburg, 1981).**

Country	Minimum number of rhizobia	Number of contaminants allowed
France	10^6 per seed	0
Australia	$1 \times 10^9 \, g^{-1}$	$< 10^6$
England	$2 \times 10^9 \, g^{-1}$	$< 10^6$
Netherlands	$4 - 25 \times 10^9 \, g^{-1}$	--
New Zealand	$1 \times 10^8 \, g^{-1}$	--
South Africa	$5 \times 10^8 \, g^{-1}$	$< 10^6$
Canada	$10^3 - 10^5$ per seed *	No regulation
Thailand	$5 \times 10^7 \, g^{-1}$	--
Rwanda	$1 \times 10^9 \, g^{-1}$	$< 0.001\%$ of rhizobial count

* 10^3, 10^4, 10^5 for small, intermediate and large seeds respectively

In South Africa, the minimum requirement is at least 5×10^8 rhizobial cells per gram of peat. However, inoculants show an average of 1.8×10^9 to 3.5×10^9 cells per gram. A minimum requirement of 10^9 cells g^{-1} could therefore be a more realistic standard (Strijdom and Jansen van Rensburg, 1981). Further requirements include that only strains supplied by the (presently called) Nitrogen Fixation Unit of the Plant Protection Research Institute of the Agricultural Research Council are used and that an expiry date of six months for each inoculant is set (Strijdom, 1979). All legume inoculants must be registered with the Department of Agriculture (Strijdom, 1998).

3.3. RHIZOBIA AS PLANT GROWTH PROMOTING RHIZOBACTERIA

Plant growth promoting rhizobacteria (PGPR) have the ability to promote plant growth or yield by direct or indirect mechanisms. Numerous reports exist which indicate the ability of rhizobia to colonize the roots of non-legumes efficiently. Höflich *et al.* (1995), described the stimulation of

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growth in the Graminae and crucifers under field conditions by a strain of *R. leguminosarum* biovar trifolii. Noel *et al.* (1996) demonstrated the growth-promotive effect of *Rhizobium* strains of lettuce and canola under gnotobiotic conditions. Strains of *R. leguminosarum* bv. phaseoli colonized the roots of maize and lettuce and were able to survive in the rhizosphere (Chabot *et al.*, 1996b). In all these instances, colonization only took place on the external parts of the roots, and nodulation and nitrogen fixation were absent.

The plant growth-promoting properties of rhizobia may be due to the production of plant-growth regulators such as IAA and cytokinin, as well as increased phosphorus supply to plants through solubilization and/or mineralization of soil organic and inorganic phosphorus (Chabot *et al.*, 1996a). The PGPR ability of rhizobia is of agronomical importance. This is evident with improved rhizobium-legume symbiosis when inoculating the preceding maize crop with rhizobium in crop-rotation (Chabot *et al.*, 1996b). The potential for the use of nitrogen fixing species in alley-cropping systems where arable crops are grown between rows of planted trees and shrubs that are coppiced to provide green manure, needs to be examined (National Research Council, 1994).

4. CONCLUDING REMARKS

One of the major challenges facing us today is to increase the production of food rapidly enough to meet the demands of the growing world population, without degrading soil and related resources. A sustainable agriculture requires that all nutrients removed from the system, must be replenished and in the long-term, the only option is to restore the potassium and phosphorus supplies by using fertilizers. Nitrogen fertilizers are expensive and often difficult to obtain, especially in developing countries in Africa, but nitrogen can be directly fixed from the atmosphere by rhizobial-legume symbiosis. Biological nitrogen fixation shows the greatest promise for improving soil fertility and increasing crop yield. The major limitation to the implementation of this technology, is the difficulty in identifying efficient rhizobial strains which are able to compete successfully with indigenous strains.

Future research on rhizobium-legume symbiosis should include the following :

- Isolation of symbionts from leguminous hosts not investigated previously.

- Initial screening of new isolates should include methods such as SDS-PAGE of whole cell proteins, RFLP analysis of ribosomal sequences, REP-PCR, RAPD analysis, MLEE and numerical taxonomy.

- For the description of new taxa DNA relatedness, sequence analysis of rDNA and a proper phenotypic description are needed.

- Selection and development of improved strains of rhizobia by increasing the host range and improving the nitrogen fixation efficiency, as well as development of plant cultivars with an improved nitrogen fixation capacity.

- Exploitation of the rhizobial-legume symbiosis for the production of food- and forage legumes, as well as fuelwood.

- Promoting improved inoculation and agricultural practices favouring the productivity of the nodulated legumes.

CHAPTER 3

CHAPTER 3

ISOLATION OF PUTATIVE RHIZOBIA FROM INDIGENOUS LEGUMES AND CHARACTERIZATION WITH SODIUM DODECYL-SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

3.1. INTRODUCTION

The family Fabaceae comprises 18 000 species and is the third largest family of flowering plants. This family of plants ranges from huge forest trees to small ephemerals, and extends in a wide range of terrestrial habitats. This versatility enhances the economic importance of legumes. As the human population increases, the pressure on the effective use of marginal land increases and the potential of food and forage legumes has been emphasized in this respect (Polhill *et al.*, 1981).

Certain members of the family Fabaceae are able to join nitrogen-fixing rhizobia in a symbiotic association and specialized structures, the root nodules, are formed. Within these nodules the conversion of inert atmospheric nitrogen to a biologically available substrate, ammonia, takes place (Bohlool *et al.*, 1992, Burton, 1979). This association shows great promise for increasing crop yield and improving soil fertility (Gutteridge and Shelton, 1994).

Until 1981, only about 15% of the legume species had been examined for nodulation by rhizobia (Allen and Allen, 1981). As a result of the investigation of a wider range of leguminous hosts and the advances in molecular techniques for the classification of bacteria, the taxonomy of the root-nodulating bacteria fell into a state of disarray (Young, 1996). In 1984, only two genera, *Rhizobium* and *Bradyrhizobium*, were described for the fast- and slow-growing species respectively. Three new genera, *Azorhizobium* for stem-nodulating isolates of *Sesbania rostrata* (Dreyfus *et al.*, 1988), *Sinorhizobium* (De Lajudie *et al.*, 1994) and *Mesorhizobium* (Jarvis *et al.*, 1997), and numerous new species have been described since.

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The inclusion of South African rhizobia is needed to ensure an accurate taxonomy. Insufficient information on South African rhizobia led to a comprehensive study of indigenous root-nodulating bacteria. Rhizobia were isolated from a diverse range of leguminous hosts and diverse geographic regions, and compared to authentic rhizobial strains of several species with SDS-PAGE analysis of the whole cell proteins (Dagutat, 1995). It was shown that although some of the isolates investigated were related to previously described taxa, some isolates were clearly distinct from previously described rhizobial species. Rhizobia were isolated from three plant genera that were previously described as non-nodulating and subsequently the need for continued investigation of legumes from diverse geographic and climatic environments was emphasized. Further characterization of these isolates should include multilocus enzyme electrophoresis, phenotypic characterization, genotypic characterization, as well as symbiotic performance (Dagutat, 1995).

The aim of this study was to expand the collection of rhizobia from indigenous legumes and to characterize these putative rhizobial isolates, together with isolates from the study by Dagutat (1995), with authentic rhizobial strains with SDS-PAGE analysis of the whole cell proteins. Type and/or reference strains of species of *Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Azorhizobium* and *Agrobacterium*, a genus closely related to *Rhizobium* were included in this study. Characterization with SDS-PAGE served as a basis for the selection of representative isolates for further phenotypic and genotypic analysis.

3.2. MATERIALS AND METHODS

3.2.1. Isolation from nodulated leguminous species

Specimens from all three subfamilies of the Fabaceae were collected (Table 4) and the associated nodules examined for the presence of rhizobia. Plants were identified by the National Botanical Institute (Private Bag X101, Pretoria, 0001).

Nodules were excised and surface sterilized with 5% (v/v) H_2O_2 for 1 to 4 minutes according to size and washed repeatedly with sterilized distilled water. Nodules were squashed individually in 250 - 500 µl sterile distilled water. Loopsful of these suspensions were streaked onto yeast extract mannitol (YM) agar plates, containing 1% (m/v) mannitol (UniVar), 0.5% (m/v) K_2HPO_4 (Merck), 0.02% (m/v) $MgSO_4.7H_2O$ (Merck), 0.01% (m/v) NaCl (NT Chemicals), 0.04% (m/v) yeast extract (Biolab) and 1.5% (m/v) bacteriological agar (Biolab) supplemented with Congo red (Allen, 1959). The excess suspension was poured onto YM agar plates as well. Plates were incubated at 26°C and examined after 3, 5 and 7 days for growth. Colonies were purified by further streaking on the same medium, at least three times or until pure cultures were obtained. Pure cultures were transferred to YM agar without Congo red (Vincent, 1970), and living cells and Gram-stained cells were examined microscopically.

3.2.2. Test for the presence of *Agrobacterium*

3.2.2.1. Reaction in litmus milk

Litmus milk broth (Biolab) was prepared according to the manufacturer's instructions and divided into test tubes prior to sterilization at 121°C for 5 minutes. Each test tube was inoculated with a loopful of culture. Inoculated tubes were incubated at 26°C for 6 weeks. Cultures were examined each week as to colour and consistency of the medium, and presence or absence of gas production (Harley and Prescott, 1993).

3.2.2.2. Production of 3-ketolactose

Strains were initially grown on agar slopes containing 1% (m/v) yeast extract (Biolab), 2% (m/v) glucose (Merck), 2% (m/v) $CaCO_3$ (Merck) and 2% (m/v) bacteriological agar at 26°C for 72 h. Loopsful of the subsequent growth were transferred (spot-inoculation) to agar plates containing 1% (m/v) lactose (BDH), 0.1% (m/v) yeast extract (Biolab) and 2% (m/v) bacteriological agar (Biolab). Plates were incubated for 72 h at 26°C and then flooded with Benedict's qualitative reagent. Flooded plates were left at room temperature for 1 hour.

3.2.3. Maintenance of cultures

Cultures were maintained on YM agar slants and long term storage of the strains was carried out in glycerol. Yeast mannitol broth (YMB) cultures were incubated at 25 - 28°C for 5 - 7 days with rigorous shaking and mixed 1:1 with 50% (v/v) glycerol (Merck) in sterile cryotubes and stored at -20°C and -70°C.

3.2.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

3.2.4.1. Strains used

Thirty five reference strains (Table 5) of the genera *Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Azorhizobium* and *Agrobacterium* obtained from the Laboratorium voor Microbiologie Gent Culture Collection (State University, Belgium) and United States Department of Agriculture-ARS National Rhizobium Culture Collection, 66 indigenous rhizobial strains isolated in a previous study (Table 6) (Dagutat, 1995) and six strains received from the Agricultural Research Council (Table 6), and 24 strains isolated in this study (Table 4) were used in SDS-PAGE analysis of the whole cell proteins.

3.2.4.2. Preparation of whole cell protein extracts

Whole cell protein extracts were prepared as described by Dagutat (1995). Strains were grown on YEB medium at 26°C for 5 days and transferred to fresh YEB twice before sample preparation started. YEB contained 0.5% peptone (m/v) (Biolab), 0.1% (m/v) yeast extract (Merck), 0.5% (m/v) beef extract (Biolab), 0.5% (m/v) sucrose (Merck) and 2% (m/v) bacteriological agar (Biolab). Cells were harvested with a sterile spatula, transferred to 1.5 ml Eppendorf tubes and washed twice in 750 µl 0.2 M sodium phosphate buffer (pH 6.88 (Merck)) by centrifugation and resuspension. Cell pellets were suspended in 300-600 µl sample treatment buffer (STB) [0.5 M Tris-HCl pH 6.8, 5% (v/v) 2-ß-mercaptoethanol (BDH), 10% (v/v) glycerol (Merck), 2% (m/v) SDS (UniVar)] depending on pellet size and heated to 95°C. A Cole-Parmer ultrasonic homogenizer (Series 4710) was used at 50% maximum output (40 Watt) to disrupt cells. Equal volumes of STB were added, mixed and centrifuged at 15 000 rpm for 8 minutes. The supernatant was transferred to clean Eppendorf centrifuge tubes and frozen at -20°C overnight. To dispose of

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excess slime, samples were heated to 95°C for 30 minutes, cooled to room temperature and centrifuged for 10 minutes at 15 000 rpm. The supernatant was transferred to clean Eppendorf centrifuge tubes and stored at -20°C overnight.

3.2.4.3. Polyacrylamide gel electrophoresis

The method of Laemmli (1970), as modified by Kiredjian et al. (1986), was used. A monomer solution containing 29.2% (m/v) acrylamide (BDH Electran) and 0.8% (m/v) N^1-N^1-bismethylene acrylamide (BDH Electran) was used for the preparation of a 12 % separation gel (1.5 M Tris-HCl pH 8.68, conductivity 17.0 mS) and 5% stacking gel (0.5 M Tris-HCl pH 6.68, conductivity 30.9 mS). Electrophoresis of the 1.5 mm thick gels was done in a BioRad Protean II gel apparatus at a constant current of 5 Watt through the stacking gel and 10 Watt through the separation gel (at 10°C). Gels were stained in Coomassie Blue solution prepared from a 2% Coomassie Brilliant Blue R (UniLab) stock solution [12.5% (m/v) Coomassie Stock solution, 50%(v/v) methanol (BDH Analar), 10% (v/v) acetic acid (NT Chemicals)]. Overnight destaining of the gels in a solution containing 25% (v/v) methanol (BDH Analar) and 10% (v/v) acetic acid (NT Chemicals) followed.

Table 4.   **Putative rhizobia isolated in this study and their host legumes.**

Isolate	Host legume	Subfamily*	Isolate	Host legume	Subfamily*
UP2a	Lessertia capitata	P	UP23b	Indigofera verrucosa	P
UP3a	Lotononis falcata	P	UP24a	Lessertia annularis	P
UP9	Medicago polymorpha	P	UP24b	Lessertia annularis	P
UP10	Senna petersiana	C	UP26b	Crotalaria damarensis	P
UP11e	Bolusanthus speciosus	P	UP26c	Crotalaria damarensis	P
UP14a	Acacia sp.	M	UP27a	Crotalaria damarensis	P
UP15b	Indigofera schinzii	P	UP27b	Crotalaria damarensis	P
UP16b	Desmodium repandum	P	UP27c	Crotalaria damarensis	P
UP16c	Desmodium repandum	P	UP30a	Indigofera filipes	P
UP17b	Indigofera heterophylla	P	UP30c	Indigofera filipes	P
UP19	Trifolium sp.	P	UP31a	Chamaecrista mimosoides	C
UP22b	Medicago sativa	P	UP32c	Vigna unguiculata	P

*   Subfamily of the Fabaceae: P = Papilionoideae,   C = Caesalpinioideae,   M = Mimosoideae

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Table 5.   **List of reference strains used in SDS-PAGE analysis.**

	Strain no. *	Host plant	Geographic origin
Rhizobium leguminosarum	LMG 4260	*Vigna unguiculata*	NS
Rhizobium leguminosarum	LMG 6294t1	*Lathyrus* sp.	St. Petersburg
Rhizobium leguminosarum	LMG 6294t2	*Lathyrus* sp.	St. Petersburg
R. leguminosarum bv. *viciae*	USDA 2370^T	*Pisum sativum*	NS
R. leguminosarum bv. *trifolii*	USDA 2046	NS	NS
Rhizobium galegae	USDA 4128^T	*Galega orientalis*	USSR
Rhizobium galegae	LMG 6215	*Galega orientalis*	USSR
Rhizobium tropici IIB	USDA 9030^T	*Phaseolus vulgaris*	NS
Rhizobium hainanensis	USDA 3588^T	NS	NS
Rhizobium sp.	LMG 6463	*Sesbania rostrata*	Senegal
Rhizobium sp.	LMG 8311	*Acacia farnesiana*	Senegal
Rhizobium sp.	USDA 2947	NS	NS
Bradyrhizobium japonicum	USDA 6^T	NS	NS
Bradyrhizobium japonicum	LMG 4265	*Ulex europaeus*	NS
Bradyrhizobium elkanii	LMG 6134^T	*Glycine max*	NS
Bradyrhizobium elkanii	USDA 76	NS	NS
Bradyrhizobium sp.	LMG 8319	*Macrotyloma africanus*	Zimbabwe
Azorhizobium caulinodans	LMG 6465	*Sesbania rostrata*	Senegal
Sinorhizobium meliloti	LMG 6133^T	*Medicago sativa*	NS
Sinorhizobium meliloti	LMG 6131	*Medicago sativa*	NS
Sinorhizobium meliloti	USDA 1002	*Medicago sativa*	NS
Sinorhizobium meliloti	USDA 1954	*Trigonella suavissima*	NS
Sinorhizobium fredii	LMG 6217^T	*Glycine max*	Honan, China
Sinorhizobium saheli	LMG 7837^T	*Sesbania cannabina*	Senegal
Sinorhizobium teranga	LMG 7834^T	*Acacia laeta*	Senegal
Mesorhizobium loti	LMG 4268t2	*Lotus americanus*	NS
Mesorhizobium loti	LMG 6123	*Lotus divaricatus*	New Zealand
Mesorhizobium loti	LMG 4264	*Lupinus densiflorus*	NS
Mesorhizobium huakuii	USDA 4778^T	*Astragalus sinicus*	China
Mesorhizobium huakuii	LMG 14107	NS	NS
Agrobacterium radiobacter	LMG 140^T	NS	NS
Agrobacterium tumefaciens	LMG 187^T	*Lycopersicon lycopersicum*	NS
Agrobacterium rhizogenes	LMG 150^T	NS	NS
Agrobacterium stellatum	LMG 122	NS	Baltic Sea
Agrobacterium rubi	LMG 156^T	*Rubus ursinus*	USA

*   **LMG**, Laboratorium voor Microbiologie Gent Culture Collection, State University Gent, Belgium.

USDA, United States Department of Agriculture-ARS National Rhizobium Culture Collection.

T   Type strain

NS   not stated

Table 6. **List of indigenous rhizobia isolated by Dagutat (1995) and isolates received from the Agricultural Research Council used in SDS-PAGE analysis.**

Isolate	Host legume	Subfamily[#]	Isolate	Host legume	Subfamily[#]
2	*Rhynchosia nervosa*	P	60	*Rhynchosia monophylla*	P
3a	*Trifolium* sp.	P	65a2	*Acacia* sp.	M
4a1	*Teramnus labialis*	P	66a1t1	*Acacia robusta*	M
4c	*Teramnus labialis*	P	68a	*Mucuna coriacea*	P
5b	*Crotalaria distans*	P	68d	*Mucuna coriacea*	P
6a	*Vigna vexillata*	P	70a	*Crotalaria brachycarpa*	P
7b	*Alysicarpus rugosus*	P	70b2	*Crotalaria brachycarpa*	P
8	*Medicago lupulina*	P	72f	*Tephrosia purpurea*	P
12c1	*Indigofera rhytidocarpa*	P	73a1	*Chamaecrista mimosoides*	C
12c2	*Indigofera rhytidocarpa*	P	74a	*Indigofera arrecta*	P
13b	*Lotononis bainesii*	P	79c	*Acacia caffra*	M
13c1	*Lotononis bainesii*	P	82a	*Tephrosia purpurea*	P
13c2	*Lotononis bainesii*	P	85a	*Acacia xanthoploea*	M
14b	NS	--	85b	*Acacia xanthoploea*	M
15b	*Desmodium tortuosum*	P	85c	*Acacia xanthoploea*	M
15c	*Desmodium tortuosum*	P	87a	*Rhynchosia hirta*	P
24a	*Desmodium tortuosum*	P	91a1	*Tephrosia* sp.	P
24b	*Desmodium tortuosum*	P	94	*Vigna subterranea*	P
25aa	*Neonotonia wightii*	P	98d2	*Bolusanthus speciosus*	P
26c	*Strongylodon macrobotrys*	P	101d	*Zornia capensis*	P
29d	*Alysicarpus rugosus*	P	102a	*Chamaecrista biensis*	C
33b	*Crotalaria* sp.	P	102b1	*Chamaecrista biensis*	C
34	*Neonotonia wightii*	P	103b	*Indigofera hilaris*	P
36b1	*Neonotonia wightii*	P	104a1	*Chamaecrista* sp.	C
36b2	*Neonotonia wightii*	P	108a2	*Indigofera swaziensis*	P
36d	*Neonotonia wightii*	P	109a2	*Acacia dealbata*	M
36d2	*Neonotonia wightii*	P	114d	*Pseudarthria hookeri*	P
39a	*Chamaecrista abrus*	C	123c	*Desmodium repandum*	P
40a	*Sesbania bispinosa*	P	125e	*Indigofera woodii*	P
42b	*Neonotonia wightii*	P	128a	*Melolobium obcordatum*	P
46c2	*Acacia sieberana* var *woodii*	M	PL1*	*Medicago lupinifolia*	P
48a	*Tephrosia purpurea*	P	PL3*	*Tephrosia grandiflora*	P
48b	*Tephrosia purpurea*	P	PL6*	*Indigofera porrecta*	P
49a	*Indigofera melanadenia*	P	PL10a	*Argyrolobium tomentosum*	P
49b	*Indigofera melanadenia*	P	PL10b*	*Argyrolobium tomentosum*	P
55a	*Rhynchosia monophylla*	P	PL12b*	*Argyrolobium tomentosum*	P

NS   Not stated

*   Received from the Plant Protection Research Institute, Agricultural Research Council, Roodeplaat.

Subfamily of the Fabaceae: P = Papilionoideae, C = Caesalpinioideae, M = Mimosoideae

3.2.4.4. Analysis of gels

Electrophoretic patterns of the proteins were scanned with a Hoefer GS300 densitometer (Hoefer Scientific Instruments, San Francisco). Data were analyzed with the GelCompar 4.0 Program (Applied Maths, Kortrijk, Belgium). 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. To ensure reproducibility of electrophoresis, the protein pattern of *Psychrobacter immobilis* LMG 1125 was used as reference pattern on each gel (six tracks per gel). One *P. immobilis* protein profile was selected as standard and a correlation (r) of 94% (where r = r x 100%) of this standard with the six reference protein profiles on successive gels was accepted as reproducible.

3.3. RESULTS

3.3.1. Isolation from nodulated legumes

In this study, rhizobia were isolated from nodules of all three subfamilies of the Fabaceae (Table 3) and in several cases, more than one rhizobial strain were isolated from the same nodule, e.g. UP26a and UP26b from *Crotalaria damarensis*.

3.3.2. Presence of *Agrobacterium*

3.3.2.1. Reaction in litmus milk

Rhizobium strains are weakly proteolytic and show slow digestion of litmus milk. Differences in the digestion of litmus milk by rhizobia and agrobacteria are given in Table 7. Non-rhizobial contaminants show rapid change in litmus milk coupled with decolourization and curdling thereof (Vincent, 1970). Most of the isolates produced an alkaline reaction with or without a clear "serum zone". Although a few produced an acidic reaction, these isolates were not excluded as the changes were not rapid.

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3.3.2.2. Production of 3-ketolactose

This method can be used to differentiate *Agrobacterium* strains as rhizobial strains do not produce 3-ketoglycosides (Jordan, 1984). Development of a yellowish-brown zone (Cu_2O) was interpreted as positive, i.e. production of 3-ketolactose (Holding and Collee, 1971). None of the isolates tested positive for the production of 3-ketolactose.

Table 7. **Differentiation of strains with reaction in litmus milk (Jordan, 1984).**

Bacterial group	Reaction in litmus milk
Fast-growing rhizobial species	Weak alkaline reaction with clear "serum zone" or no reaction. *S. meliloti* is inclined to produce an acidic reaction.
Slow-growing *Bradyrhizobium*	Alkaline reaction without the production of a "serum zone".
Agrobacterium biovar 1	Alkaline reaction with brown "serum zone".
Agrobacterium biovar 2	Acidic reaction.

3.3.3. SDS-PAGE of whole cell proteins

In this study, SDS-PAGE analysis was used for the characterization of a large group of isolates from which representative strains were selected for further analysis. Similar to findings of Dagutat (1995) and Moreira *et al.* (1993), the quality of protein profiles of the slow-growing isolates was lower than those of the fast growers with less well-defined protein bands.

The isolates and reference strains investigated grouped into three major sections which could be further divided into 15 clusters (Fig. 3). (Separation into three sections in this case is to facilitate discussion of the results and does not imply a closer relationship between the isolates in the three sections respectively). Section I constituted the bradyrhizobial group and contained 56 of the indigenous strains and four *Bradyrhizobium* reference strains. Two closely related *Rhizobium* strains and one *Sinorhizobium* reference strain which did not show close similarity to any other

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strain were also included in this section. Section II formed a *Rhizobium - Bradyrhizobium - Mesorhizobium* - section with reference strains from these three genera and 23 indigenous strains. Section III constituted a *Rhizobium - Sinorhizobium - Mesorhizobium* section in which all the *Agrobacterium* strains formed a separate cluster. Seventeen indigenous strains were included.

Section I

Most of the slow-growing indigenous strains clustered in this section together with *Bradyrhizobium* strains. This section was further divided into five cluster (clusters 1 to 5), two of which did not contain any reference strains.

Cluster 1

Cluster 1 consisted of 16 slow growing indigenous isolates and one reference strain, *Bradyrhizobium japonicum* USDA 6 with a similarity of 83.7%. Three distinct subgroups were distinguished, with Clusters 1a and 1b associated at 86.4% similarity and Cluster 1c joined these two at 83.7% similarity.

> **Cluster 1a** contained six indigenous isolates and one *Bradyrhizobium* reference with a similarity of 88.9%: 5b (*Crotalaria distans*), 8 (*Medicago lupulina*), 34 (*Neonotonia wightii*), 104a1 (*Chamaecrista* sp.), and 49b (*Indigofera melanadenia*) and 48b (*Tephrosia purpurea*) which were very closely related at 96.5% similarity. Isolate 8 (*Medicago*) showed the highest similarity (94.8%) with *B. japonicum* USDA 6. **Cluster 1b** consisted of four indigenous isolates, 73a1 (*Chamaecrista mimosoides*), PL6 (*Indigofera porrecta*), 82a and 91a1 both from *Tephrosia* spp., with 87.4% similarity. **Cluster 1c** comprised six indigenous isolates with 84.6% similarity from various legume hosts: 46c2 (*Acacia sieberana* var *woodii*), UP26c and 33b (*Crotalaria* spp.), UP11e (*Bolusanthus speciosus*), 123c (*Desmodium repandum*) and 103b (*Indigofera hilaris*)

Cluster 2

This constituted the largest cluster with 20 rhizobial isolates and three reference strains with a similarity of 84.9%. Four subgroups could be distinguished.
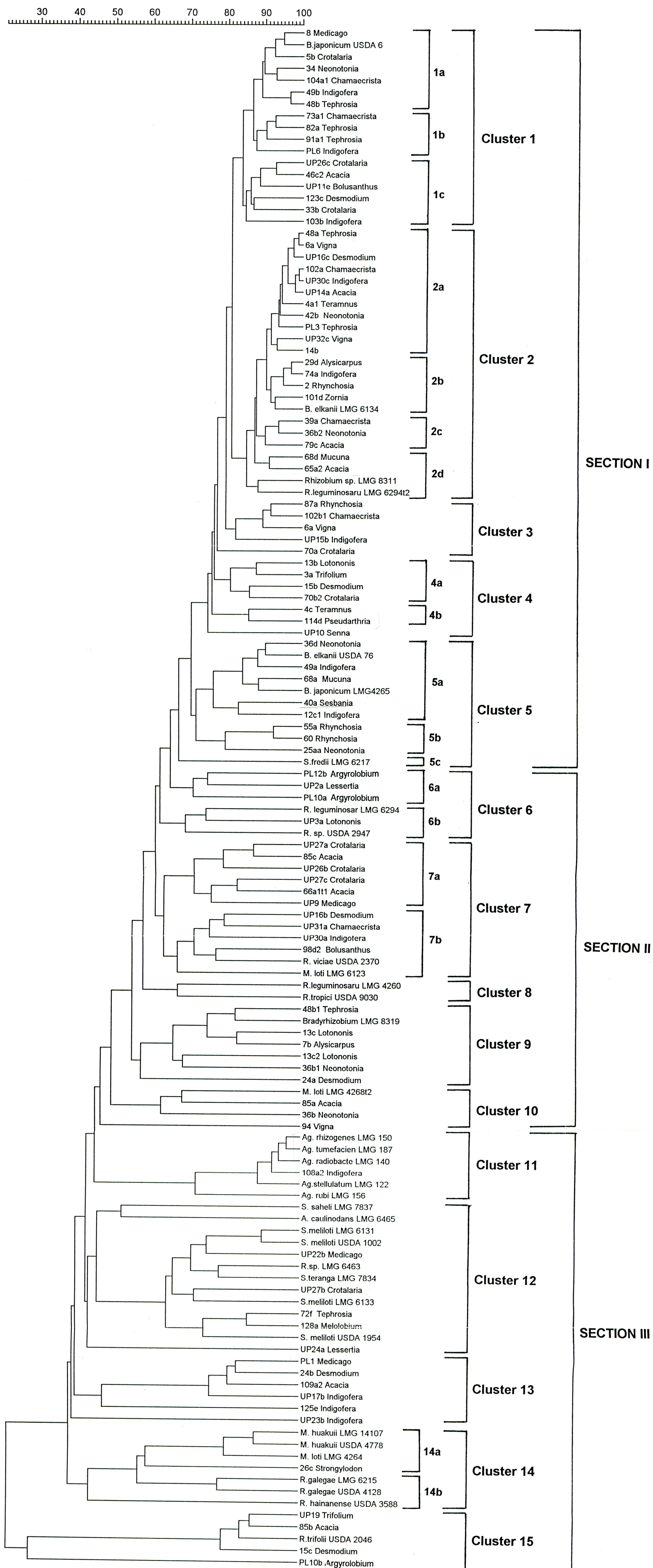
Fig. 3. Dendrogram based on UPGMA analysis of the correlation coefficients (r) between protein profiles of indigenous isolates and reference strains of the genera *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium, Meso-rhizobium* and *Agrobacterium*. Correlation (r), where r = r x 100% are represented on the x-axis.

Cluster 2a contained eleven indigenous isolates from a variety of leguminous hosts with a similarity of 92% constituted this group. No reference strain was included. Isolates 6a (*Vigna vexillata*), 48a (*Tephrosia purpurea*) and UP16c (*Desmodium repandum*), and UP30c (*Indigofera filipes*), 102a (*Chamaecrista biensis*) and UP14a (*Acacia* sp.) showed very close relatedness with 97.5% and 97.9% similarity between the two groups respectively. Isolates 4a1 (*Teramnus labialis*), 42b (*Neonotonia wightii*), PL3 (*Tephrosia grandiflora*), 14b and UP32c (*Vigna unguiculata*) were also contained in this closely related group of isolates. **Cluster 2b** consisted of four indigenous isolates and one reference strain, *Bradyrhizobium elkanii* LMG 6134 with 91.4% similarity. Isolate 101d (*Zornia*) showed a similarity of 92.6% to the *Bradyrhizobium* reference strain. Isolates 74a (*Indigofera arrecta*), 29d (*Alysicarpus rugosus*) and 2 (*Rhynchosia nervosa*) were closely associated at a similarity of 94.8% within this cluster. **Cluster 2c** contained three indigenous isolates 36b2 (*Neonotonia wightii*), 39a (*Chamaecrista abrus*) and 79c (*Acacia caffra*) with a similarity of 90%. **Cluster 2d** consisted of two groups with a similarity of 84.9%. Indigenous strains 68d (*Mucuna coriacea*) and 65a2 (*Acacia* sp.) showed 91.1% similarity and two *Rhizobium* reference strains, *R. leguminosarum* LMG 6294t2 and *Rhizobium* sp. (*Acacia*) LMG 8311 showed 86% similarity.

Cluster 3

Cluster 3 joined clusters 1 and 2 at a similarity of 79.4% and consisted of five indigenous isolates with a slow growth rate. Isolates 87a (*Rhynchosia hirta*), 102b1 (*Chamaecrista biensis*) and 6a (*Vigna vexillata*) showed 90% similarity and UP15b (*Indigofera schinzii*) joined these isolates at 82.1% similarity. Isolate 70a (*Crotalaria*) showed no close association with any other strain investigated and joined the rest of cluster 3 at 77.1% similarity.

Cluster 4

Two subgroups were distinguished in cluster 4 which contained no reference strain.

> **Cluster 4a** consisted of four indigenous strains isolated from various legumes. Isolates 70b2 (*Crotalaria brachycarpa*), 15b (*Desmodium tortuosum*), 13b (*Lotononis bainesii*) and 3a (*Trifolium* sp.) exhibited a similarity of 80.7%. In **cluster 4b**, isolates 114d (*Pseudarthria hookeri*) and 4c (*Teramnus labialis*), joined cluster 4a at 76.3% similarity.

Isolate UP10 isolated from *Senna petersiana* with no close association with any other strain joined the other members of cluster 4 at 75.7% similarity.

Cluster 5

This cluster constituted a *Bradyrhizobium* branch with two reference strains and eight indigenous isolates. Three subgroups were distinguished.

Cluster 5a with the two reference strains, *B. japonicum* LMG 4265 and *B. elkanii* USDA 76 and isolates 36d (*Neonotonia wightii*), 49a (*Indigofera melanadenia*) and 68a (*Mucuna coriacea*) with a similarity of 84%. Isolates 40a (*Sesbania bispinosa*) and 12c1 (*Indigofera rhytidocarpa*) had a similarity of 76.1% to these isolates. **Cluster 5b** comprised two slow-growing isolates from *Rhynchosia monophylla*, 55a and 60, which showed a similarity of 92.4% and were joined by 25aa (*Neonotonia wightii*) at 79.4% similarity. **Cluster 5c** consisted of only one strain, *Sinorhizobium fredii* LMG 6217 which was clearly distinct from not only the other *Sinorhizobium* strains which all clustered in cluster 12, but also the indigenous isolates.

Section II

Five clusters (clusters 6 to 10) comprised this section which contained *Rhizobium, Bradyrhizobium* and *Mesorhizobium* reference strains and some of the indigenous isolates. None of these clustered showed similarity higher than 71.1%.

Cluster 6

This group contained four indigenous isolates and two *Rhizobium* reference strains and was further divided into two subgroups.

Cluster 6a consisted of three indigenous isolates UP2a (*Lessertia capitata*), PL12b and PL10a (*Argyrolobium tomentosum*) with 70.8% similarity and in **Cluster 6b** isolate UP3a (*Lotononis falcata*) and *Rhizobium leguminosarum* LMG 6294 showed 74.3% similarity, and were joined by *Rhizobium* sp. USDA 2947 at 68.7% similarity.

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Cluster 7

Cluster 7a contained six isolates, 66a1t1 (*Acacia robusta*), UP27c, UP27a and UP26b (*Crotalaria damarensis*), UP9 (*Medicago polymorpha*) and 85c (*Acacia xanthoploea*) which exhibited 71.1% similarity. Cluster 7b consisted of four indigenous isolates from different leguminous hosts, UP31a (*Chamaecrista mimosoides*), UP16b (*Desmodium repandum*), UP30a (*Indigofera filipes*) and 98d2 (*Bolusanthus speciosus*) and one reference strain *Rhizobium leguminosarum* bv. viciae USDA 2370 with a 71.1% similarity. *Mesorhizobium loti* LMG 6123 joined these strains at 66.6% similarity. Cluster 7b joined cluster 7a at a similarity of 62.9%.

Cluster 8

Two *Rhizobium* reference strains, *R. leguminosarum* LMG 4260 and *R. tropici* USDA 9030 showed 66.7% and grouped apart from any of the other strains investigated. This cluster joined cluster 7 at 60.7% similarity.

Cluster 9

Cluster 9 represented a *Bradyrhizobium* group with six indigenous isolates and one reference strain. *Bradyrhizobium* sp. LMG 8319 and 48b1 (*Tephrosia purpurea*) showed 82.5% similarity, and 7b (*Alysicarpus rugosus*) and 13c (*Lotononis bainesii*) 85.5%, and these four isolates showed a similarity of 74.8%. Isolates 36b1 (*Neonotonia wightii*), 13c2 (*Lotononis bainesii*) and 24a (*Desmodium tortuosum*) were loosely associated with this group.

Cluster 10

Indigenous isolates 85a (*Acacia xanthoploea*) and 36b (*Neonotonia wightii*) and *Mesorhizobium loti* LMG 4268t2 showed a similarity of 62.4% and was joined by isolate 94 (*Vigna subterranea*) at a similarity of 49.0%. The latter isolate showed no close similarity to any other strain investigated.

Section III

This section contained the *Sinorhizobium* and *Agrobacterium* clusters, as well as *Rhizobium, Mesorhizobium* and *Azorhizobium* reference strains, and few of the indigenous isolates and was further divided into five clusters (clusters 11 to 15).

Cluster 11

All the *Agrobacterium* reference strains were grouped into one cluster which was clearly separate from the rhizobial genera. Isolate 108a2 (*Indigofera swaziensis*) produced a weak alkaline reaction in litmus milk and did not produce 3-ketolactose and was therefore not excluded from the study, but showed 93% similarity with three of the *Agrobacterium* reference strains. *Agrobacterium rubi* LMG 156 showed the least relatedness to the other *Agrobacterium* strains.

Cluster 12

Cluster 12 represented the *Sinorhizobium* branch of the dendrogram with six reference strains of this genus enclosed in this cluster. *S. meliloti* strains LMG 6131 and USDA 1002 showed 89.8% similarity in their electrophoregrams, with isolate UP22b (*Medicago sativa*) closely associated with these two strains. Two other *S. meliloti* groups were distinguished within this branch. *S. meliloti* LMG 6133 and UP27b (*Crotalaria damarensis*) showed 71.6% similarity, and 128a (*Melolobium obcordatum*), 72f (*Tephrosia purpurea*) and *S. meliloti* USDA 1954 showed 74.2% similarity. *S. teranga* LMG 7834 and *Rhizobium* sp. LMG 6463 were combined into one group with 78.3% similarity. *S. saheli* LMG 7837 and *Azorhizobium caulinodans* LMG 6465 joined this group at a similarity of 45.6%. Indigenous isolate UP24a (*Lessertia annularis*) was loosely associated with this *Sinorhizobium* branch at 43.2% similarity.

Cluster 13

Four indigenous isolates 24b (*Desmodium tortuosum*), PL1 (*Medicago lupiniflora*), 109a2 (*Acacia dealbata*) and UP17b (*Indigofera heterophylla*) formed a closer related cluster with 75.8% similarity. Associated with this group was isolate 125e (*Indigofera woodii*) which joined the other isolates at a similarity value of 47.1%. Isolate UP23b (*Indigofera verrucosa*) showed no close similarity with any other strain and joined the rest of cluster 13 at 39.8% similarity.

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Cluster 14

Two distinct subgroups could be distinguished.

Cluster 14a represented a *Mesorhizobium* branch in the dendrogram with three reference strains, *M. huakuii* LMG 14107 and USDA 4778, and *M. loti* LMG 4264, in close association (79.8%). Isolate 26c (*Strongylodon macrobotrys*) showed 58.8% similarity with these strains. Three *Rhizobium* reference strains, *R. galegae* LMG 6215 and USDA 4128, and *R. hainanensis* USDA 3588 constituted **cluster 14b**. The two *R. galegae* strains showed 78.2% similarity with each other and joined the *Mesorhizobium* group at a similarity value of 56.7%. The *R. hainanensis* strain had no close similarity with any of the indigenous strains and joined the *R. galegae* group at a similarity of 43.5%.

Cluster 15

This group was clearly separate from the other 14 clusters and showed 21.6% similarity with the isolates in these clusters. A closely associated group of isolates could be distinguished: *R. trifolii* USDA 2046, 85b (*Acacia xanthoploea*), UP19 (*Trifolium* sp.) and 15c (*Desmodium tortuosum*) showed 79.3% similarity. Isolate PL10b (*Argyrolobium tomentosum*) was associated with this group at 27.6% similarity.

3.4. DISCUSSION

The collection of indigenous rhizobia was expanded with the isolation of new rhizobia from diverse legumes in this study. In this study, as in the study by Dagutat (1995), the majority of nodulated leguminous hosts investigated belonged to the subfamily Papilionoideae. Only a small percentage of legumes has been investigated for nodulation by rhizobia. Estimates of nodulation range from 94 - 98% of the species of the Papilionoideae, 92 - 96% of the species of the Mimosoideae and only 30 - 34% of the species of the Caesalpinioideae (Corby *et al.*, 1983).

In Allen and Allen (1981), *Senna petersiana* is reported as a non-nodulated species in the subgenus *Senna* of the genus *Cassia* and only 5 - 19% of *Senna* species are nodulated by rhizobia (Bryan *et al.*, 1996). The *Senna* host investigated in this study showed thickening of the lateral

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roots and classical nodule types were absent. However, these swollen parts of the roots were treated the same way as nodules from other legumes investigated and strain UP10 which produced white mucous colonies on YMA with large pleomorphic cells that are characteristic of bacteroids, was isolated. In litmus milk, a weak alkaline reaction was produced after six weeks and 3-ketolactose was not produced. On the basis of these results, this isolate was included in the study as a putative rhizobial isolate.

It has been shown that infection threads are formed in the root hairs of non-nodulating legumes such as *Glenditsia triacanthos* L. (Allen and Allen, 1981), *Cassia fistula* and *C. grandis* and *Senna tora* (*Cassia tora* in Allen and Allen, 1981). According to Allen and Allen (1981) these root hairs were not part of a symbiosis as the infection thread observed only grew into the outer root cortices. However, distinct bacteroids were observed in the roots of among other, *G. triacanthos* (Bryan *et al.*, 1996). In a study done by Bryan *et al.* (1996), cells resembling rhizobial bacteroids were found in the swollen roots of six non-nodulating species investigated and the hypothesis that symbiotic nitrogen fixation does occur in the roots of these legumes was confirmed. It is estimated that the rate of acetylene reduction (thus, by implication nitrogen fixation) in these non-nodulating legumes may be one to two orders of a magnitude lower than in nodulated legumes.

Although the root cortices of *Senna petersiana* were not examined in this study, the isolation of strain UP10 is significant. This strain show similarity with slow-growing bradyrhizobial strains and has to be tested further with regard to symbiotic performance. The presence of effective rhizobial bacteria in the roots of non-nodulated legumes is of great importance as these legumes grow well in many nitrogen-limited soils, often dominate in poor soils and give higher yields than nodulating species in these circumstances (Bryan *et al.*, 1996).

As in the study by Dagutat (1995), where rhizobia were isolated from three genera (*Cassia, Schizolobium* and *Bauhinia*) of the subfamily Caesalpinioideae previously reported as non-nodulating, the need to examine a wide variety of legumes from diverse geographic regions in order to improve knowledge of the associated rhizobia, was once again emphasized.

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More than one rhizobial type was isolated from the same nodule, which once again reflects on the dubious nature of symbiotic performance as a taxonomic trait. The isolation of diverse rhizobia from the same leguminous host and even the same nodule has been reported by several authors (Dagutat, 1995; Dreyfus and Dommergues, 1981; Jenkins *et al.*, 1987).

Most of the indigenous isolates investigated were members of the genus *Bradyrhizobium*, but close similarity to the genera *Rhizobium, Sinorhizobium* and *Mesorhizobium* was also found. None of the isolates showed any similarity with *Azorhizobium caulinodans*, which supported the results of Dagutat (1995). Neither host plant specificity nor geographic origin played any role in the systematic grouping of strains, as most of the strains did not cluster systematically according to these factors. Closely related groups of isolates which were not related to any of the reference strains included, could warrant the description of new taxa if further analysis supports SDS-PAGE results.

A higher similarity was observed between the slow-growing isolates in clusters 1 and 2, compared to that of the faster-growing isolates in clusters 12, 13 and 14. The former isolates produced protein profiles that were not as well defined, with less distinct protein bands. Thus, in the analysis of the electrophoregrams, fewer values are taken into account in determining the similarity.

Representative strains of the genus *Rhizobium* were dispersed throughout the dendrogram (clusters 2, 6b, 7b, 8, 10, 14b, 15) which supported the heterogeneous character of this group of bacteria (Willems and Collins, 1993; Young, 1996). Isolate 98d2 from the indigenous tree legume *Bolusanthus speciosus* showed affinity to *R. leguminosarum* bv. viciae. This species was described for symbionts of *Vicia, Pisum, Lathyrus* and *Lens* spp. (Jordan, 1984). Isolates from *Trifolium, Desmodium* and *Acacia* showed affinity with *Rhizobium leguminosarum* bv. trifolii which was initially described for rhizobial species nodulating *Trifolium* species and a unique symbiotic relationship exists (Allen and Allen, 1981; Jordan, 1984). This corroborated results of Dagutat (1995) that *R. leguminosarum* bv. trifolii species is not restricted to strains isolated from *Trifolium* spp.

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In this study, *R. hainanensis* showed similarity to *R. galegae* strains. However, on the basis of 16S rDNA sequence analysis *R. hainanensis* is closely related to *R. tropici* (Gao *et al.*, 1994). This group has not been validated as a *bona fide* species and therefore the exact taxonomic position is not clear. None of the indigenous strains showed any close similarity to this species.

None of the indigenous isolates were closely related to *R. galegae* and this species constituted a separate taxonomic group within the genus *Rhizobium*. This supports results of Dagutat (1995) and Lindström (1989). However, controversy exists over the exact taxonomic position of *R. galegae* in the alpha subgroup of the Proteobacteria. Based on full-length 16S rDNA sequence analysis, this species is on the same phylogenetic branch as *Agrobacterium* (Terefework *et al.*, 1998; Young, 1996). Partial 16S rDNA sequencing and PCR-RFLP analysis of the 16S intergenic spacer (IGS) region showed *R. galegae* to be closely related to *M. loti* (Nour *et al.*, 1994; Young *et al.*, 1991). Results of SDS-PAGE analysis in this study appear to support the latter study.

In this study, *A. caulinodans* showed closer similarity to *S. saheli*, which is also able to nodulate stems of *Sesbania rostrata* (Boivin *et al.*, 1997). *Azorhizobium caulinodans* was described for strains nodulating the roots and stems of *Sesbania rostrata* (Dreyfus *et al.*, 1988). None of the indigenous isolates showed similarity to *A. caulinodans*. The only indigenous isolate from *Sesbania*, 40a (*Sesbania bispinosa*) grouped with the slow-growing *Bradyrhizobium* strains in cluster 5a. On the basis of DNA-rRNA hybridization analysis, *A. caulinodans* represents a separate lineage within the rhizobia that is more closely related to *Bradyrhizobium* than to *Rhizobium* (Dreyfus *et al.*, 1988). In the study done by Dagutat (1995), *A. caulinodans* LMG 6465 showed similarity to the slow growing *Bradyrhizobium* group.

The relationship between *S. fredii* and *S. meliloti* was not shown by these results, probably as a result of the misrepresentation of strains of *S. fredii*. The only strain included in this study formed a sub-cluster (5c) and showed no association with any of the strains investigated. All the other *Sinorhizobium* strains grouped in one cluster (cluster 12) together with *Rhizobium* sp. LMG 6463 isolated from *Sesbania rostrata* in Senegal. *Sinorhizobium teranga* and *S. saheli* were described by De Lajudie *et al.* (1994) for rhizobial isolates which nodulate *Acacia* and *Sesbania* hosts. *Rhizobium* sp. LMG 6463 (= ORS 22) grouped in cluster T, the *S. teranga* group. Our results

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confirmed the identity of this isolate. Indigenous strains isolated from *Medicago, Crotalaria, Tephrosia* and *Melolobium* showed similarity to *S. meliloti* (cluster 12).

Our results demonstrated a high degree of relatedness between *M. loti* (LMG 4264) and *M. huakuii* (LMG 14107 and USDA 4778, cluster 14a). *M. loti - M. huakuii* represents a separate phylogenetic branch that is intermediate between the *Rhizobium - Sinorhizobium - Agrobacterium* complex of the alpha subgroup of the Proteobacteria (Jarvis *et al.*, 1997; Lindström, 1996; Young, 1996). The other *M. loti* strains were dispersed in the dendrogram (Fig 3.) with strain LMG 6123 in cluster 7 and LMG 6268t2 in cluster 10. In the study by De Lajudie *et al.* (1998), *M. loti* reference strains were recovered in different positions in the dendrogram of whole cell protein analysis and some of these strains (including LMG 6123 used in our analysis) formed a rRNA cluster apart from the majority of *M. loti* strains. Our results corroborated results of De Lajudie and co-workers that this species needs further examination to define different groups and to secure its taxonomic status. One indigenous strain from *Strongylodon* exhibited similarity to the *M. loti - M. huakuii* lineage, as well as isolates from *Acacia* and *Neonotonia*.

The majority of indigenous isolates investigated showed a high degree of relatedness to *Bradyrhizobium* reference strains, including isolates from the tree legumes *Chamaecrista mimosoides, C. biensis, C. abrus, Acacia sieberana* var *woodii, A. caffra* and *Bolusanthus speciosus*. However, isolates from *Acacia dealbata, A. xanthoploea* and *A. robusta* grouped with the fast-growing rhizobial species. This diversity within the rhizobia from tree legumes was also demonstrated by Dagutat (1995). Although all tree nodulating rhizobia were initially classified as *Bradyrhizobium*, subsequent studies showed that both fast- and slow-growing rhizobia nodulate tree legumes (Dagutat, 1995; Dreyfus and Dommergues, 1981; Trinick, 1980; Zhang *et al.*, 1991). Isolates from *Acacia* and *Chamaecrista* species tended to group together in the same clusters. This was also observed in the study by Dagutat (1995).

In conclusion, our results supported the current division of root- and stem-nodule bacteria into five genera and the close relationship of the genus *Agrobacterium* to the rhizobial genera. Most of the indigenous strains investigated belonged to the slow-growing *Bradyrhizobium* genus, whilst some showed affinity to *Rhizobium, Sinorhizobium* and *Mesorhizobium*. None of the indigenous

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isolates showed similarity to *Azorhizobium*. SDS-PAGE proved a rapid tool for characterization of a large group of closely related strains. Discrimination is possible at species level, but it is not useful for discrimination at genus and higher levels. This is evident from the occurrence of clusters containing potential *Bradyrhizobium* strains among clusters containing *Rhizobium* species (Dupuy *et al.*, 1994). A database was established from which representative isolates were selected for further analysis to elucidate their taxonomic position.

CHAPTER 4

CHAPTER 4

CHARACTERIZATION OF PUTATIVE INDIGENOUS RHIZOBIAL ISOLATES WITH PCR-RFLP ANALYSIS OF THE 16S rDNA

4.1. INTRODUCTION

Rhizobia are heterotrophic soil bacteria capable of entering a symbiotic association with leguminous plants in temperate and tropical regions. Nodules are formed on the roots and/or stems in which the conversion of atmospheric dinitrogen to ammonia takes place. In 1984, only two genera were recognized (Jordan, 1984). Currently rhizobia comprise five genera and approximately 24 species have been described. The taxonomy of these symbiotic bacteria develops continually. Changes that have taken place are mainly due to the isolation of strains from legumes not previously investigated and to new approaches in bacterial taxonomy, especially with regard to molecular methods and the polyphasic approach (Young, 1996). Both phylogenetic and phenotypic investigation of a large number of strains is needed prior to any proposed changes in the taxonomy of this group of bacteria. Phenotypic traits should include cultural, morphological, physiological and symbiotic characteristics, whereas DNA-DNA hybridization is regarded as the standard method for species designation. Phylogenetic approaches such as 16S rDNA sequence analysis and DNA-rRNA hybridization are valuable tools for classification at species and higher levels (Graham *et al.*, 1991).

Insufficient information on the taxonomy of South African rhizobia led to the isolation of rhizobia from a diverse range of leguminous hosts and geographic regions (Dagutat, 1995). This was the first in depth study of indigenous root-nodulating bacteria. Characterization with SDS-PAGE analysis of the whole cell proteins, showed that although some of the isolates investigated were related to previously described taxa, some isolates were clearly distinct from previously described rhizobial species. It was suggested that further study of these isolates should include multilocus enzyme electrophoresis, phenotypic characterization, genotypic characterization such as

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restriction fragment length polymorphism analysis (RFLP) of PCR amplified 16S rDNA and RAPD analysis, as well as symbiotic performance (Dagutat, 1995).

16S rRNA molecules are useful for inferring phylogenetic relationships because of the universal distribution, structural and functional conservation and sufficient size thereof. Alternating sequences of highly variable regions and invariant, conserved regions on the primary structure allows investigation of phylogenetic distances from domain to species level (Ludwig and Schleifer, 1994). *Rhizobium* species contain at least three copies of the rRNA operon and *Bradyrhizobium* strains generally have a single copy (Geniaux *et al.*, 1993; Huber and Selenska-Pobell, 1994; Kündig *et al.*, 1995).

In a study done by Laguerre *et al.* (1994), the use of RFLP analysis of PCR amplified 16S rDNA was investigated for differentiation of *Rhizobium* strains into species. Nine endonucleases were investigated and a minimum of four enzymes was found to be necessary to achieve this. Strains of *Veilonella* (Sato *et al.*, 1997), *Salmonella* (Shah and Romick, 1997) and *Leptospira* (Ralph *et al.*, 1993) have been successfully characterized with this method.

The aim of this study was to determine the genetic relationship between rhizobial strains isolated from indigenous legumes with PCR-RFLP of 16S rDNA, using the method as described by Laguerre *et al.* (1994), and to evaluate the use of this method for characterization of indigenous rhizobia. One hundred and thirty one strains were first characterized with SDS-PAGE of the whole cell proteins (previous chapter) after which 73 representative strains were further analyzed with RFLP analysis of PCR amplified 16S rDNA.

4.2. MATERIALS AND METHODS

4.2.1. Strains used

Representative strains of the SDS-PAGE clusters (Fig. 3, previous chapter) were selected for further analysis of the 16S rDNA (Table 8) .

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4.2.2. Maintenance of cultures

Cultures were maintained on YM agar slants and long term storage of the strains was carried out in glycerol. Yeast mannitol broth (YMB) cultures were incubated at 25 - 28°C for 5 - 7 days with rigorous shaking and mixed 1:1 with a 50% (v/v) glycerol (Merck) in sterile cryotubes and stored at -20°C and -70°C.

4.2.3. Extraction of genomic DNA

Genomic DNA was extracted using a modified method as described by Somasegaran and Hoben (1994). Selected strains were used to inoculate 5 ml yeast-mannitol broth (YMB) in screw-cap tubes and incubated for 5 - 7 days at 28 - 30°C on a rotary shaker. One ml of the resulting turbid cultures was used to inoculate 5 ml tryptone yeast (TY) broth [0.5% (m/v) tryptone (Difco), 0.3% (m/v) yeast extract (Biolab), 0.13% (m/v) $CaCl_2.6H_2O$ (UniLab)] and incubated with vigorous shaking at 28 - 30°C overnight. Cells were pelleted by centrifugation at 8 000 rpm for 8 minutes. Pellets with a volume of 25 - 30 µl were washed twice with 750 µl phosphate buffer (pH 6.88, Merck) by resuspension and centrifugation. Washed cells were resuspended in 750 µl Tris-HCl (pH 7.2) and centrifuged at 8 000 rpm for 6 minutes. The supernatant was discarded and cells were resuspended in the residual Tris buffer by vortexing. 750 µl ice-cold acetone was added and immediately vortexed to prevent the cells from clumping and placed on ice for 5 minutes. After centrifugation at 8 000 rpm for 5 minutes, the acetone was discarded and remaining liquid aspirated using a vacuum-pump. The pellet was left at room temperature for 5 minutes to air dry. Cells were resuspended in 40 µl Tris-EDTA buffer (pH 8.0) and 50 µl of freshly prepared 2 mg/ml lysozyme added and incubated at 25°C for 10 minutes. 60 µl of a 2 mg/ml sarkosyl-pronase in 0.1 M EDTA (pH 7.0) was added and tubes incubated at 37°C overnight or until the suspension was clear. After incubation, 60 µl of 7.5 M ammonium acetate was added and several gentle extractions with equal volumes of phenol:chloroform (Sambrook *et al.*, 1989) using a large-bore pipette. The aqueous DNA phase was adjusted to 0.3 M NaOAc with a 3 M NaOAc stock solution and two volumes ice cold (-20°C) absolute ethanol were added and incubated for 2 hours at -70°C to allow precipitation of the DNA. Precipitated DNA was pelleted by centrifugation for 30 minutes at 8 000 rpm and washed twice with 70% ethanol. Excess ethanol

was removed through vacuum-drying. Pellets were dissolved in 50 µl sterile double distilled water. Contaminating RNA was digested with RNaseI (Epicentre Technologies) for 90 minutes at 37°C.

4.2.4. PCR amplification of 16S rDNA

Primers fD1 and rD1 (Table 9) were used for the amplification of rhizobial 16S rDNA. The oligonucleotides were synthesized by Boehringer Mannheim. Amplification was carried out in 50µl reaction volumes containing approximately 50 ng of pure genomic DNA, 100 pmol of both fD1 and rD1, 10x PCR buffer (20 mM Tris-HCl, pH 8.0; 100 mM KCl; 0.1 mM EDTA; 1 mM DTT; 0.5% Tween20; 0.5% NonidetP-40; 50% glycerol), 100 µM each dATP, dCTP, dTTP and dGTP and 0.5 U *TaKaRa Taq* polymerase (TaKaRa Biomedicals, Japan). Amplification was carried out in a Hybaid Omnigene Thermocycler with the following temperature profile: an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles consisting of denaturation at 94°C for 1 minute; annealing at 55°C for 1 minute and extension at 72°C for 2 minutes, and a final extension step at 72°C for 3 minutes. Aliquots (5 µl) of the PCR-products were examined by horizontal electrophoresis using 0.9% (m/v) agarose gels (Promega), in 1x TAE buffer (40 mM Tris-HCl; 20 mM NaOAc; 1 mM EDTA pH 8.5) stained with ethidium bromide (10 mg/ml). The molecular weight standard used was Boehringer Mannheim Molecular Weight Marker VI.

Table 9. Primers used for the PCR amplification of rhizobial 16S rDNA (Weisburg *et al.*, 1991).

Primer*	Sequence (5' to 3')
fD1	ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG
rD1	cccgggatccaagcttAAGGAGGTGATCCAGCC

4.2.5. Restriction fragment analysis

The method as described by Laguerre *et al.* (1994), with minor changes was used for RFLP analysis of PCR amplified 16S rDNA sequences. 15 µl aliquots of PCR products were digested

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with endonucleases *Hin*fI, *Cfo*I, *Msp*I and *Rsa*I (Boehringer Mannheim) with excess of the enzymes (5 U per reaction) at 37°C for 90 minutes. Analysis of restricted DNA by horizontal electrophoresis in 2% (m/v) agarose (Promega) in 1X TAE buffer, was carried out at 80V for 135 minutes with 20.5 x 10 cm gels (Hybaid Maxi Gel System) and stained with ethidium bromide (10 mg/ml). Boehringer Mannheim Molecular Weight Marker VI was included as molecular weight standard in two lanes per 22 lane gel. Migration distances of the restricted fragments were measured and fragment sizes determined with the SEQAID software program (Molecular Genetics Laboratory, Kansas State University). The RAPDistance Package (Australian National University, Canberra, Australia) was used to calculate a distance matrix using the Dice coefficient (Nei and Li, 1979). A dendrogram was constructed from this matrix using the unweighted pair group method with arithmetic mean (UPGMA) with the PHYLIP software package.

4.3. RESULTS

Strains representing each of the SDS-PAGE clusters (Fig. 3, previous chapter) were selected and total genomic DNA extracted. 16S rDNA of these strains were amplified using primers fD1 and rD1, which were derived from conserved regions of the 16S rRNA genes and allow nearly full-length amplification of the 16S rDNA (Weisburg *et al.*, 1991). A single band of approximately 1 500 bp was produced in all cases (Fig. 4). This corresponded to the expected size of the bacterial 16S rRNA genes.
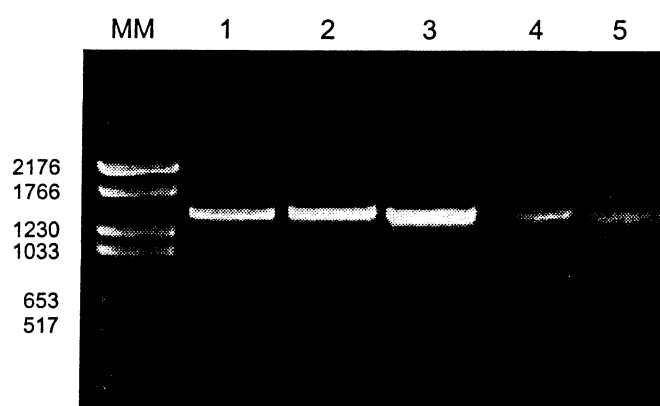
Fig. 4. 16S rDNA amplification products with primers fD1 and rD1, in 0.9 % agarose gel stained with ethidium bromide. Lanes: (MM) Boehringer Mannheim Molecular Weight Marker VI, (1) Bradyrhizobium elkanii LMG 6134, (2) UP31a, (3) 60, (4) 82a, (5) Mesorhizobium huakuii USDA 4778.

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Restriction fragments, produced after digestion of the amplified 16S rDNA with four endonucleases, of 100 bp or less were not taken into account as such small fragments were not well resolved by electrophoresis in 2% agarose gels. Therefore, summing of the restriction fragments sizes ranged from 1 300 to 1 600 bp.

Distinct restriction patterns for each of the endonucleases used could be recognized: 10 patterns could be distinguished after digestion with *Hinf*I, eight with *Cfo*I, seven with *Msp*I and six with *Rsa*I (Fig. 5). Patterns obtained for strains used after digestion with the four endonucleases are listed in Table 8.

To determine the genetic relationship between amplified 16S rRNA genes, a distance matrix was constructed using a total of 63 restriction fragments per strain. This represented the total number of fragments present in the restriction patterns obtained. For each strain, the presence or absence of a specific fragment was scored as 1 or 0. The Dice coefficient (Nei and Li, 1979) was used to construct a distance matrix from these data and the UPGMA algorithm used to construct a dendrogram (Fig. 6). The dendrogram could be divided into three major sections which were further divided into 12 groups. Section I contained the *Bradyrhizobium* reference strains, one *Mesorhizobium* strain and 31 of the indigenous isolates investigated. Section II comprised a group of isolates not closely related which included one of the *Agrobacterium* reference strains. Section III some of the indigenous isolates, one *Agrobacterium* strain and the *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* reference strains.

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Fig. 5. Restriction patterns produced after digestion of PCR amplified 16S rDNA with *Hin*fI (fig. 5a), *Cfo*I (fig. 5b), *Msp*I (fig. 5c) and *Rsa*I (fig. 5d).
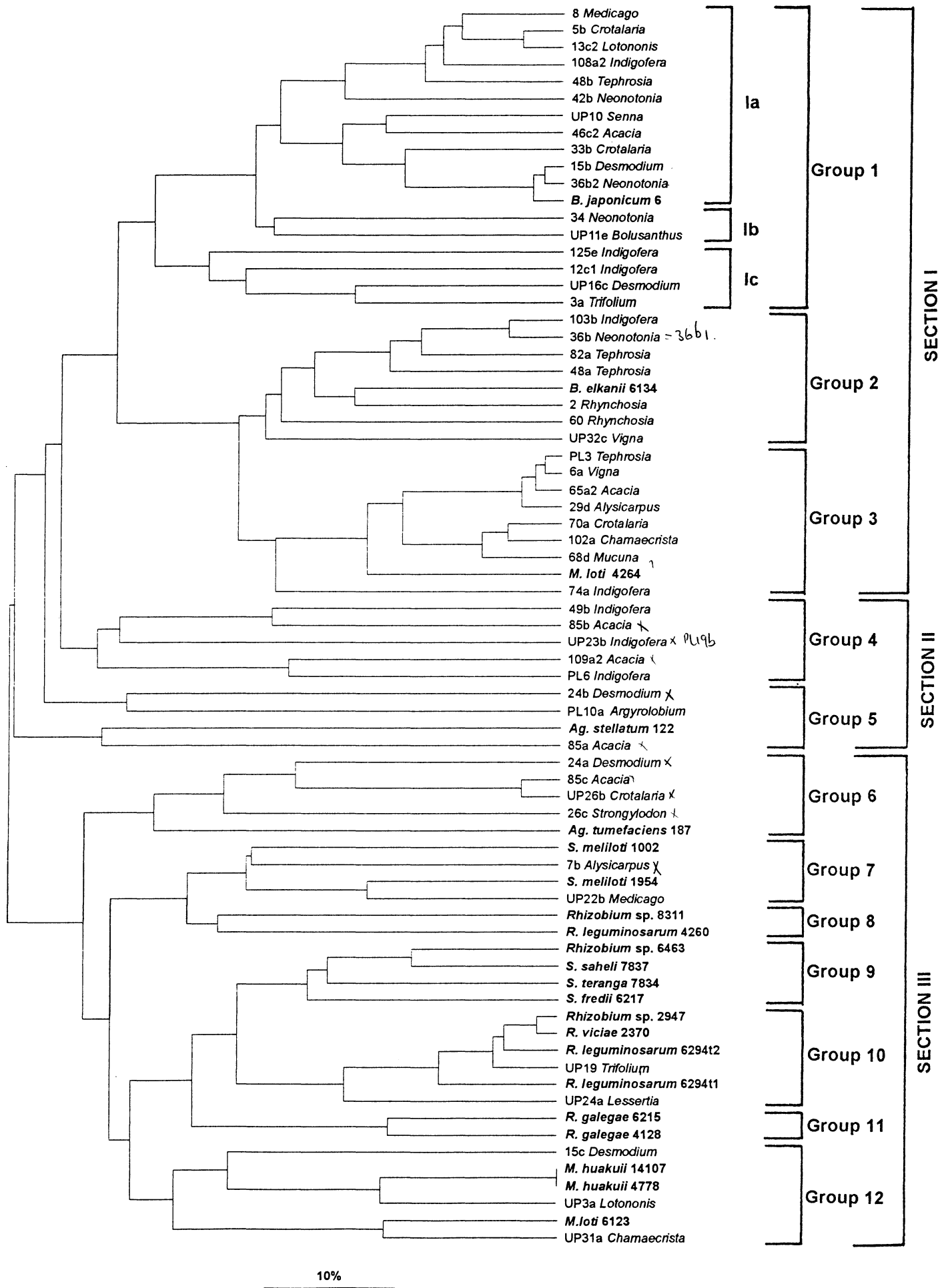
Fig. 6. Dendrogram based on analysis of restriction patterns after digestion of PCR-amplified 16S rDNA with *Hinf*I, *Cfo*I, *Msp*I and *Rsa*I. Genetic relationships were determined with the Dice coefficient and strains grouped with UPGMA.

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The majority of the indigenous isolates investigated together with three reference strains were included in this section which could be further divided into three groups.

Group 1

Seventeen indigenous strains and *Bradyrhizobium japonicum* USDA 6 contained in this group, could be further divided into three subgroups.

In **group 1a** isolates 15b and 36b2 were very closely related to *B. japonicum* USDA 6 and exhibited identical restriction patterns. Closely related to this group were isolates 33b and 46c2 that belonged to SDS-PAGE cluster 1c and showed a 83.7% similarity with *B. japonicum* USDA 6; and UP10 which showed no close similarity to any of the strains with SDS-PAGE analysis (cluster 4). Within group 1a, another closely related cluster was distinguished. Isolates 5b, 8, 48b and 13c2 showed a very close association and exhibited similar restriction patterns. With SDS-PAGE analysis, isolates 5b, 8 and 48b exhibited the same close association to *B. japonicum* USDA 6 (cluster 1a) and 13c2 clustered with *Bradyrhizobium* sp. LMG 8319 (cluster 10). Isolates 108a2 and 42b were closely related to these strains and 42b differed only with regard to the *Hin*fI restriction analysis. With SDS-PAGE analysis, isolate 42b showed similarity to the slow-growing strains in cluster 2a (Fig. 3). Isolate 108a2 which clustered with *Agrobacterium* in Fig. 3, showed a high degree of relatedness to slow-growing strains with 16S rDNA analysis. **Group 1b** consisted of two indigenous strains which were only loosely associated and were in the same SDS-PAGE cluster (cluster 1). **Group 1c** consisted of four isolates with a low degree of relatedness that were scattered through the SDS-PAGE cluster. All the isolates in group 1 had identical patterns for *Cfo*I and with a few exceptions, similar patterns for *Hin*fI and *Msp*I (Table 8).

Group 2

Bradyrhizobium elkanii LMG 6134 and six indigenous isolates in which 103b and 36d showed a close relationship with identical restriction patterns constituted group 2. All the isolates in this group were grouped in *Bradyrhizobium* clusters (clusters 1, 2 and 5) with SDS-PAGE analysis.

These isolates shared identical patterns for *Rsa*I and *Hinf*I, and near identical patterns for *Msp*I (Table 8).

Group 3

Two very closely related clusters consisting of PL3, 6a, 65a2, 29d, and 70a, 102a, 68d, respectively could be distinguished within this group. Loosely related to these two groups were *M. loti* LMG 4264 and isolate 74a. These isolates (except LMG 4264 and 70a) all grouped in SDS-PAGE cluster 2. *M. loti* LMG 4264 clustered with *M. huakuii* strains in the SDS-PAGE cluster (cluster 14a) and 70a was contained in cluster 3 (Fig. 3). These strains exhibited identical restriction patterns for *Hinf*I, *Cfo*I and *Msp*I.

Section II

Section II consisted of two groups of isolates that were not closely related. These isolates produced diverse combinations of patterns with no conformity.

Group 4

Five indigenous strains, 49b, 85b, UP23b, 109a2 and PL6, with no close association to each other or any of the reference strains comprised group 4. In Fig. 3 isolates 49b and 48b had near identical protein profiles (cluster 1a). With PCR-RFLP analysis, these two isolates exhibited different patterns for *Hinf*I and *Msp*I and identical patterns for the other two restriction endonucleases used (Table 8).

Group 5

Isolates 24b and PL10a and *Agrobacterium stellatum* LMG 122 and 85a were only distantly related and were distributed through the SDS-PAGE dendrogram (clusters 9, 15, 11 and 10 respectively; Fig. 3). These isolates showed no similarity in their restriction patterns.

Section III

This section represented the *Rhizobium - Sinorhizobium - Mesorhizobium* branch with an *Agrobacterium* strain closely associated to it. Seven groups were distinguished within this section.

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Group 6

Four indigenous isolates (85c, UP26b, 26c, 24a) and *Agrobacterium tumefaciens* LMG 187 constituted this relatively distantly related group, with only isolates 85c and UP26b very closely related with identical restriction patterns. These two isolates both clustered in SDS-PAGE cluster 7a with a similarity of 79%. Isolate 26c showed 58.8% similarity with the *M. loti-M. huakuii* SDS-PAGE cluster. Isolate 24a was loosely associated with slow-growing strains in SDS-PAGE cluster 9.

Group 7

This group represented a *Sinorhizobium* branch with reference strains *S. meliloti* USDA 1002 and USDA 1954 and two indigenous isolates. UP22b isolated from *Medicago* showed a close relationship with the latter and was also present in the *Sinorhizobium* SDS-PAGE cluster (cluster 12) with a similarity of 75% to *S. meliloti* LMG 6131 and USDA 1002. Also included in this group was isolate 7b which showed a closer similarity to *Bradyrhizobium* strains in Fig. 3 (cluster 9).

Group 8

Reference strains *R. leguminosarum* LMG 4260 and *Rhizobium* sp. (*Acacia*) LMG 8311 were only distantly related and showed no close relationship to any of the indigenous strains investigated. These two strains exhibited unique combinations of restriction patterns (Table 8). These two strains were contained in clusters 8 and 2b of the SDS-PAGE dendrogram respectively. These clusters represented *Rhizobium* branches.

Group 9

This group represented the second *Sinorhizobium* branch with *S. saheli* LMG 7837, *S. teranga* LMG 7834 and *S. fredii* LMG 6217 and none of the indigenous isolates. Included in this group was *Rhizobium* sp. (*Sesbania*) LMG 6463 which was also clustered in the *Sinorhizobium* SDS-PAGE cluster (Cluster 12). These isolates had, with a few exceptions, identical restriction patterns (Table 8).

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Table 8. Restriction patterns of selected *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Agrobacterium* spp. and indigenous rhizobial strains used in RFLP analysis of PCR-amplified 16S rDNA.

Strain	SDS-PAGE cluster	RFLP group	Restriction pattern* of amplified 16S rDNA with:			
			*Hin*fI	*Cfo*I	*Msp*I	*Rsa*I
8	1a	1a	H6	C1	M2	R1
5b	1a	1a	H6	C1	M2	R1
13c2	9	1a	H6	C1	M2	R1
108a2	11	1a	H6	C1	M2	R1
48b	1a	1a	H6	C1	M2	R1
42b	2a	1a	H9	C1	M2	R1
15b	4a	1a	H6	C1	M2	R1
36b2	2c	1a	H6	C1	M2	R1
B. japonicum 6	1a	1a	H6	C1	M2	R1
33b	1c	1a	H6	C1	M2	R1
UP10	4	1a	H6	C1	M2	R2
46c2	1c	1a	H6	C1	M2	R2
34	1a	1b	H6	C1	M1	R1
UP11e	1c	1b	H6	C1	M7	R6
125e	13	1c	H4	C1	M2	R3
12c1	5a	1c	H6	C1	M2	R2
3a	4a	1c	H5	C1	M2	R2
UP16c	2a	1c	H5	C1	M2	R2
48a	2a	2	H9	C1	M2	R2
103b	1c	2	H9	C6	M2	R2
36d	5	2	H9	C6	M2	R2
82a	1b	2	H9	C7	M2	R2
B. elkanii 6134	2b	2	H9	C6	M3	R2
2	2b	2	H9	C7	M6	R2
60	5b	2	H9	C7	M5	R2
UP32c	2a	2	H3	C7	M2	R2
M. loti 4264	14a	3	H9	C6	M2	R5
70a	3	3	H9	C6	M2	R2
102a	2b	3	H9	C6	M2	R2
68d	2d	3	H9	C6	M2	R2

Table 8 (continued). Restriction patterns of *Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Agrobacterium* spp. and indigenous rhizobial strains with RFLP analysis of PCR-amplified 16S rDNA.

Strain	SDS-PAGE cluster	RFLP group	Restriction pattern* of amplified 16S rDNA with:			
			*Hinf*I	*Cfo*I	*Msp*I	*Rsa*I
PL3	2a	3	H9	C6	M2	R1
6a	2a	3	H9	C6	M2	R1
65a2	2d	3	H9	C6	M2	R1
29d	2b	3	H9	C6	M2	R1
74a	2b	3	H9	C6	M2	R1
49b	1a	4	H4	C1	M6	R1
85b	15	4	H4	C2	M5	R1
UP23b	13	4	H4	C1	M3	R2
109a2	13	4	H4	C1	M7	R1
PL6	1b	4	H6	C1	M7	R1
24b	13	5	H1	C4	M4	R2
PL10b	15	5	H8	C1	M4	R4
Ag. stellatum 122	11	5	H4	C5	M2	R3
85a	10	5	H3	C4	M5	R4
24a	9	6	H1	C8	M4	R3
85c	7a	6	H1	C8	M4	R3
UP26b	7a	6	H1	C8	M4	R3
26c	14a	6	H1	C8	M2	R4
Ag. tumefaciens 187	11	6	H8	C2	M4	R6
S. meliloti 1002	12	7	H6	C4	M5	R4
7b	9	7	H1	C5	M5	R5
S. meliloti 1954	12	7	H6	C5	M5	R4
UP22b	12	7	H6	C5	M5	R5
Rhizobium sp. 8311	2d	8	H6	C2	M5	R4
R. leguminosarum 4260	8	8	H1	C7	M5	R4

Table 8 (continued). Restriction patterns of *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Agrobacterium* spp. and indigenous rhizobial strains with RFLP analysis of PCR-amplified 16S rDNA.

Strain	SDS-PAGE cluster	RFLP group	Restriction pattern* of amplified 16S rDNA with:			
			*Hinf*I	*Cfo*I	*Msp*I	*Rsa*I
Rhizobium sp. 6463	12	9	H6	C7	M6	R4
S. saheli 7837	12	9	H6	C5	M6	R4
S. teranga 7834	12	9	H6	C5	M6	R4
S. fredii 6217	5	9	H6	C5	M6	R5
Rhizobium sp. 2947	6b	10	H1	C5	M3	R4
R. viciae 2370	7b	10	H1	C5	M3	R4
R. leguminosarum 6294t2	2d	10	H2	C5	M3	R4
UP19	15	10	H1	C5	M3	R4
R. leguminosarum 6294t1	6b	10	H1	C5	M3	R4
UP24a	12	10	H1	C5	M3	R5
R. galegae 6215	14b	11	H1	C7	M2	R4
R. galegae 4128	14b	11	H2	C5	M2	R4
15c	15	12	H8	C3	M7	R5
M. huakuii 14107	14a	12	H10	C5	M7	R5
M. huakuii 4778	14a	12	H10	C5	M7	R5
UP3a	6b	12	H7	C5	M7	R5
M. loti 6123	7	12	H6	C7	M6	R5
UP31a	7b	12	H6	C7	M7	R5

*	Patterns detected after digestion of PCR amplified 16S rDNA with different endonucleases (Fig. 3)
S.	*Sinorhizobium*
M.	*Mesorhizobium*
R.	*Rhizobium*
B.	*Bradyrhizobium*
Ag.	*Agrobacterium*

Group 10

This *Rhizobium* group consisted of four reference strains and two indigenous isolates. *R. leguminosarum* bv. *viciae* USDA 2370, *R. leguminosarum* LMG 6294t2 and LMG 6294t1, *Rhizobium* sp. USDA 2947 and isolates UP19 (*Trifolium*) and UP24a (*Lessertia*) showed close relationship. These isolates were distributed throughout the SDS-PAGE dendrogram (Fig. 3). UP19 showed in the SDS-PAGE analysis 84.2% similarity to another *Rhizobium* species, *R. trifolii* USDA 2046. All these isolates produced the same restriction patterns with *Cfo*I, *Msp*I and near identical patterns with *Hinf*I and *Rsa*I (Table 8).

Group 11

Group 11 represented the *R. galegae* branch with only the two reference strains LMG 6215 and USDA 4128. These two strains produced identical restriction patterns for *Msp*I and *Rsa*I, and differed with one restriction fragment for *Hinf*I and *Cfo*I. This is similar to the SDS-PAGE clustering (cluster 14b) of these strains. None of the indigenous isolates showed any similarity to these strains.

Group 12

Three *Mesorhizobium* reference strains and three indigenous isolates constituted this group. The two *M. huakuii* strains LMG 14107 and USDA 4778 exhibited exactly the same restriction patterns. Closely related to these strains were UP3a (*Lotononis*) and 15c (*Desmodium*) which clustered with SDS-PAGE analysis together with *Rhizobium* reference strains in clusters 6 and 15 respectively. *M. loti* LMG 6123 and UP31a were also included in this group which were both grouped in cluster 7 of the SDS-PAGE dendrogram.

4.4. DISCUSSION

Amplification of rhizobial 16S rDNA was achieved with primers fD1 and rD1. Weisburg *et al.* (1991), described several oligonucleotide primers which can be used for the enzymatic amplification of a wide range of bacteria. Ribosomal primers fD1 and rD1 (Table 9) were suggested for use when members of the Proteobacteria are investigated. The use of rD1 as reverse

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primer ensures amplification of the maximum number of nucleotides of 16S rDNA as it is closer to the 3' end of the 16S rRNA gene. These primers have been used successfully for the amplification of rhizobial 16S rDNA by several authors (Laguerre *et al.*, 1994; Terefework *et al.*, 1998; Van Berkum *et al.*, 1996; Van Berkum *et al.*, 1998).

The combination of *Cfo*I (GCG/C), *Hinf*1 (G/ANTC), *Msp*I (C/CGG) and *Rsa*I (GT/AC) was used for digestion of PCR amplified 16S rDNA. This was sufficient for the discrimination of rhizobial strains at species level. As indicated by Laguerre *et al.* (1994), a minimum of four restriction endonucleases was necessary when implementing PCR-RFLP analysis of the 16S rDNA, and the combinations *Cfo*I, *Hinf*1, *Msp*I and *Nde*II or *Cfo*I, *Hinf*1, *Rsa*I and *Nde*II or *Msp*I were proposed. In our study, *Nde*II was found to be difficult to work with as it produced complex restriction patterns, with multiple restriction fragments (sometimes in excess of 10 fragments) which hampered interpretation of the results, especially when numerous small fragments were produced. Restriction analysis with *Rsa*I produced less complex patterns which could be interpreted more accurately.

The results of PCR-RFLP analysis of the 16S rDNA largely supported the results of SDS-PAGE of whole cell proteins. The majority of indigenous isolates which were included in the SDS-PAGE *Bradyrhizobium* clusters (clusters 1, 2, 5, and 9), grouped in PCR-RFLP groups 1, 2 and 3. The *Bradyrhizobium* strains investigated were clearly separate from strains of the genera *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*.

In the PCR-RFLP dendrogram (Fig. 6), representative strains of the genus *Rhizobium* showed a closer relationship than exhibited with SDS-PAGE analysis, with three *Rhizobium* clusters (groups 8, 10 and 11). The close phylogenetic relationship between *Agrobacterium* and *Rhizobium* could not be deduced as these strains exhibited completely different restriction patterns and did not cluster closely together with RFLP analysis of the 16S rDNA. *Rhizobium* and *Agrobacterium* species are phylogenetically closely related (Willems and Collins, 1993; Young *et al.*, 1991).

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Variation of the 16S rDNA sequence within the rhizobial genera were adequate to distinguish individual species. As with SDS-PAGE analysis, *R. galegae* (group 11) clustered separately with no close association to any of the other strains. With PCR-RFLP analysis, these strains did not show close similarity to the *Mesorhizobium* strains as was the case with SDS-PAGE analysis. Thus, 16S rDNA analysis confirmed the status of *R. galegae* as a distinct lineage within the root- and stem-nodulating bacteria (Dagutat, 1995; Lindström, 1989; Terefework *et al.*, 1998). It is important to note that part of the 16S rRNA gene and the 16S IGS region of *R. galegae* have features in common with corresponding regions of the mesorhizobial ribosomal operons (Eardly *et al.*, 1996; Nour *et al.*, 1994; Young *et al.*, 1991)

With PCR-RFLP analysis the lineages of *M. loti* - *M. huakuii* (group 12) as a separate phylogenetic branch within the rhizobial genera could be distinguished. This corroborated results of Laguerre *et al.* (1994) and Terefework *et al.* (1998), who found no relationship between *Mesorhizobium* strains and any other rhizobial or *Agrobacterium* strains with 16S rDNA PCR-RFLP analysis. The heterogeneity of *M. loti* strains as discussed by De Lajudie *et al.* (1998) and in the previous chapter, was demonstrated with 16S rDNA analysis as well. In this case, *M. loti* LMG 6123 showed a higher degree of relatedness with *M. huakuii* strains and *M. loti* LMG 4264 grouped outside this phylogenetic branch.

The *S. meliloti* - *S. fredii* lineage within the rhizobial genera was not clear with our results. Two *Sinorhizobium* groups (groups 7 and 9) were distinguished. Terefework *et al.* (1998) found that with 23S rDNA PCR-RFLP analysis, the genera *Agrobacterium*, *Rhizobium* and *Sinorhizobium* were less distinct from each other than with 16S rDNA analysis.

At species level, identical restriction patterns were generated for *S. meliloti* (USDA 1002 and USDA 1954) and *M. huakuii* (LMG 14107 and USDA 4778). Such species-specific patterns were also demonstrated by Vandamme *et al.* (1996).

RFLP analysis of PCR amplified 16S rDNA could not distinguish at the same taxonomic level as SDS-PAGE. The closely related group of isolates in SDS-PAGE cluster 2a (similarity of 93.5%), were distributed through the three *Bradyrhizobium* PCR-RFLP groups. This was also evident

with characterization of *Sinorhizobium* strains. Laguerre *et al.* (1994) also noted that this method is limited with regard to differentiation of closely related species. According to Terefework *et al.* (1998), the 16S rDNA gene is useful for the assessment of phylogenies down to genus level. It must be borne in mind that the *rrn* operons represent only a small part of the bacterial genome which limit discrimination of closely related strains by this technique (Selenska-Pobell *et al.*, 1996). The limited discrimination power of 16S rDNA can also be attributed to the conserved nature of this gene.

Thus, PCR-RFLP analysis of 16S rDNA corroborated results of SDS-PAGE of whole cell proteins, and was useful for differentiation of indigenous rhizobial isolates at species level. Close relationships between strains could not be deduced with this method. DNA sequences that are less highly conserved can be analyzed to increase discrimination at intraspecies level. PCR-RFLP analysis of the variable region of the intergenic spacer regions between the 16S and 23S rDNA is a useful tool to achieve this (Jensen *et al.*, 1993; Navarro *et al.*, 1992). However, Terefework *et al.* (1998), noted that IGS existed in multiple, variable copies in many strains and subsequently is not suitable for PCR-RFLP analysis. PCR-fingerprinting methods such as repetitive extragenic palindromic (REP)- and enterobacterial repetitive intergenic consensus (ERIC)- PCR and randomly amplified polymorphic DNA (RAPD) analysis can also be used for differentiation at strain level (De Bruijn, 1992; Dooley *et al.*, 1993; Harrison *et al.*, 1992; Judd *et al.*, 1993; Selenska-Pobell *et al.*, 1996).

CHAPTER 5

CHAPTER 5

PHENOTYPIC CLASSIFICATION OF PUTATIVE INDIGENOUS
RHIZOBIA WITH THE BIOLOG SYSTEM

5.1. INTRODUCTION

Taxonomy of the root- and stem-nodulating bacteria is in a state of transition. Characterization of strains isolated from leguminous hosts not investigated previously, is one of the main factors contributing to the constant revision of rhizobial systematics (Young, 1996). A set of minimal standards for the description of new taxa has been set which includes phenotypic and genotypic criteria (Graham *et al.*, 1991). This polyphasic approach is necessary for the delineation of taxa at all levels (Vandamme *et al.*, 1996).

Classical phenotypic studies can be laborious and difficult to interpret when slowly growing bacteria are used (Dupuy *et al.*, 1994). Phenotypic test systems which allow numerous tests to be done in one experiment, has long been in demand (Bochner, 1989b; Grimont *et al.*, 1996). One such system, the Biolog MicroPlate System (Biolog Inc., Hayward, CA, USA) test the ability of micro-organisms to utilize 95 different substrates which include sugars, alcohols, organic acids, amino acids and polymeric chemicals as sole carbon source (Bochner, 1989b).

The utilization pattern of each organism represents a metabolic fingerprint which can be compared with fingerprints of other strains to determine phenotypic relationships (Bochner, 1989b). Theoretically, with 95 tests than can be either positive or negative, there are 2^{95} ($= 4 \times 10^{28}$) possible metabolic fingerprints (Bochner, 1989a). This is an efficient and rapid method for the characterization of large numbers of strains and the construction of a database is possible. The Biolog system has been used for the identification of plant-pathogenic bacteria (Jones *et al.*, 1993) and in a polyphasic study of *Vibrio vulnificus* (Arias *et al.*, 1997) and bradyrhizobia nodulating *Acacia albida* (Dupuy *et al.*, 1994).

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The purpose of this investigation was to characterize putative rhizobial isolates from a wide variety of leguminous hosts phenotypically with the Biolog system and to evaluate the use of this system for routine identification.

5.2. MATERIALS AND METHODS

5.2.1. Strains used

Sixty seven putative rhizobial strains isolated from a diverse range of indigenous host legumes (listed in Table 10) were selected from the SDS-PAGE dendrogram (Fig. 3, Chapter 3) for further phenotypic analysis. Reference strains of the genera *Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium,* and *Agrobacterium* (Table 11) were included.

5.2.2. Maintenance of cultures

Cultures were maintained on YM agar slants, which contained 1% mannitol (UniVar), 0.05% K_2HPO_4 (Merck), 0.02% $MgSO_4.7H_2O$ (Merck), 0.01% NaCl, 0,04% yeast extract (Biolab) and 1.5% bacteriological Agar (Biolab). Long term storage of the strains was carried out in glycerol. Yeast mannitol broth (YMB) cultures were incubated at 25 - 28°C for 5 - 7 days and mixed 1:1 with a 50% (v/v) glycerol (Merck) in sterile cryotubes and stored at -20°C and -70°C.

5.2.3. The Biolog system

Oxidation of 95 different carbon sources (Appendix A, divided into 11 categories by Garland and Mills, 1991) was determined with commercially available microtiter plate tests. Because of the low level activity expected, the procedure was adapted as follows: strains were grown on YMA plates for 5 - 7 days at 26°C and transferred to R_2A as recommended by the manufacturers, and incubated at 26°C for 72 h. R_2A contained 0.05% proteose peptone (Difco), 0.05% yeast extract (Biolab), 0.05% Casamino acids (Difco), 0.05% glucose (BDH), 0.05% soluble starch (Biolab), 0.03% sodium pyruvate (Merck), 0.03% K_2HPO_4 (Merck), 0.005% $MgSO_4.H_2O$ (Merck) and

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1.5% bacteriological agar (Biolab). Cells were harvested using a sterile inoculation needle and resuspended in sterile physiological saline (0.85% (m/v) NaCl) to an optical density of 0.20 to 0.40 at 590 nm. The resulting suspension was transferred to sterile multichannel pipette reservoirs and GN Biolog MicroPlates were inoculated (150 µl per well). Paper towel was moistened and placed together with the microtiter plate in its original foil bag to prevent dehydration of the plates. Plates were incubated at 26°C for 4 days. Results were recorded manually as positive or negative for each well at 24 h intervals. Development of a purple colour (due to oxidization of the substrate) compared with the control well containing no substrate, was recorded as positive. Visual readings were recorded as 1 when positive and 0 when negative and entered into the Bionum computer program (Applied Maths, Kortrijk, Belgium), and similarity between strains was calculated using the Simple Matching coefficient (S_{SM}). The resulting similarity matrix was imported in the GelCompar 4.0 computer program (Applied Maths) and cluster analysis was done using UPGMA.

Microplates, agar plates and saline were stored at 4°C and equilibrated at room temperature before use.

Table 10. List of putative rhizobia isolated from indigenous rhizobia characterized with the Biolog system.

Isolate*	Host legume	Isolate*	Host legume
2	*Rhynchosia nervosa*	98d2	*Bolusanthus speciosus*
3a	*Trifolium* sp.	101d	*Zornia capensis*
5b	*Crotalaria distans*	102a	*Chamaecrista biensis*
6a	*Vigna vexillata*	103b	*Indigofera hilaris*
7b	*Alysicarpus rugosus*	104a1	*Chamaecrista* sp.
8	*Medicago lupulina*	108a2	*Indigofera swaziensis*
12c1	*Indigofera rhytidocarpa*	109a2	*Acacia dealbata*
12c2	*Indigofera rhytidocarpa*	114d	*Pseudarthria hookeri*
13c1	*Lotononis bainesii*	125e	*Indigofera woodii*
13c2	*Lotononis bainesii*	128a	*Melolobium obcordatum*
15c	*Desmodium tortuosum*	PL3	*Tephrosia grandiflora*
24a	*Desmodium tortuosum*	PL6	*Indigofera porrecta*
26c	*Strongylodon macrobotrys*	PL10a	*Argyrolobium tomentosum*
29d	*Alysicarpus rugosus*	PL10b	*Argyrolobium tomentosum*
33b	*Crotalaria* sp.	UP3	*Lotononis falcata*
34	*Neonotonia wightii*	UP10	*Senna petersiana*
36b1	*Neonotonia wightii*	UP11e	*Bolusanthus speciosus*
36b2	*Neonotonia wightii*	UP15b	*Indigofera schinzii*
36d	*Neonotonia wightii*	UP16b	*Desmodium repandum*
42b	*Neonotonia wightii*	UP17b	*Indigofera heterophylla*
46c2	*Acacia sieberana* var *woodii*	UP19	*Trifolium* sp.
48a	*Tephrosia purpurea*	UP22b	*Medicago sativa*
60	*Rhynchosia monophylla*	UP23b	*Indigofera verrucosa*
65a2	*Acacia* sp.	UP24a	*Lessertia annularis*
68d	*Mucuna coriacea*	UP24b	*Lessertia annularis*
70a	*Crotalaria brachycarpa*	UP26b	*Crotalaria damarensis*
70b2	*Crotalaria brachycarpa*	UP27b	*Crotalaria damarensis*
72f	*Tephrosia purpurea*	UP27c	*Crotalaria damarensis*
74a	*Indigofera arrecta*	UP30a	*Indigofera filipes*
82a	*Tephrosia purpurea*	UP30c	*Indigofera filipes*
85a	*Acacia xanthoploea*	UP31a	*Chamaecrista mimosoides*
85c	*Acacia xanthoploea*	UP32c	*Vigna unguiculata*

*   Source of strains: Without suffix: Dagutat (1995)

With suffix PL: Isolated by PL Steyn and received from the Agricultural Research Council

With suffix UP: Isolated in this study (chapter 3)

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Table 11.   **List of reference strains characterized with the Biolog system.**

	Strain no. [*]	Host plant	Geographic origin
Rhizobium leguminosarum	LMG 4260	*Vigna unguiculata*	NS
Rhizobium leguminosarum	LMG 6294t1	*Lathyrus* sp.	St. Petersburg
R. leguminosarum bv *viciae*	USDA 2370^T	*Pisum sativum*	NS
R. leguminosarum bv. *trifolii*	LMG 6119	*Trifolium repens*	New Zealand
Rhizobium galegae	USDA 4128^T	*Galega otientalis*	USSR
Rhizobium tropici	USDA 9030^T	*Phaseolus vulgaris*	NS
Rhizobium etli	USDA 9041	NS	NS
Rhizobium sp.	LMG 6463	*Sesbania rostrata*	Senegal
Rhizobium sp.	LMG 8311	*Acacia farnesiana*	Senegal
Rhizobium sp.	USDA 2947	NS	NS
Bradyrhizobium japonicum	LMG 4265	*Ulex europaeus*	NS
Bradyrhizobium japonicum	USDA 6^T	NS	NS
Bradyrhizobium elkanii	USDA 76^T	*Glycine max*	NS
Bradyrhizobium sp.	LMG 8319	*Macrotyloma africanus*	Zimbabwe
Sinorhizobium meliloti	LMG 6133^T	*Medicago sativa*	NS
Sinorhizobium meliloti	USDA 1002	*Medicago sativa*	
Sinorhizobium fredii	LMG 6217^T	*Glycine max*	Honan, China
Sinorhizobium fredii	LMG 8317		Shanghai, China
Sinorhizobium saheli	LMG 7837	*Sesbania cannabina*	Senegal
Mesorhizobium loti	LMG 4268t1	*Lotus americanus*	NS
Mesorhizobium loti	LMG 4268t2	*Lotus americanus*	NS
Mesorhizobium loti	LMG 6123	*Lotus divaricatus*	New Zealand
Mesorhizobium huakuii	USDA 4778^T	*Astragalus sinicus*	China
Agrobacterium radiobacter	LMG 140	NS	NS
Agrobacterium tumefaciens	LMG 187	*Lycopersicon lycopersicum*	NS
Agrobacterium rhizogenes	LMG 150	NS	NS
Agrobacterium stellatum	LMG 122	NS	Baltic Sea

[*]   LMG: Laboratorium voor Microbiologie Gent Culture Collection, State University Gent, Belgium.

USDA: United States Department of Agriculture-ARS National Rhizobium Culture Collection.

[T]   Type strain

NS   not stated
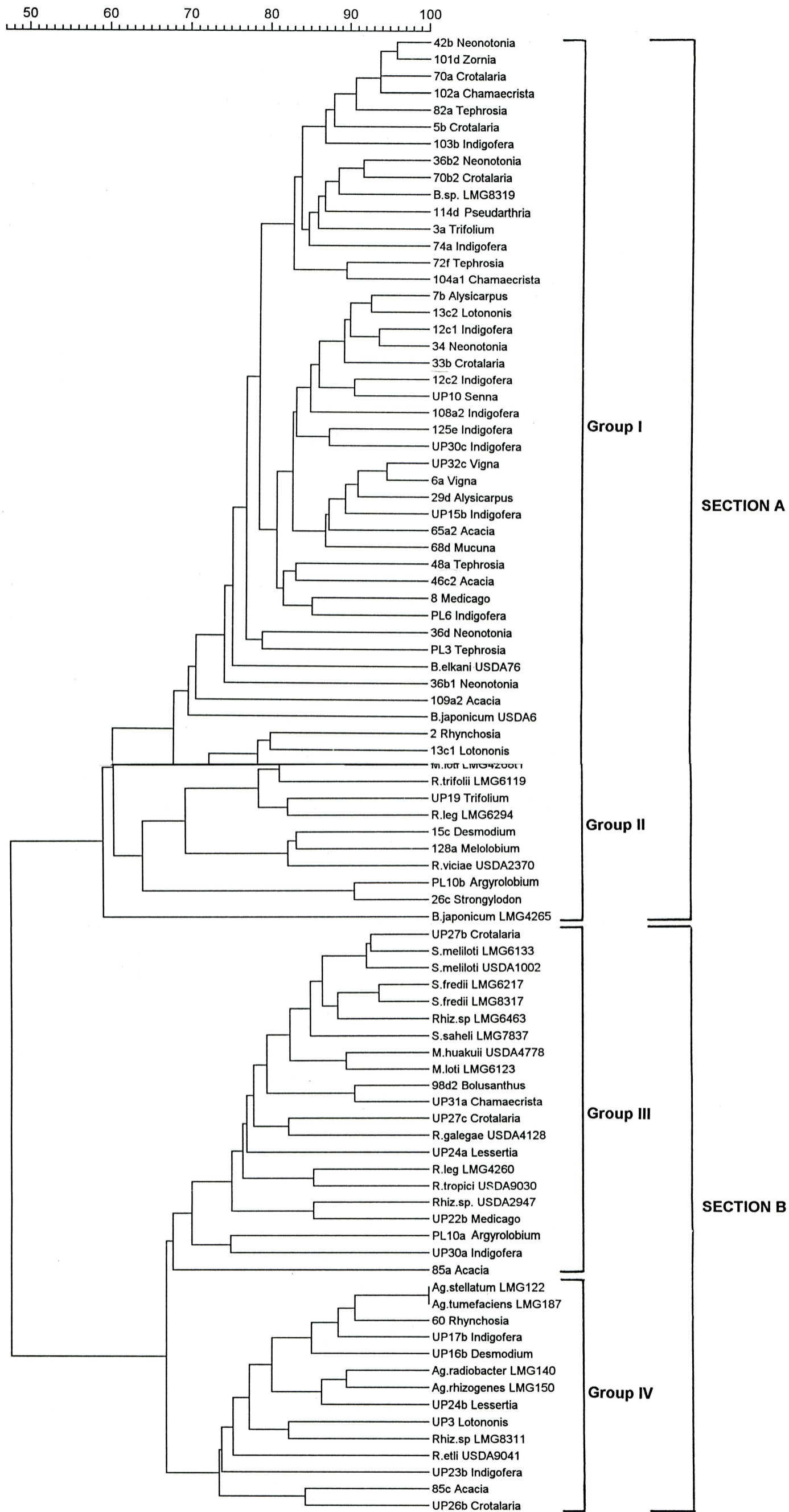
Fig. 9. Dendrogram based on UPGMA analysis of S_{SM} values from Biolog results after 96 h.

5.3. RESULTS

Growth of rhizobia on R₂A was associated with a decrease in extracellular polysaccharide production. Rhizobial isolates proved rather inert in the oxidation of the substrates. No significant changes were detected after 24 h and faster-growing isolates showed marked changes after 48 h.

5.3.1. Analysis of substrate utilization patterns after 72 h

Results of the numerical analysis of substrate utilization patterns after 72 h are represented in Figure 7 and the results of the 95 substrate oxidation tests which were performed are given in Table 12. The substrates utilized by almost all of the rhizobial strains investigated were carbohydrates L-arabinose and L-fucose, bromo succinic acid and succinamic acid, both esters (methyl-pyruvate and mono-methyl succinate), and five carboxylic acids (acetic acid, formic acid, β-hydroxybutyric acid, D,L-lactic acid, succinic acid).

Isolates in the dendrogram (Fig. 7) were seperated into two major sections with a similarity of 47.5 % and were further divided into six groups. Section I contained the four *Bradyrhizobium* reference strains, one *Rhizobium* reference strain and 70% of the indigenous rhizobial strains investigated. Reference strains of the genera *Rhizobium, Sinorhizobium, Mesorhizobium* and *Agrobacterium* and 30% of the indigenous rhizobial isolates were incorporated into Section II.

Section I

Section I contained most of the slow-growing indigenous isolates and was further divided into three groups.

Group 1 was a large cluster containing 23 indigenous isolates and two reference strains. Four subgroups were distinguished at a similarity of 81.3%.

> **Subgroup 1a** contained 12 slow-growing strains and two *Bradyrhizobium* reference strains with 84.4% similarity. Within this subgroup was a cluster of five closely related indigenous isolates with a similarity of 90.6% isolated from various host legumes: 42b (*Neonotonia wightii*), 101d (*Zornia capensis*), UP32c (*Vigna unguiculata*), UP15b

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(*Indigofera schinzii*) and 70a (*Crotalaria brachycarpa*). Related to this group at 87.6% similarity was another cluster of indigenous strains: two isolates from *Crotalaria*, 5b and 33b, and strain 108a2 isolated from *Indigofera swaziensis*. Isolate 36b2 from *Neonotonia wightii* showed 90.6% similarity to *Bradyrhizobium* sp. LMG 8319. Also included in this subgroup were isolates 12c1 (*Indigofera rhytidocarpa*) and 34 (*Neonotonia wightii*) with no near relatives. Isolate 68d (*Mucuna coriacea*) showed a similarity of 87.5% with *Bradyrhizobium elkanii* USDA 76 and joined the other isolates in subgroup 1a at 85.8% similarity. **Subgroup 1b** consisted of five isolates with a similarity of 86.5% isolated from different legume hosts: 7b (*Alysicarpus rugosus*), 13c2 (*Lotononis bainesii*), UP11e (*Bolusanthus speciosus*) and 125e and UP30c from *Indigofera woodii* and *Indigofera filipes* respectively. Isolates 7b and 13c2 had a close similarity of 95.8%. These isolates showed no similarity to any of the reference strains used. **Subgroup 1c** was a tight cluster with 90.3% similarity and comprised four isolates from a variety of leguminous hosts: 65a2, 2 (*Rhynchosia nervosa*), and 29d (*Alysicarpus rugosus*) and 6a (*Vigna vexillata*) which showed 97.9% similarity. **Subgroup 1d** comprised only two isolates 8 (*Medicago lupulina*) and UP10 (*Senna petersiana*) with a similarity of 87.5% which joined the other subgroups of Group 1 at 83.5% similarity.

Group 2 comprised 16 indigenous isolates and one reference strain and could be further divided into four subgroups.

Subgroup 2a consisted only of indigenous strains isolated from various host legumes: the tree legumes *Acacia dealbata* (109a2) and *Acacia sieberana* var. *woodii* (46c2), *Pseudarthria hookeri* (114d), *Tephrosia purpurea* (72f and 82a), *Chamaecrista* sp. (104a1) and *Indigofera arrecta* (74a). These isolates had a similarity of 85.4% and the highest similarity between any two strains of this subgroup was 89.6%. **Subgroup 2b** comprised four strains with a 89.1% similarity and included *B. japonicum* USDA 6, isolates 102a (*Chamaecrista biensis*), 48a (*Tephrosia purpurea*) and 103b (*Indigofera hilaris*). The latter two isolates showed 94.8% similarity. **Subgroup 2c** joined subgroup 2b at 87.4% similarity and consisted of four indigenous isolates: 70b2 (*Crotalaria brachycarpa*), PL3, 3a (*Trifolium* sp.) and 12c2 (*Indigofera rhytidocarpa*). **Subgroup 2d** contained only two isolates, 36d (*Neonotonia wightii*) and UP17b (*Indigofera*

heterophylla) with a similarity of 94.8%. Subgroups 2b, 2c and 2d joined subgroup 2a at 86.7% similarity.

Group 3 consisted of six indigenous strains and two reference strains with no close similarity and two subgroups could be distinguished within this group.

Subgroup 3a comprised two isolates, 13c1 (*Lotononis bainesii*) and PL6 with 78.1% similarity. **Subgroup 3b** contained *Rhizobium viciae* USDA 2370, isolates 15c (*Desmodium tortuosum*) and 128a (*Melolobium obcordatum*) with a 82.8% similarity. Other isolates of Group 3 were 36b1 (*Neonotonia wightii*), 24a and *Bradyrhizobium japonicum* LMG 4265.

Section II

Nineteen (30%) of the isolates investigated and 22 reference strains constituted Section II. Three groups were distinguished with a similarity of 61.6%.

Group 4 comprised 11 reference strains of the genera *Rhizobium, Sinorhizobium, Mesorhizobium* and *Agrobacterium* and 10 indigenous isolates which were further divided into four subgroups.

Subgroup 4a consisted mostly of reference strains of the genus *Sinorhizobium* with a similarity of 82.5%. Two *S. meliloti* strains USDA 1002 and LMG 6133 and isolate UP27b (*Crotalaria damarensis*) formed a tight cluster with 92.7% similarity. *S. fredii* LMG 6217 and *Rhizobium* sp. LMG 6463 showed a 89.6% similarity and joined the above mentioned cluster at 87.5% similarity. Isolate UP3 (*Lotononis falcata*) and *S. fredii* LMG 8317 showed 84.0% and 82.5% similarity respectively with the other isolates of this group. Included in this subgroup was *Rhizobium galegae* USDA 4128 which joined other strains in this group at 80,5% similarity. None of the indigenous isolates showed similarity in metabolic activity with this strain. **Subgroup 4b** constituted a *Mesorhizobium* branch with two reference strains *M. huakuii* USDA 4778 and *M. loti* LMG 6123 and one indigenous isolate, UP16b (*Desmodium repandum*) with a similarity of 87.0%. Two indigenous isolates 98d2 (*Bolusanthus speciosus*) and UP31a (*Chamaecrista mimosoides*) clustered at a value of 90.6% similarity. These strains joined the *Sinorhizobium* group

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(subgroup 4a) at 79.1% similarity. Strain UP24a (*Lessertia annularis*) was loosely associated (77.2%) with other strains of subgroup 4b. **Subgroup 4c** constituted two reference strains from different genera. *Sinorhizobium saheli* LMG 7837 and *Rhizobium etli* USDA 9041 showed a 79.2% similarity. **Subgroup 4d** consisted of three indigenous strains, 85c (*Acacia xanthoploea*), UP27c and UP26b (*Crotalaria damarensis*) and the reference strain *Agrobacterium tumefaciens* LMG 187. Isolate 85a (*Acacia xanthoploea*) joined other strains of group 4 at a similarity value of 68.5%.

Group 5 contained *Rhizobium* and *Agrobacterium* reference strains and three indigenous isolates. Two subgroups with a similarity of 75.9% were distinguished.

Subgroup 5a comprised three *Agrobacterium* reference strains and two indigenous isolates, UP24b (*Lessertia annularis*) and 60 (*Rhynchosia monophylla*) with a similarity of 85.4%. The three reference strains *A. radiobacter* LMG 140, *A. rhizogenes* LMG 150 and *A. stellatum* LMG 122 formed a tight cluster within this subgroup with 93.7% similarity. **Subgroup 5b** consisted of *Rhizobium* reference strains only. *Rhizobium* sp.(*Acacia*) LMG 8311, *R. leguminosarum* LMG 4260 and *R. tropici* USDA 9030 showed 79.7% similarity. Isolate, UP23b isolated from *Indigofera verrucosa* joined the rest of Group 5 at 73.7% similarity.

Isolate PL10a joined isolates of groups 4 and 5 at a similarity of 63.7%.

Group 6 joined Groups 4 and 5 at a similarity of 61.6% and comprised *Rhizobium* and *Mesorhizobium* reference strains and five indigenous isolates. Four subgroups were distinguished.

Subgroup 6a consisted of two clusters with 77.1% similarity: *M. loti* LMG 4268t1 and LMG 4268t2 showed 85.4% similarity, and UP19 (*Trifolium* sp.) and *R. leguminosarum* LMG 6294 showed 85.4% similarity. **Subgroup 6b** was a cluster of *Rhizobium* sp. USDA 2947 and isolate UP22b from *Medicago sativa* with 79.2% similarity. Two strains, UP30a (*Indigofera filipes*) and *R. trifolii* LMG 6119 with no close similarity constituted **Subgroup 6c** and joined subgroups 6a and 6b at a similarity of 75.0% and 70.5% respectively. **Subgroup 6d** consisted of two very closely related indigenous strains PL10b and 26c (*Strongylodon macrobotrys*) with a similarity of 96.9% which joined the rest of Group 6 at 65.8% similarity.

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Table 12. Oxidation of 95 different carbon sources of the Biolog GN system for the five Biolog groups.

Carbon source(s)	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
methylpyruvate	+	+	+	+	+	+
succinamic acid	+	+	+	d	d	+
mono-methylsuccinate	+	d	+	+	+	d
Tween 80	+	+	d	d	+	-
Tween 40	+	+	d	d	d	-
ß-hydroxybutyric acid	+	+	d	d	+	d
L-leucine	+	-	d	d	d	d
L-arabinose	+	d	+	+	+	+
D,L- lactic acid	+	d	d	+	+	+
succinic acid; bromosuccinic acid	+	d	d	+	+	d
formic acid; glucuronamide	+	d	+	d	+	d
L-fucose	+	d	d	+	d	d
γ-hydroxybutyric acid	+	d	d	-	-	-
citric acid; D-gluconic acid	+	d	d	d	+	d
D-saccharic acid	+	d	d	d	+	-
α-cyclodextrin; 2,3-butanediol	-	-	-	-	-	-
2-amino ethanol; D,L-α-glycerol phosphate; putrescine	-	-	-	-	d	-
D-glucuronic acid	-	-	-	-	d	d
cellobiose; D-sorbitol	-	-	-	+	+	+
N-acetyl- galactosamine; D-melibiose, D-raffinose; L-serine; L-threonine	-	-	-	d	+	-
adonitol; i-erytritol; α-D-lactose; lactulose; D,L-carnitine	-	-	-	d	d	d
L-ornithine	-	-	-	+	+	-
glucose-1-phosphate; glucose-6-phosphate	-	-	-	d	d	-
xylitol	-	-	-	d	d	+

+   more than 90% of the isolates were positive
-     more than 90% of the isolates were negative
d   different results (positive or negative)

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Table 12 (continued). Oxidation of 95 different carbon sources of the Biolog GN system for the five Biolog groups.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
sucrose	-	-	-	+	d	+
N-acetyl-glucosamine; gentobiose; m-inositol; ß-methyl-D-glucoside; D-trehalose; L-alanine; L-alanyl-glycine	-	-	-	+	+	d
α-D-glucose; maltose; turanose	-	-	d	+	+	+
L-histidine	-	-	d	+	+	-
L-proline; urocanic acid	-	-	d	+	+	d
hydroxy-L-proline	-	-	d	+	d	d
itonic acid	-	-	d	-	-	-
D-serine	-	-	d	-	+	-
D-glucosamic acid	-	-	d	-	d	-
L-asparagine	-	-	d	d	+	-
cis-aconitic acid; glycyl-L-glutamic acid; inosine; uridine	-	-	d	d	+	d
γ-amino butyric acid	-	-	d	d	d	-
dextrin; glycogen; glycyl-L-aspartic acid, thymidine	-	-	d	d	d	d
L-aspartic acid	d	-	-	+	+	d
p-hydroxy phenylacetic acid; phenyl-ethylamine	d	-	-	-	d	-
malonic acid	d	-	-	d	d	d
D-galactose; D-mannitol	d	-	d	+	+	+
D-arabitol	d	-	d	+	d	+
D-mannose; L-rhamnose; glycerol	d	-	d	+	+	d
D-galacturonic acid	d	-	d	-	+	d
alaninamide; L-glutamic acid	d	-	d	d	+	d
D-alanine	d	-	d	d	+	-

+	more than 90% of the isolates were positive
-	more than 90% of the isolates were negative
d	different results (positive or negative)

Table 12 (continued)   Oxidation of 95 different carbon sources of the Biolog GN system for the five Biolog groups.

|	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
L-pyroglutamic acid	d	-	d	d	d	d
propionic acid	d	d	+	d	d	d
L-phenylalanine	d	d	-	-	+	-
acetic acid	d	d	+	d	+	d
D-fructose; D-psicose	d	d	d	+	+	+
sebacic acid	d	d	d	-	-	-
α-keto glutaric acid	d	d	d	-	d	-
α-keto valeric acid	d	d	d	d	-	-
D-galactonic acid lactone	d	d	d	d	+	d
α-keto butyric acid	d	d	d	d	-	d
α-hydroxybutyric acid; quinic acid	d	d	d	d	d	d

+   more than 90% of the isolates were positive
-      more than 90% of the isolates were negative
d   different results (positive or negative)

Numerical analysis of the substrate utilization patterns of the 27 reference strains used in the study is represented in Figure 8. Three distinct clusters could be distinguished at 60% similarity. Clusters I and II constituted the *Rhizobium - Agrobacterium - Sinorhizobium - Mesorhizobium* branch and Cluster III the *Bradyrhizobium* branch. *Mesorhizobium* strains were contained in cluster Ia and II. The *Sinorhizobium* strains constituted a separate branch and were all contained in cluster Ia. Cluster Ib constituted only *Rhizobium* and *Agrobacterium* strains.
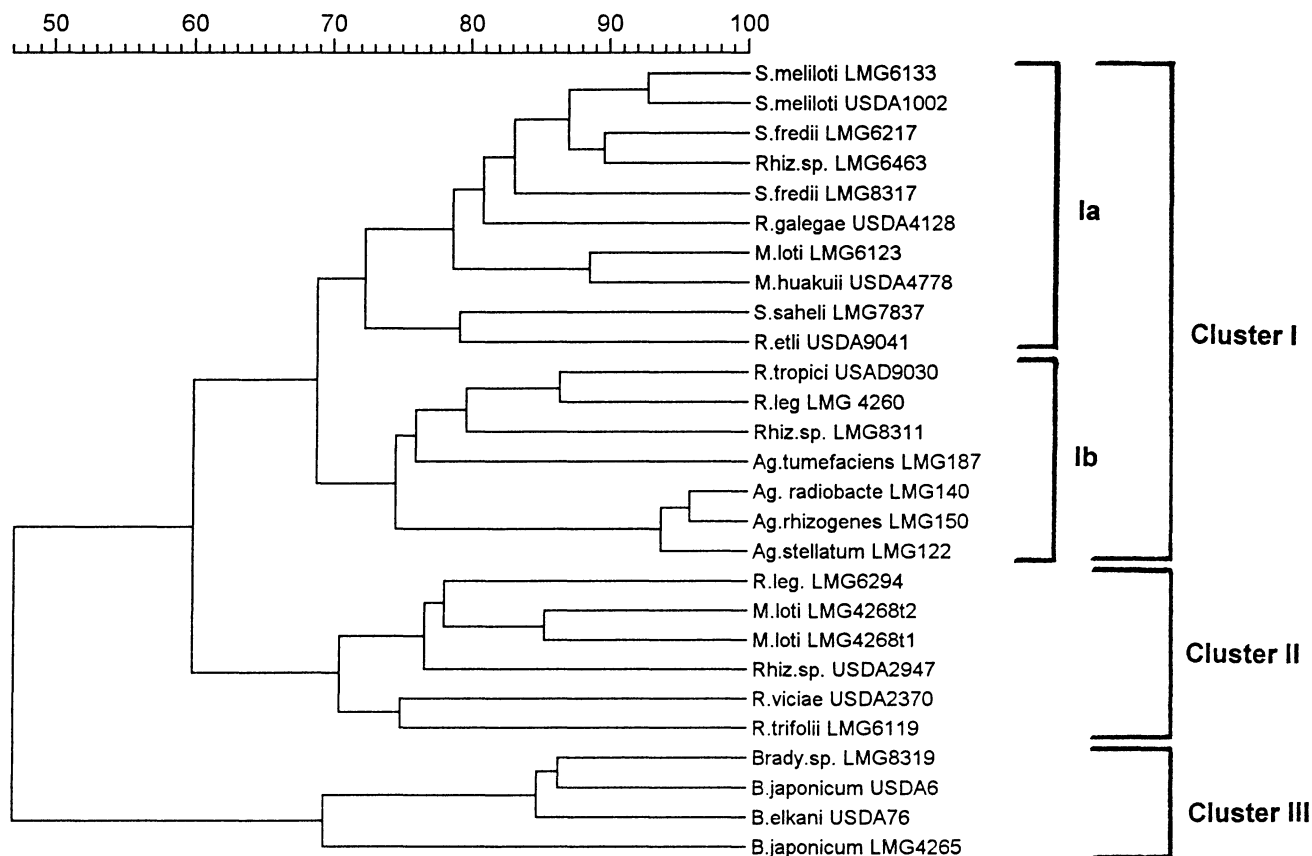
Fig. 8. Classification of reference strains with the Biolog system after 72 h, presented as a dendrogram based on UPGMA analysis of S_{SM} values.

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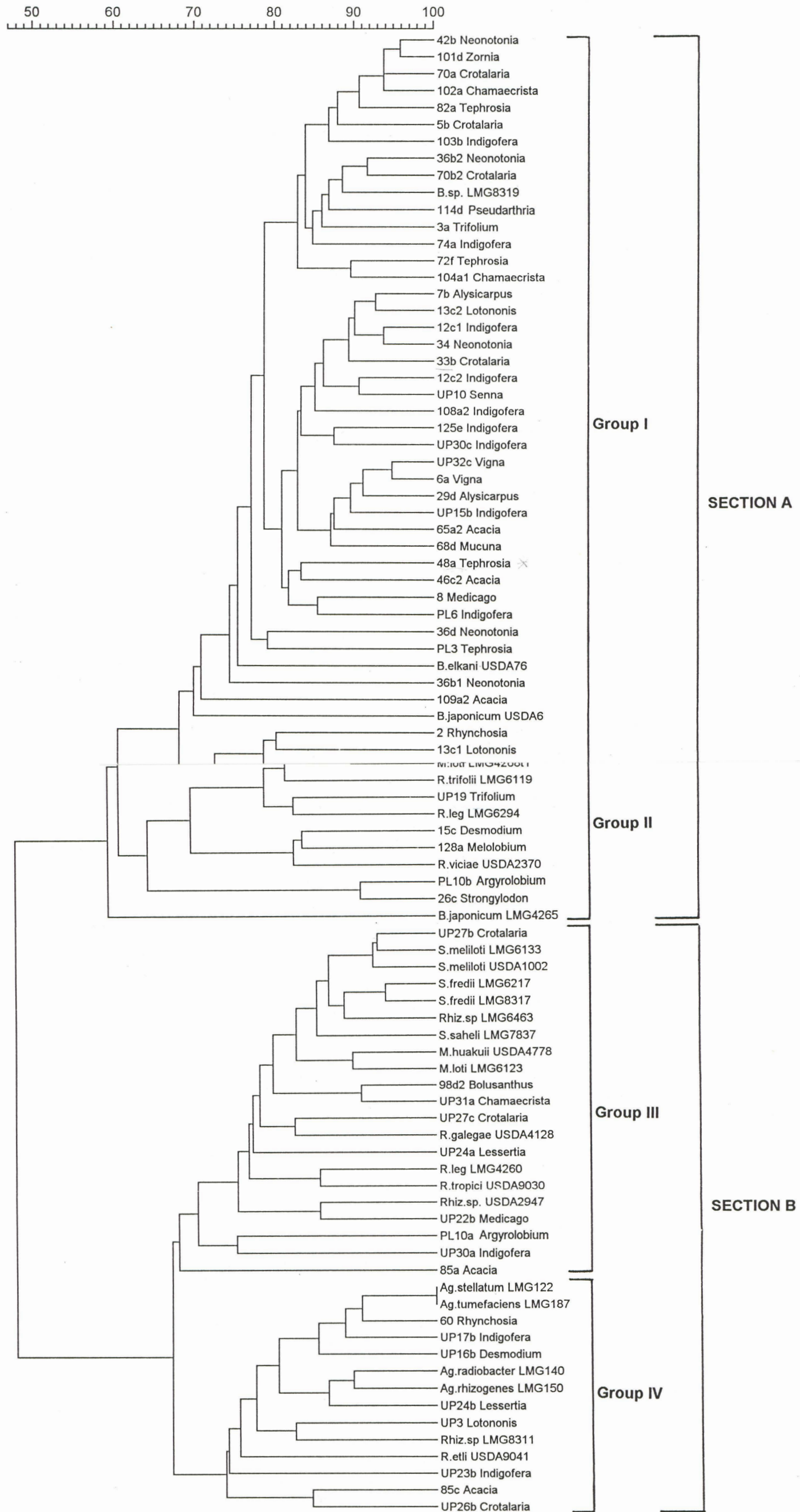
Fig. 9. Dendrogram based on UPGMA analysis of S_{SM} values from Biolog results after 96 h.

5.3.2. Effect of incubation time

To determine the effect of incubation time on the characterization of the isolates studied, results of substrate utilization after 96 h were also analyzed. This rendered a dendrogram (Fig. 9) with similar grouping as Fig. 7, although some changes were observed. Two major sections could once again be distinguished at a similarity level of 47.7% and is only discussed briefly.

Section A comprised two groups with a similarity of 59.2%.

Group I constituted 42 of the indigenous isolates and the other three *Bradyrhizobium* reference strains *B. elkanii* USDA 76, *B. japonicum* USDA 6 and *Bradyrhizobium* sp. LMG 8319. All the isolates of Groups 1 and 2 and some of the isolates of Group 3 (Fig. 7) were included in this group. Only one exception was observed in Group 2: isolate UP17b, clustered in Group IV. Slight changes in the clustering of these isolates were observed and similarity to the *Bradyrhizobium* reference strains were lower than in Fig. 7. None of the isolates in Groups 4, 5 and 6 (Fig. 7) were included in this *Bradyrhizobium* group.

Group II represented the most prominent change in the clustering of the isolates. Included in this group were *Mesorhizobium* and *Rhizobium* reference and five indigenous isolates. Group 6a and *R. trifolii* LMG 6119 (Group 6, Fig. 7) clustered in the 96 h dendrogram with members of Group 3 (Fig. 7) including *R. viciae* USDA 2370. Included in this group was *B. japonicum* LMG 4265 which also formed a separate line in Fig. 7.

Section B could be divided into two groups with a similarity of 66.9%.

Group III comprised *Sinorhizobium*, *Mesorhizobium*, and *Rhizobium* reference strains and some of the indigenous isolates. The *Sinorhizobium* lineage was more apparent with a closer relationship of *Sinorhizobium saheli* with the *S. meliloti* - *S. fredii* branch. Included in this group was the *Mesorhizobium* branch of *M. loti* - *M. huakuii*, several *Rhizobium* reference strains as well as nine indigenous isolates which clustered in Groups 4, 5 and 6 (Fig. 1). In this dendrogram *R. galegae* USDA 4128 clustered with the *Rhizobium* group.

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Group IV consisted of the four *Agrobacterium* reference strains, two *Rhizobium* reference strains and eight indigenous isolates which clustered in Groups 4 and 5 (Fig. 7). No differences in the utilization patterns of *A. stellatum* LMG 122 and *A. tumefaciens* LMG 187 could be observed after 96h and these two strains showed 100% similarity.

5.4. DISCUSSION

5.4.1. Characterization after 72 h incubation

The use of the Biolog system proved a rapid, easy method for the characterization of rhizobial isolates. One of the biggest advantages of the Biolog system is its simplicity. Prefilled and dried plates are easily inoculated with a cell suspension, no need for the addition of colour-developing reagents or oil overlays, and no follow-on tests are needed (Bochner, 1989a; Klingler *et al.*, 1992). Identification of isolates is usually achieved through comparison of substrate utilization patterns with a library of patterns in the Biolog software program. Identification is accepted as correct if the similarity index of the genus and species name is 0.750 or greater at 4 h, or 0.500 or greater at 24h (Biolog, 1994). Rapid identification of numerous isolates has been achieved in this manner in several studies (Jones *et al.*, 1993; Klingler *et al.*, 1992). Because of the constant progress in the taxonomy of the rhizobia and the limited rhizobial species in the Biolog database (Microlog 3.50), the substrate utilization patterns were used in cluster analysis in our study. Each substrate was viewed as a phenotypic trait and recorded qualitatively.

Comparison of reference strains used in this study resulted in a dendrogram (Fig. 8) which showed good agreement with analysis of rhizobia found in literature. The close relationship between the genera *Rhizobium* and *Agrobacterium* (Dreyfus *et al.*, 1988; Jordan, 1984; Young, 1996) was demonstrated and the constitution of the genus *Bradyrhizobium* as a separate branch, distinct from the other stem- and root-nodule bacteria was confirmed (Willems and Collins, 1993; Young, 1996).

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Most of the indigenous strains investigated showed a high degree of relatedness with the genus *Bradyrhizobium*. Some of these strains formed tight clusters (e.g. group 1b and 1c, Fig. 7) with no similarity to the reference strains used. Isolate UP10 from *Senna petersiana* grouped with bradyrhizobia strains.

The genus *Rhizobium* is a very heterogeneous group of bacteria (Willems and Collins, 1993; Young, 1996) which could be clearly seen from our analysis. Reference strains of this genus were scattered throughout the dendrogram. Several of the indigenous isolates showed a high degree of correlation with strains of this genus. The close relationship between the genera *Rhizobium* and *Agrobacterium* was demonstrated with the clustering of reference strains of these two genera into one group (group 5, Fig. 7).

Phenotypic analysis of carbon source utilization confirmed results from SDS-PAGE analysis (Dagutat, 1995) and DNA homology (Lindström, 1989) that *R. galegae* constitutes a separate lineage within the genus *Rhizobium*. In this study *R. galegae* USDA 4128 showed closer similarity to the *Sinorhizobium* group and none of the indigenous isolates investigated showed any similarity with *R. galegae*. The latter was also demonstrated in previous chapters and by Dagutat (1995).

The *Sinorhizobium* strains constituted a separate branch with all the *S. fredii* and *S. meliloti* reference strains clustered in one group (group 4a, Fig. 7). Included in this group was *Rhizobium* sp. LMG 6463 isolated from *Sesbania rostrata* that was classified as *S. teranga* by De Lajudie *et al.* (1994). Our results supports classification of this strain as *Sinorhizobium*. One *Sinorhizobium* strain, *S. saheli* LMG 7837, did not show a high degree of similarity with other *Sinorhizobium* strains, and was closely related to *R. etli* USDA 9041.

Mesorhizobium strains were distributed into two groups. *M. loti* LMG 6123 and *M. huakuii* USDA 4778 in group 4b and *M. loti* LMG 4268t1 and LMG 4268t2 (group 6a). Included in the former mesorhizobial group were isolates from *Desmodium*, *Bolusanthus* and *Chamaecrista*. The close similarity of isolate 98d2 from *Bolusanthus speciosus*, a tree legume, is of importance. This

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confirmed the results of Dagutat (1995), who once again questioned the use of host plant specificity as a standard for use in rhizobial taxonomy.

5.4.2. Effect of incubation time

In this study, plates were incubated for 96 h and visual readings were made at 24h intervals. We found that analysis of the substrate utilization patterns after 96 h resulted in a dendrogram similar to analysis at 72 h. Isolates which showed a high degree of relatedness to *Bradyrhizobium* strains, still grouped with *Bradyrhizobium* strains. Better classification of the *Sinorhizobium* group was achieved after 96 h with *S. saheli* showing a higher similarity to other sinorhizobial strains. *Agrobacterium* has a faster growth rate and these isolates showed rapid oxidation of the substrates after 24 h. After incubation for 96 h, these isolates were positive for most of the substrates, and type strains *Agrobacterium stellatum* LMG 122 and *A. tumefaciens* LMG 187 could not be distinguished at 96 h. Standardization of incubation time and multiple readings at regular intervals are thus necessary.

Several factors affect colour development and therefore substrate utilization patterns (Kersters *et al.*, 1997). The production batch of Biolog MicroPlates, incubation time and inoculum density have all been shown to play a role. Incorrect identification and identification that changes with incubation time have also been pinpointed as one of the system's drawbacks by other authors (Klingler *et al.*, 1992). In the study done by Klingler *et al.* (1992), it was observed that classification of enteric species was improved by incubating microplates for 6 to 8 h in stead of 4 h. It has been demonstrated by several authors that growth occurs in the Biolog MicroPlates wells and that substrate oxidization responses are nonlinear and often exhibit a lag phase, an exponential phase and a stationary phase (Garland and Mills, 1991; Haack *et al.*, 1995). Because of the differences in generation time of the rhizobia, standardization of growth period and incubation time can be difficult (Vandamme *et al.*, 1996). One solution to overcome the problem of nonlinear colour development is to do multiple readings over a time course of incubation (Kersters *et al.*, 1997). When both fast- and slow-growing isolates are investigated, it might be more valuable to analyze results after 96 h to ensure oxidation of carbon substrates by slow-growing isolates. In the study done by Dupuy *et al.* (1994), Biolog plates were incubated for 5 days at 33°C for the phenotypic characterization of slow-growing bradyrhizobia.

The differentiation power of this system is difficult to assess because of the numerous d responses (i.e. either positive or negative) obtained as demonstrated in Table 12. This was also observed for the bradyrhizobia from *Acacia albida* by Dupuy *et al.* (1994), who identified only three characteristics that could be used for differentiation between clusters. In our study no distinct differentiating feature could be recognized by which the groups could be distinguished from each other. Fast- growing rhizobia utilize a wider range of carbon sources than slow-growing strains, and have more glycolytical pathways at their disposal (Vincent, 1974). This was evident from the data in Table 12. Groups 1 and 2 (slow-growing bradyrhizobial strains), exhibited negative results for more substrates than groups 4 to 6.

The use of the Biolog GN MicroPlate system is a rapid method which can be used as an initial screening method for the phenotypic characterization of rhizobia whereafter representative strains can be selected for rDNA sequence analysis. A database of the metabolic fingerprints of indigenous rhizobial isolates has been established. The main limitation of this method is the high cost involved. Biolog MicroPlates have to be imported from the United States of America, and it is not economically feasible to be used for routine investigation at this stage.

5.4.3. Comparison of Biolog results with SDS-PAGE of whole cell proteins and PCR-RFLP analysis of 16S rDNA

Comparison of indigenous rhizobia and authentic rhizobial strains with the Biolog GN MicroPlate system, SDS-PAGE of whole cell proteins and RFLP analysis of PCR-amplified 16S rDNA, yielded three dendrograms with a high degree of overall agreement. Precise agreement of the different groupings was, however, not achieved. Most of the indigenous isolates investigated showed a high degree of relatedness to the genus *Bradyrhizobium* and some of the isolates to the fast-growing genera of *Rhizobium, Mesorhizobium* and *Sinorhizobium*.

With a few exceptions, all the isolates in Biolog groups 1 and 2 clustered in SDS-PAGE *Bradyrhizobium* clusters 1, 2, 5 and 9, and clusters 3 and 4 which contained slow-growing isolates but none of the reference strains (Fig. 3). Exceptions were 108a2 which grouped in cluster 11 together with the *Agrobacterium* strains and 109a2, which grouped in cluster 13

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without any reference strains. Biolog group 1 and 2 isolates were all contained in RFLP groups 1, 2 and 3, except isolate 7b which clustered in group 7, and 109a2 in group 4 (Fig. 6). These isolates constituted the *Bradyrhizobium* group of the indigenous isolates.

Biolog group 3 contained isolates that were only loosely associated and did not show any close relationship to each other with either SDS-PAGE or PCR-RFLP analysis of the 16S rDNA. These isolates grouped in different clusters in both these dendrograms.

Biolog group 4a represented the *Sinorhizobium* branch and results correlated well with those of SDS-PAGE (cluster 12). With both these methods the *Sinorhizobium* reference strains clustered in a single cluster/group. With RFLP analysis these strains clustered into two groups (group 7 and 9), and not on a single branch within the dendrogram.

The *Mesorhizobium* Biolog group (4b) contained isolates from SDS-PAGE clusters 7 and 14 (the *Mesorhizobium* branches), and one isolate, UP24a, from cluster 12. All these isolates also clustered in the *Mesorhizobium* RFLP branch (group 12), with UP24a as the only exception.

In group 4d, indigenous isolates UP26b, UP 27c and 85c, showed the same similarity with both SDS-PAGE and RFLP analysis clustering in cluster 7a and group 6 respectively. However, with Biolog and RFLP analysis the *Agrobacterium tumefaciens* reference strains showed similarity to these isolates. The genus *Rhizobium* cluster together with *Agrobacterium* in the alpha subclass of the *Proteobacteria* and analysis of 16S rDNA placed *Rhizobium galegae* on the same phylogenetic branch as *Agrobacterium* (Elkan, 1992; Jarvis *et al.*, 1996; Jordan, 1984). Taxonomy of the genus *Agrobacterium* is in the same state of disarray as that of the rhizobia and extensive changes were proposed for the genus *Agrobacterium*, which included separate species status for biovars 1 and 2, rejection of the name *A. tumefaciens*, revised description of strains with *A. radiobacter* and *A. rhizogenes* for biovar 1 and 2 strains respectively and recognition of *A. radiobacter* as the type species (Sawada *et al.*, 1993). This proposal was subsequently rejected and revision of the classification and nomenclature of this genus as well as the rhizobial genera is unavoidable (De Lajudie *et al.*, 1994). Our results confirmed the status of the *Agrobacterium* lineage imbedded in the rhizobial lineages.

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Group 5 of the Biolog dendrogram represented a *Rhizobium - Agrobacterium* branch. With SDS-PAGE and RFLP analysis these isolates showed diverse groupings, all within *Rhizobium* groups. Biolog group 6 contained strains that clustered in different groups of the SDS-PAGE and RFLP dendrograms and no definite similarities could be distinguished.

The clustering of strains together with one method and separation into several clusters by another method, were identified as one of the difficulties of the polyphasic approach. Inconsistencies across various methods could restrict taxonomic conclusions to be drawn from a combination of methods (Van Rossum *et al.*, 1995).

Close relationships between strains were best resolved with SDS-PAGE analysis. This was in agreement with findings of other authors (Laguerre *et al.*, 1994; Terefework *et al.*, 1998). The value of SDS-PAGE as a method for grouping large numbers of closely related strains has been proven repeatedly, and the biggest disadvantage of this method with regard to general identification, is that it only yields discriminative information at species or lower level (Vandamme *et al.*, 1996). With RFLP analysis, discrimination is possible at species and higher levels, but is limited in discrimination of closely related species (Laguerre *et al.*, 1994).

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CHAPTER 6

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CONCLUDING REMARKS

- The present classification of rhizobia into five separate genera was supported by the polyphasic analysis of reference strains using SDS-PAGE of whole cell proteins, 16S rDNA PCR-RFLP and the Biolog GN MicroPlate system. The genus *Bradyrhizobium* was clearly separated from the four genera representing the fast-growers, and the recognition of *Sinorhizobium* as a separate genus was substantiated.

- Most of the indigenous isolates investigated by SDS-PAGE, the Biolog system and RFLP analysis of the PCR-amplified 16S rDNA, were slow-growers belonging to the genus *Bradyrhizobium*. The other isolates were closely related to species of the genera *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*.

- Some methods allowed detailed characterization and separation of strains within a species, while other methods only elucidated interspecies or intergeneric differences.

    - SDS-PAGE analysis proved useful for differentiation of closely related strains into groups and identification with reference strains, and provided differentiation at intraspecies level.

    - PCR-RFLP of the 16S rRNA genes proved a rapid tool for differentiation of rhizobia at species and higher level.

    - The Biolog system provided differentiation of rhizobia at species level.

- One of the difficulties of a polyphasic approach was highlighted in this study: some isolates which clustered together by one method, were contained in separate clusters when another method was used.

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- Incubation time of Biolog plates play a role in characterization of isolates and has to be taken into account in determining the relationship among strains, especially when a diverse group of bacteria such as the root- and stem-nodulating bacteria is investigated.

- Continued isolation of rhizobia from leguminous hosts, especially legumes not previously investigated, is necessary to elucidate the taxonomy of rhizobia.

- Reportedly non-nodulating legumes should be examined for possible nodulation and the presence of rhizobia.

- Symbiotic performance is only another phenotypic property and should not play a decisive roll in the determination of rhizobial taxa.

- More than one type of rhizobial isolate can occupy the same nodule.

CHAPTER 7

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APPENDIX A

APPENDIX A

CARBON SOURCES IN THE BIOLOG GN MICROPLATES
(Biolog, 1994; Garland and Mills, 1991)

Carbohydrates	Carboxylic acids	Amino acids
N-acetyl-D-galactosamine	Acetic acid	D-Alanine
N-acetyl-D-glucosamine	cis-Aconitic acid	L-Alanine
Adonitol	Citric acid	L-Alanyl-glycine
L-Arabinose	Formic acid	L-Asparagine
D-Arabitol	D-Galactonic acid lactone	L-Aspartic acid
Cellobiose	D-Galacturonic acid	L-Glutamic acid
i-Erythritol	D-Gluconic acid	Glycyl-L-aspartic acid
D-Fructose	D-Glucosaminic acid	Glycyl-L-glutamic acid
L-Fucose	D-Glucoronic acid	L-Histidine
D-Galactose	α-Hydroxybutyric acid	Hydroxy-L-proline
Gentiobiose	ß-Hydroxybutyric acid	L-Leucine
α-D-Glucose	γ-Hydroxybutyric acid	L-Ornithine
m-Inositol	p-Hydroxyphenylacetic acid	L-Phenylalanine
α-Lactose	Itaconic acid	L-Proline
Lactulose	α-Ketobutyric acid	L-Pyroglutamic acid
Maltose	α-ketoglutaric acid	D-Serine
D-Mannitol	α-Ketovaleric acid	L-Serine
D-Mannose	D,L-Lactic acid	L-Threonine
D-Melibiose	Malonic acid	D,L-Carnitine
ß-Methylglucoside	Propionic acid	γ-Aminobutyric acid
Psicose	Quinic acid	
D-Raffinose	D-Saccharic acid	**Alcohols**
L-Rhamnose	Sebacic acid	2,3-Butanediol
D-Sorbitol	Succinic acid	Glycerol
Sucrose		
D-Trehalose	**Brominated chemicals**	
Turanose	Bromosuccinic acid	
Xylitol		

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Polymers	Aromatic chemicals	Phosphorylated chemicals
Glycogen	Inosine	D,L-α-Glycerol phosphate
α-Cyclodextrin	Urocanic acid	Glucose-1-phosphate
Dextrin	Thymidine	Glucose-6-phosphate
Tween 80	Uridine	
Tween 40		

Amides	Amines	Esters
Succinamic acid	Phenylethylamine	Mono-methylsuccinate
Glucuronamide	2-Aminoethanol	Methylpyruvate
Alaninamide	Putrescine	