CHAPTER 7

Characterisation of methylotrophic bacteria which nodulate *Lotononis bainesii.*

ABSTRACT

Nodule isolates from a shrubby legume, *Lotononis bainesii*, was characterised by 16S rRNA gene sequencing and morphologically by substrate utilisation patterns. The symbiotic genome of these isolates was analysed by partial sequencing of the *nifH* gene. Based on the results of numerical taxonomy, the isolates formed a closely related cluster, showing no correspondence to any of the known rhizobial clusters. Analysis of nearly full-length 16S rDNA sequences demonstrated that these isolates were related to *Methylobacterium nodulans* (Sy *et al.* 2001). In the absence of *nifH* sequence data for the genus *Methylobacterium*, the *nifH* phylogeny showed these isolates to be related to *Azospirillum brasilense.* The facultative methylotrophic nature of these isolates was also demonstrated by their ability to grow in the presence of methanol as a sole carbon source.

Keywords: *Lotononis bainesii,* 16S rDNA sequencing, *nifH* gene, *Methylobacterium*

INTRODUCTION

Symbiotic nitrogen fixing bacteria, commonly referred to as rhizobia, are able to establish a symbiotic association with most leguminous plants. As a result of this symbiotic association, specialised organs, called nodules, are induced on the host plant. Within such nodules atmospheric nitrogen is reduced to ammonia the benefit of the host plant. These nitrogen-fixing nodulating rhizobia have been assigned to different genera within the α- subclass of the *Proteobacteria* and include: *Rhizobium* (Frank, 1889), *Sinorhizobium* (Chen *et al.*, 1988), *Mesorhizobium* (Jarvis *et al.*, 1997), *Allorhizobium* (de Lajudie *et al.*, 1998), *Bradyrhizobium* (Jordan, 1982) and *Azorhizobium* (Dreyfus *et al.*, 1988). These rhizobial genera are very diverse with some being phylogenetically closer related to other non-symbiotic genera, than to each other (Young, 1996; van Berkum & Eardly, 1998; Young, 2001). Recently, Sy *et al.* (2001) reported the existence of an additional rhizobial branch involving bacteria of the genus *Methylobacterium*. These rhizobia were isolated from *Crotalaria* species and were able to grow facultatively on methanol, a common trait for *Methylobacterium* species, but unique to the known rhizobial species. Analysis of the 16S rDNA gene, nodulation ability, as well as amplification of the *nodA* gene confirmed these isolates to be nodulating *Methylobacterium* species for which the name *Methylobacterium nodulans* was proposed. According to Holland (1997), *Methylobacterium* species are usually isolated from water and leaf surfaces and are known as pink-pigmented facultative methylotrophs. However, the presence of the photosynthetic pigment, bacteriochlorophyll *a*, was not detected in *M. nodulans* (Sy *et al.*, 2001).

Lotononis species are herbs and shrubs of the subfamily Papilionoideae with more than 140 species commonly occurring under diverse climatological and geographical conditions. Their distribution is chiefly in southern Africa extending to the Mediterranean, with a few species in southern Europe and central Asia (van Wyk, 1991). *Lotononis bainesii* has proven its value as a pasture legume in regions in Australia. In addition to *L. bainesii*, other *Lotononis* species such as *L. divaricata*, *L. tenella* and *L. laxa* also have potential value as grazing plants since many are well adapted to the arid regions (Shearing, 1994). In 1958 Norris described a pigmented nodulating strain obtained from the roots of *Lotononis bainesii*. The chemical structure of this pigment was subsequently determined by Kleinig & Broughton (1982) and found to be similar to that of *Pseudomonas* species. Dagutat (unpublished results) compared

the protein profiles of a collection of bacterial isolates obtained from the root nodules of *Lotononis bainesii*. These isolates formed a closely related cluster, clearly separated from the rhizobial reference strains. Initial partial sequence 16S rDNA sequencing performed as part of this study revealed that these isolates were indeed a group of unknown taxonomic status. However, with the report of the methylotrophic nodulating bacteria (Sy *et al.*, 2001) it became evident that these pigmented *Lotononis* isolates were related to the genus *Methylobacterium*, showing high homology to *Methylobacterium nodulans.*

This primary objective of this study was therefore to characterise the nine isolates from the root nodules of *Lotononis bainesii* obtained from different localities in southern Africa. Using nearly full length 16S rDNA and partial *nif*H sequencing and substrate utilisation patterns, it was possible to show that these pigmented nodulating strains were facultative methylotrophs, related to the genus *Methylobacterium,* more specifically *M. nodulans.*

MATERIALS AND METHODS

Bacterial strains

Strains analysed (Table 7.1) in this study was obtained from the rhizobial collection of the ARC-Plant Protection Research Institute (Private Bag X134, Pretoria 0001, South Africa). These isolates were previously obtained from the root nodules of *Lotononis bainesii.* Reference cultures (Table 7.2) of the different rhizobial genera were obtained from the bacterial culture collection of the Laboratorium voor Microbiologie (LMG), State University Gent, Belgium and the United States Department of Agriculture (USDA), Soybean and Alfalfa Research Laboratory, Maryland, USA.

Maintenance of bacterial cultures

Strains were maintained on yeast extract mannitol (YM) agar, containing (w/v): 1% mannitol, 0.5% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.01%, NaCl, 0.04% yeast extract and 1.5% bacteriological agar. For long term storage cultures were grown (with rigorous shaking) in yeast mannitol broth (YMB) at 28°C for approximately five days. The turbid culture broth was subsequently mixed at a 1:1 ratio with sterile 50% (v/v) glycerol in sterile cryotubes and copies of each stored at both -20°C and -70°C.

Table 7.1. List of isolates analysed in this study.

Isolate	Host plant	Isolate	Host plant
xct7	*Lotononis bainesii*	xhm4	*Aspalathus linearis*
xct8	*Lotononis bainesii*	xhj7	*Aspalathus linearis*
xct9	*Lotononis bainesii*	xhj8	*Aspalathus linearis*
xct10	*Lotononis bainesii*	xhj12s	*Aspalathus linearis*
xct12	*Lotononis bainesii*	xhj15	*Aspalathus linearis*
xct13	*Lotononis bainesii*	xhj18	*Aspalathus linearis*
xct14	*Lotononis bainesii*	xhj20	*Aspalathus linearis*
xct16	*Lotononis bainesii*	xhj26	*Aspalathus linearis*
xct17	*Lotononis bainesii*	xhj27	*Aspalathus linearis*

Table 7.2. List of rhizobial reference strains analysed in this study.

Reference strains	**Strain number**	**Host plant**
Rhizobium leguminosarum	LMG 4260	*Vigna unguiculata*
Rhizobium leguminosarum	LMG 6294	*Lathyrus sp.*
R. leguminosarum bv. *trifolii*	LMG 6119	*Trifolium repens*
Rhizobium galegae	USDA 4128^T	*Galega orientalis*
Rhizobium tropici IIB	USDA 9030^T	*Phaseolus vulgaris*
Rhizobium etli	USDA 9032	*Phaseolus vulgaris*
Rhizobium sp.	LMG 6463	*Sesbania rostrata*
Bradyrhizobium japonicum	LMG 6138^T	*Glycine max*
Bradyrhizobium elkanii	LMG 6134^T	*Glycine max*
Bradyrhizobium sp.	LMG 8319	*Macrotyloma africanus*
Azorhizobium caulinodans	LMG 6465	*Sesbania rostrata*
Allorhizobium undicola	USDA 4903	*Neptunia natans*
Allorhizobium undicola	USDA 4904	*Neptunia natans*
Sinorhizobium meliloti	LMG 6133^T	*Medicago sativa*
Sinorhizobium fredii	LMG 6217^T	*Glycine max*
Sinorhizobium saheli	LMG 7837^T	*Sesbania cannabina*
Mesorhizobium loti	LMG 6125^T	*Lotus corniculatus*
Mesorhizobium huakuii	USDA 4778^T	*Astragalus sinicus*
Agrobacterium radiobacter	LMG 140^T	NS
Agrobacterium tumefaciens	LMG 187^T	*Lycopersicon lycopersicum*
Agrobacterium rhizogenes	LMG 150^T	NS
Agrobacterium aggregatum	LMG 122^T	NS

^{T} Type strain NS not stated

Numerical taxonomy

Substrate utilisation patterns of the isolates were assessed using a commercially available Biolog MicroPlate™ (Biolog, Hayward, California). These plates were specific for Gram-negative microorganisms and contained a preselected panel of 95 different carbon sources (Appendix B). Growth conditions and inoculation of the microplates were performed as prescribed by the suppliers. The utilisation of a specific carbon source was indicated by the development of a purple colour in the wells which was due to the presence of a redox dye, tetrazolium violet. Such wells were scored as one (1), while negative wells were scored as zero (0). The Dice coefficient (Nei & Li, 1979) within the Bionum computer programme (Applied Maths, Kortrijk, Belgium) was used to construct a distance matrix from this one (1)-zero (0) profiles. These distance values were subsequently analysed to generate a tree using the unweighted pair group method of arithmetic mean algorithm (UPGMA) in GelCompar 4.0 (Applied Maths, Kortrijk, Belgium). Substrate utilisation patterns of the rhizobial reference strains were obtained from a previous study (Kruger, 1998). Isolates obtained from the nodules of *Aspalathus linearis* was also included as further references.

To test the ability of the *Lotononis bainesii* isolates to utilise methanol as a sole carbon source, cells were grown on YM agar for five days and transferred to medium 72 (Appendix C), which contained 10 ml of filter-sterilised methanol per liter medium.

Genomic DNA extraction

Bacterial cultures were grown in YM broth for 4-7 days and genomic DNA extracted by the hexadecyltrimethyl ammonium bromide (CTAB) method as described by Wilson (1990). The integrity and concentration of the purified DNA samples were determined by agarose gel electrophoresis (Saambrook *et al.*, 1989).

PCR amplification, sequencing and analyses

The PCR reactions for the amplification of the 16S rRNA gene and a fragment of the *nifH* gene were carried out in a volume of 100 µl. Each reaction contained 50 pmole of each respective primer pair, 250 µM of each dNTP, 1.5 mM $MgCl_2$, approximately 50 ng genomic DNA and 0.5 U Taq DNA polymerase (Southern Cross Biotechnologies). Additionally, the reaction also contained 50 mM KCl, 10 mM Tris-HCl pH 9.0 and 0.1% Triton X-100 as

supplied in the reaction buffer. All amplification reactions were carried out on a Perkin Elmer GeneAmp PCR System 2400 thermocycler.

The 16S rRNA gene was amplified using universal primers fD1 and rP2 (Table 7.3) as described by Weisburg *et al.* (1991). However, since no PCR product cloning procedures were anticipated, linker sequences containing the restriction enzyme recognition sites were not included during the synthesis of these oligonucleotides. These primers were therefore designated fD1SHRT and rP2SHRT. The following thermal profile was used: initial denaturation step at 95 °C for 3 min, 30 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 30 sec) and extension (72 °C for 1 min). An additional extension step (72 °C for 10 min) was performed after completion of the 30 cycles. The PCR products were purified using the Qiagen PCR Purification Kit (Southern Cross Biotechnologies) according to the manufacturer's instructions.

The primers (Table 7.3) used to amplify a 750 bp fragment of the *nifH* gene were designed by comparing *nifH* sequence data of rhizobial reference strains with the following GenBank accession numbers: K10620 (*B. japonicum*) J01781 (*S. meliloti*), Z95228 (*Mesorhizobium* sp.), M15942 (*R. etli* bv *phaseoli*), L16503 (*Sinorhizobium* sp.) and M55226 (*R. leguminosarum* bv. *phaseoli*). The following thermal profile was found suitable for the amplification of the *nifH* gene: An initial denaturation step: 3 min at 95 °C; followed by 35 amplification cycles denaturation (94 °C for 30 sec), annealing (37 °C for 45 sec) and extension (72 °C for 1 min) with a final extension at 72 °C for 10 min.

Due to the difficulties experienced with the amplification of the *nifH* gene and the degeneracy of the primers used, it was necessary to clone the *nifH* PCR products prior to sequencing. The PCR product of isolate xct 7 was cloned into the pDrive cloning vector supplied in the Qiagen PCR cloning kit (Southern Cross Biotechnologies) according to the manufacturer's instructions. Plasmids were introduced into competent *E. coli* DH5α cells and recombinants isolated according to standard protocols (Saambrook, *et al.*, 1989).

Sequencing reactions were performed using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems). Each sequencing reaction was

Table 7.3. Primers used for PCR amplification and sequencing in this study

Primer	Sequence	Target Region	Reference
fD1SHRT	5' AGAGTTTGATCCTGGCTCAG 3'	16 rRNA	Weisburg *et al.* (1991)
rP2SHRT	5' ACGGCTACCTTGTTACGACTT 3'	16 rRNA	Weisburg *et al.* (1991)
16SRNAII-S	5' GTGTAGCGGTGAAATGCGTAG 3'	16 rRNA	Kuhnert, *et al.* (1996)
16SRNAVII-S	5' CTTGCGACCGTACTCCCCAGGC 3'	16 rRNA	Kuhnert, *et al.* (1996)
NifH-F	5' CGGGAAGGGCGGAATCGGCAAG 3'	*NifH*	This work
NifH-R	5' GCATGTCCTCGAGCTC(AT)TCCAT 3'	*NifH*	This work

carried out in a 5 µl volume containing approximately 100 ng template DNA, 12.5 pmole primer, and 2 µl ready reaction premix (supplied with the sequencing kit, containing the dye terminators, dNTP's, AmpliTaq DNA Polymerase, $MgCl_2$, and Tris-HCl buffer pH 9.0). The reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler and consisted of 25 cycles of denaturation (96 °C for 10 sec), annealing (50 °C for 5 sec), and extension (60 °C for 4 min). PCR primers (Weisburg *et al.*, 1991) as well as two internal primers, 16SRNAII-S and 16S RNAVII-S (Kuhnert *et al.*, 1996), were used to obtain nearly full-length 16S rDNA sequence data.

To obtain the *nifH* sequence data of xct7, recombinant plasmids were sequenced using the M13 reverse and M13 forward (-20) primers.

The ClustalX programme (Thompson *et al.*, 1997) was used to analyse the nucleic acid sequences. Additional sequence data of related α-*Proteobacteria* was obtained from GenBank and accession numbers are indicated in the relevant figures. A distance matrix was constructed by pairwise alignment of the sequences. The phylogenetic trees were constructed from the distance matrices using the neighbour-joining method of Saitou & Nei (1987). All branch lengths were proportional to the estimated divergence along each branch. The bootstrap method (Felsenstein, 1985) was used in combination with the neighbour-joining method to estimate confidence levels of the phylogenies. The phylogenetic trees were displayed using NJplot (Perrière & Gouy, 1996).

The *nifH* sequence of isolate xct 7 was also compared with *nifH* nucleotide sequences of other α- and γ-*Proteobacteria*. A distance matrix (Table 7.5) expressing these genetic distances was generated using DNAdist from Phylip version 3.5c (Felsenstein, 1989).

RESULTS

Numerical taxonomy

Substrate utilisation patterns of 95 different carbon sources were used to establish a numerical taxonomy for bacteria isolated from the root nodules of *Lotononis bainesii*. The results of the range of substrates utilised by this group of symbionts are recorded in Table 7.4. These carbon sources were previously divided into 11 categories by Garland &Mills (1991) as indicated in Table 7.4. The highest number of carbon sources tested for included carboxylic acids, amino acids and carbohydrates. Amongst the *Lotononis* isolates the full range of carboxylic acids assayed for was utilised, while only 12 out of 20 amino acids were used by the isolates. The isolates were more specific with regard to their carbohydrates as sole carbon sources since only 5 out of possible 28 sources were used.

The metabolic fingerprints generated using the Biolog system were subsequently used to generate a dendrogram (Figure 7.1) expressing the phenotypic similarities among the *Lotononis* isolates, rhizobial reference strains and other indigenous isolates obtained from *Aspalathus linearis*. Two major sections could be distinguished within this dendrogram. The first contained members of the genera *Rhizobium, Sinorhizobium, Mesorhizobium, Agrobacterium* and *Allorhizobium*, while section 2 contained the genus *Bradyrhizobium* and the *Lotononis* isolates. The overall similarity for groups within the sections is 63% and 67% for section 1 and 2 respectively. Species of the genera *Agrobacterium* and *Allorhizobium undicola* were exclusively present in clusters 2 and 3, while *Rhizobium* and *Sinorhizobium* spp. were intermixed between clusters 1 and 4. Isolates from *Aspalathus linearis* (in cluster 5) were related to *Mesorhizobium huakuii*.

The *Lotononis* isolates were distributed among three clusters with an overall similarity of 77%. Cluster 6 contained xct 14, xct 13, xct 17, xct 8, while clusters 7 and 8, contained xct 10, xct 12 and xct 7 and xct 16, respectively. Isolate xct 9 was separate from the other clusters harboring the *Lotononis* isolates. A closer examination of the substrate utilisation

Table 7.4 Oxidation patterns of the different carbon sources utilised by *Lotononis bainesii* isolates.

Carbon sources not utilised by any of the isolates are not listed. Those showing reaction indicated by positive (+) sign and no reaction by negative (-). Categories of substrates as determined by Garland & Mills, (1991).

Carbon Sources	Isolates								
	xct7	xct8	xct9	xct10	xct12	xct13	xct14	xct16	xct17
Polymers									
Tween-40	+	+	+	-	-	+	+	+	-
Tween-80	+	+	+	-	+	+	+	+	-
Carbohydrates									
L-arabinose	-	+	+	+	+	+	+	+	-
L-fucose	-	-	-	-	-	+	+	-	-
A-D-glucose	-	-	+	-	-	+	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-
xylitol	-	-	+	-	-	-	-	-	-
Esters									
methylpyruvate	+	+	+	+	+	+	+	+	+
mono-methylsuccinate	+	+	+	+	+	+	+	+	+
Carboxylic acids									
acetic acid	+	+	+	+	+	+	+	+	+
cis-aconitic acid	+	+	-	+	+	+	+	+	-
citric acid	-	+	+	+	+	+	+	+	-
formic acid	+	-	-	-	-	+	+	+	+
D-galactonic acid lactone	-	+	+	+	-	+	+	+	+
D-galacturonic acid	-	-	-	-	-	+	+	+	-
D-gluconic acid	+	+	+	+	+	+	+	+	+
D-glucosaminic acid	-	-	-	+	-	-	-	+	-
D-glucoronic acid	-	+	-	-	-	-	-	-	-
A-/ β-/ γ-hydroxybutyric acid	+	+	+	+	+	+	+	+	+
itaconic acid	-	-	-	-	-	-	-	+	-
A-keto-butyric acid	+	+	+	+	+	+	+	+	+
A-keto-glutaric acid	+	+	+	+	+	+	+	+	+
A-keto-valeric acid	+	+	+	+	+	+	+	+	+
D, L lactic acid; malonic acid	+	+	+	+	+	+	+	+	+
propionic acid, quinic acid	+	+	+	+	+	+	+	+	+
D-saccharic acid, sebacic acid	+	+	+	+	+	+	+	+	+
succinic acid	+	+	+	+	+	+	+	+	+
Bromonated chemicals									
bromo-succinic acid	+	+	+	+	+	+	+	+	+
Amides									
succinamic acid	+	+	+	+	+	+	+	+	+
alaninamide	+	+	+	+	+	+	+	+	+
Amino acids									
L-Asparagine	+	+	+	+	+	+	+	+	+
D-alanine	-	-	-	-	-	+	+	-	-
L-alanyl-glycine	-	-	+	-	-	+	-	-	-
L-aspartic acid	+	+	+	+	+	+	+	+	+
L-glutamic acid	+	+	+	+	+	+	+	+	+
glycyl-L-glutamic acid	-	-	+	+	+	+	+	-	-
L-leucine	-	-	-	-	-	-	-	-	+
L-proline	+	-	+	+	+	+	+	+	-
L-pyroglutamic acid	+	-	+	-	-	+	+	+	+
D-serine; L-serine	-	-	+	-	-	-	-	-	-
L-threonine	-	-	+	-	+	+	+	-	-
Aromatic chemicals									
urocanic acid	-	-	-	-	-	+	+	-	-
Amines									
2-amino-ethanol	-	-	+	-	-	-	-	-	-
Alcohols									
2,3- butanediol	-	-	+	-	-	-	-	-	-

pattern revealed that this isolate could uniquely use the following substrates: xylitol, D-serine, L-serine, 2-amino-ethanol and 2, 3-butanediol.

16S rDNA sequence analysis

Nearly full length (1180 bp) nucleotide sequence of the 16S rRNA gene was determined for the nine isolates obtained from the root nodules of *Lotononis bainesii*. The phylogenetic position of these isolates was inferred from comparative 16S sequence analysis and is indicated in the Figure 7.2. Representatives of the other genera of the α-*Proteobacteria* were also included in this analysis. The polyphyletic nature of the rhizobial genera, as described previously (Young, 1996) was again clearly illustrated in this analysis. The *Lotononis bainesii* isolates were clearly distinct from the other known rhizobial genera and showed high sequence homology to the *Methylobacterium* lineage of the α-*Proteobacteria*. The branching point leading to the branches, which carry the *Methylobacterium* species, was also supported by a bootstrap value of 100%. The *Lotononis* isolates had almost identical 16S rDNA sequences; with only xct 17 showing approximately 2.6% sequence difference from the other isolates. Sequence similarities of the *Lotononis* group and other described *Methylobacterium* species was on average 94%. The closest phylogenetic neighbor of the *Lotononis* isolates was *Methylobacterium nodulans* (Sy, *et al.*, 2001), showing sequence similarity values of close to 98%.

Analysis of the *nifH*-gene sequences.

A fragment of the *nifH* gene of isolate xct 7 was cloned and sequenced. Most of the available *nifH* sequence data, obtained from GenBank, were considerably shorter fragments of sequence and therefore only a 560 bp fragment of the gene was used for comparative analysis and construction of a phylogenetic tree (Fig. 7.3). 16S rDNA sequence analyses have already indicated the *Lotononis* isolates to be related the genus *Methylobacterium*, however, no *nifH* sequence data was available for members of this genus. In the absence of sufficient sequence data, the phylogenetic position of xct 7, was therefore assessed in the presence of other genera of the α- and γ– *Proteobacteria*, as well as the *nifH* sequences of other indigenous rhizobial isolates (indicated by # in Fig. 7.3). The indigenous isolates maintained their genetic affiliations as determined by 16S rDNA sequence analyses. No close relationship was evident between the *Lotononis* isolate xct 7 and any member of the known rhizobial genera included in this analysis. *Azospirillum brasilense* and xct 7 shared almost 90% *nifH*

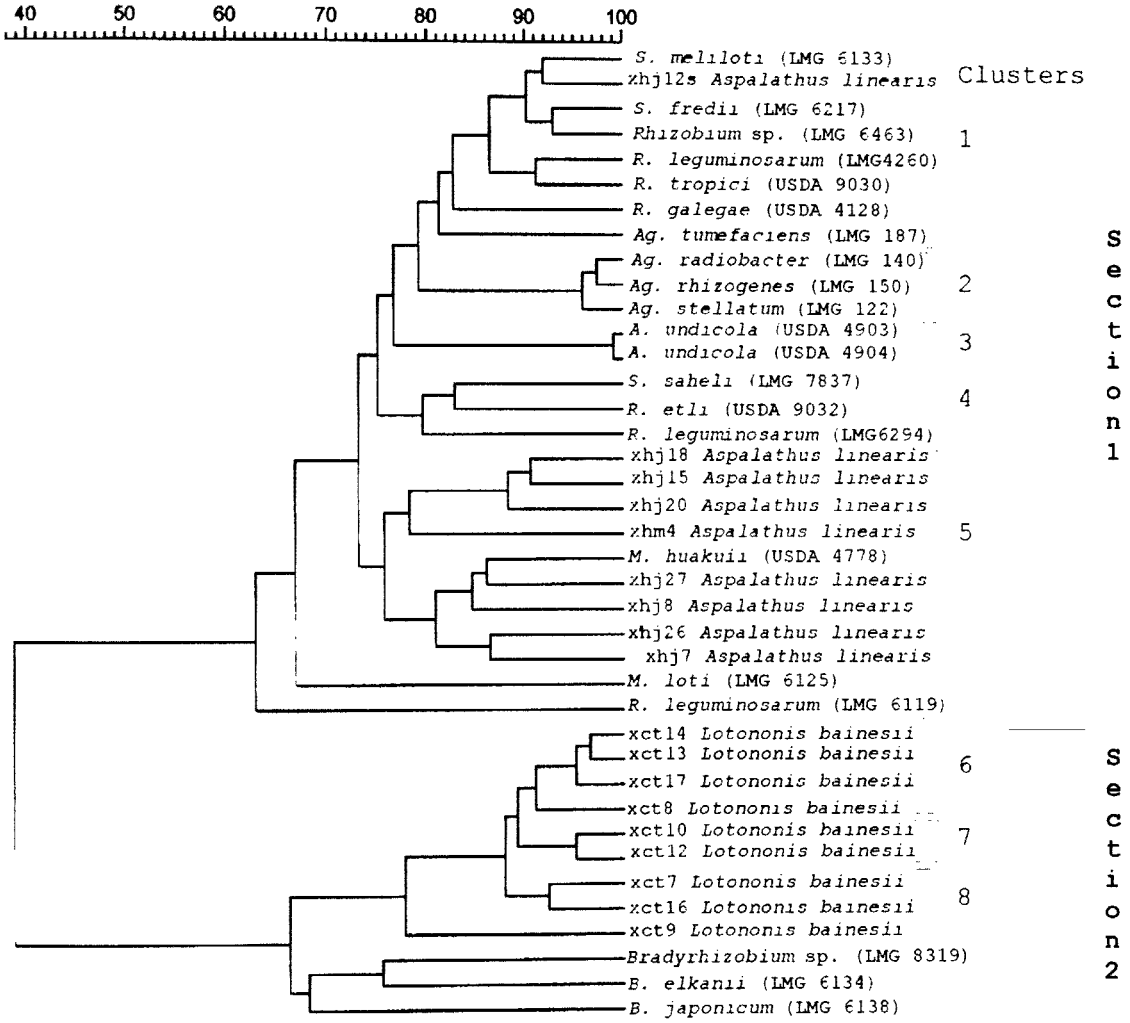

Figure 7.1. Dendrogram showing the phenotypic similarities among *Lotononis bainesii* isolates as determined by substrate utilisation patterns using the Biolog system. Rhizobial strains and isolates from Aspalathus linearis were included as references. The UPGMA method was used for cluster analysis (Sneath & Sokal, 1973). The x-axis shows the correlation between strains. *S: Sinorhizobium*; *R: Rhizobium*; *Ag: Agrobacterium*, *A: Allorhizobium*; *M: Mesorhizobium*, *B: Bradyrhizobium*. LMG strain 6119: *Rhizobium leguminosarum* bv. *trifolii*.

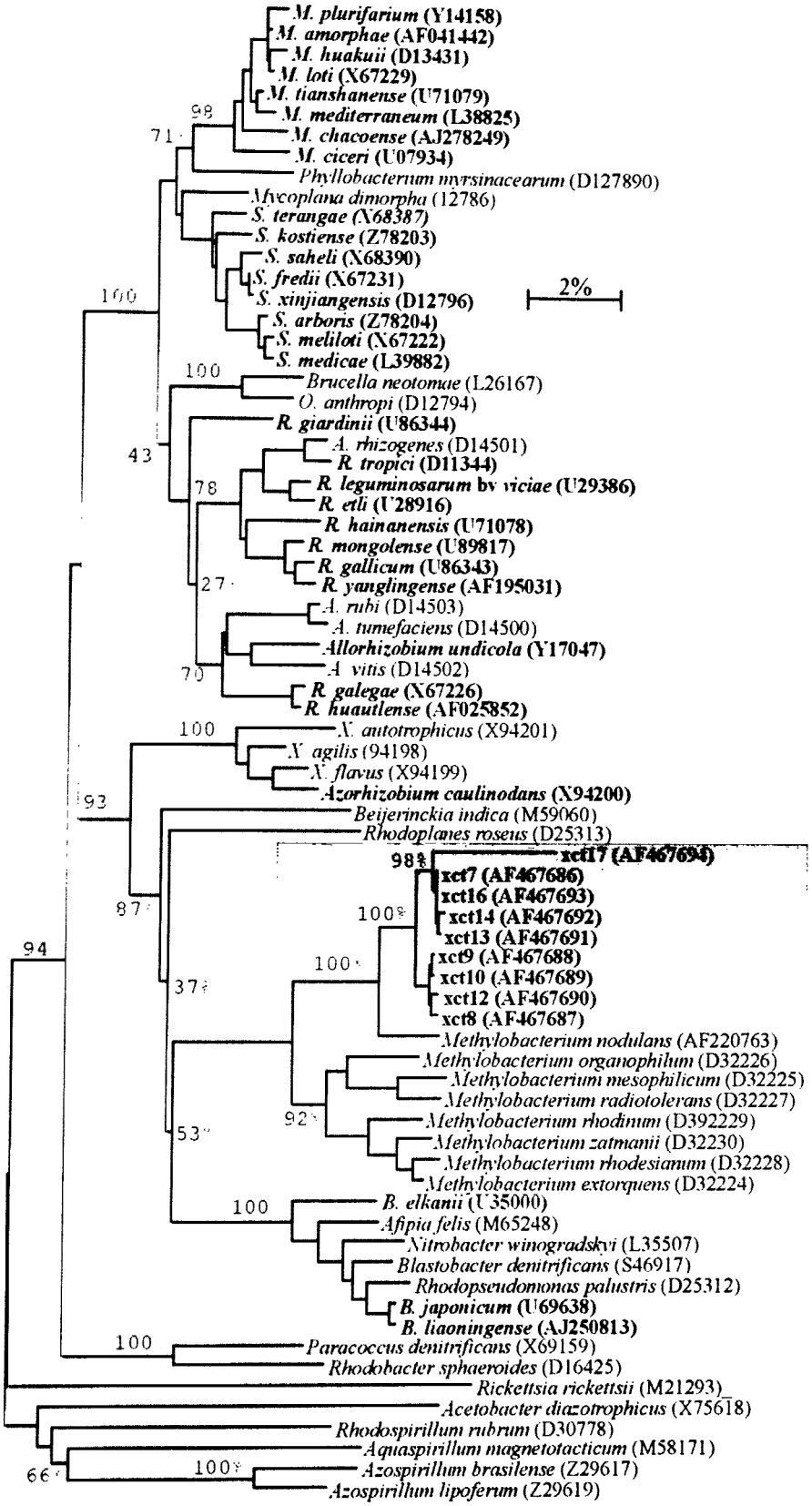

Figure 7.2 (previous page). Phylogenetic relationships of isolates nodulating *Lotononis bainesii* (shaded box) in comparison to other rhizobial genera and other α-*Proteobacteria*. This analysis was based on comparative sequence analysis of approximately 1200 bp fragment of the 16S rRNA gene. The tree was constructed using the Neighbour-joining method of Saitou & Nei (1987). Horisontal branch lengths reflect the phylogenetic distances, while vertical branch lengths are non-informative and set for clarity only. The scale bar indicates 2% nucleotide difference and bootstrap values of some of the major branching points are shown. GenBank accession numbers are indicated in brackets. *M: Mesorhizobium, S: Sinorhizobium, O: Ochrobactrum, R: Rhizobium, A: Agrobacterium, X: Xanthobacter, B: Bradyrhizobium.*

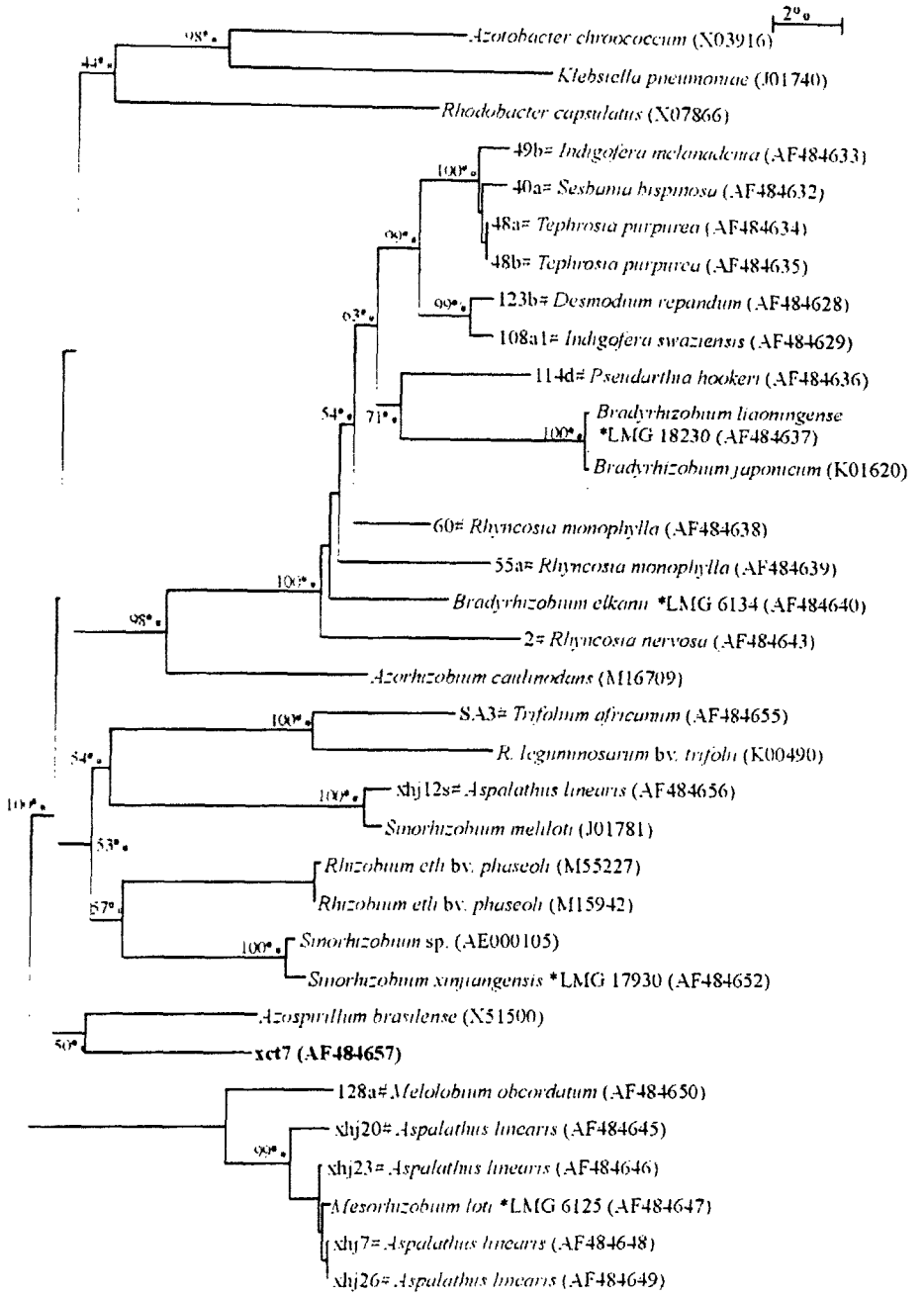

Figure 7.3. Phylogenetic tree, based on a 560 bp fragment of the *nifH* gene, expressing the relationship of *Lotononis bainesii* nodulating strain, xct7, to other rhizobial genera and other nitrogen fixing strains of the alpha- and gamma-*Proteobacteria*. The tree was generated using the Neighbour-joining method of Saitou & Nei (1987). Horisontal branches are equal to the phylogenetic distances of which the scale indicates 2%. Bootstrap values of some of the major branching points are indicated. GenBank accession numbers of the reference strains and indigenous rhizobia are indicated in brackets. The *nifH* sequences of reference strains (marked *) were determined in a related study. Rhizobial isolates (indicated by #) obtained from indigenous legumes in South Africa were included as additional references.

sequence similarity. However, the common ancestory branching point is supported by only 50% bootstrap confidence value. Therefore more *nifH* sequence data of the genus *Methylobacterium* is needed to establish the true *nifH* phylogeny of the *Lotononis* isolates.

Since *nif* genes are found in many bacteria, besides rhizobia, the *nifH* sequence of xct 7 was compared with a few representatives of the α- *Proteobacteria* which included the known rhizobial genera, *R. capsulatus* and type II methanotrophs: *Methylocystis* and *Methylosinus* species (Auman, *et al.*, 2001). The following γ- *Proteobacteria* were included: *P. stutzeri*, *M. purpuratum*, *V. diazotrophicus* and *K. pneumoniae.* These similarity values are indicated in Table 7.5. Similarity values within the rhizobial genera ranged from 59% to 96%, while the type II methanotrophs share at least 90% sequence similarity. Sequence similarity values of xct7 and the rhizobial genera ranged from 72% to 83%. When comparing the xct7 *nifH* sequence with *M. thrichosporium* (type II methanotroph) and *M. purpuratum* (γ-*Proteobacteria*), similarity values of 84% and 81% were obtained respectively. In contrast, xct7 had a lower similarity value (72%) with *B. japonicum* and *B. liaoningense.*

Table 7.5 Sequence identities, based on a 244 bp fragment of the *nifH* gene of isolate xct7 isolated from *Lotononis bainesii* and other related bacteria from the α- & γ-*Proteobacteria*.
Members of the γ-*Proteobacteria* are shaded. The rest all belong to the α –group.

	1. *Bradyrhizobium japonicum*	2. *Bradyrhizobium liaoningense**	3. *Bradyrhizobium elkanii*	4. *Azorhizobium caulinodans*	5 *Rhizobium etli* bv. *phaseoli*	6. *Rhizobium leguminosarum*	7. *Mesorhizobium loti*	8. *Methylosinus* sp. LW4	9. *Methylosinus thrichosporium*	10.. *Methylosinus* LW3	11. *Methylocystis* LW5a	12. *Azospirillum brasilense*	13. xct7	14. *Rhodobacter capsulatus*	15. *Pseudomonas stutzeri*	16. *Marichromatum purpuratum*	17. *Vibrio diazothrophicus*	18. *Klebsiella pneumoniae*
1. *B.japonicum* **(K01620)**	100	96	87	77	69	60	66	70	72	72	73	74	72	60	66	68	56	66
2. *B. liaoningense**		100	87	77	69	59	66	70	72	72	73	74	72	59	66	68	56	66
3. *B. elkanii**			100	84	77	65	69	77	80	74	82	80	82	70	74	71	60	70
4. *A. caulinodans* **(M16709)**				100	74	66	73	84	84	78	85	80	81	70	77	75	65	75
5. *R. etli* bv.*phaseoli* **(M15942)**					100	79	76	73	77	74	77	83	80	76	77	75	58	69
6. *R. leguminosarum* **(K00490)**						100	65	59	61	60	62	73	73	68	73	70	56	66
7. *Mesorhizobium loti*							100	70	72	71	74	79	83	73	70	71	57	64
8. *Methylosinus* sp. LW4 **(AF378721)**								100	95	90	90	83	82	73	74	79	60	72
9. *M. thrichosporium* **(AF378724)**									100	89	92	85	84	75	79	81	60	70
10. *Methylosinus* LW3 **(AF378720)**										100	85	81	79	75	74	80	60	70
11. *Methylocystis* LW5 **(AF378719)**											100	83	83	74	78	77	64	71
12. *Azospirillum brasilense* **(X51500)**												100	90	84	83	81	57	72
13. xct7													100	76	80	81	55	68
14. *Rhodobacter capsulatus* **(X07866)**														100	73	71	58	71
15. *Pseudomonas stutzeri* **(AJ297529)**															100	87	73	78
16. *M. purpuratum* **(AF059648)**																100	67	79
17. *Vibrio diazothrophicus* **(AF111110)**																	100	76
18. *Klebsiella pneumoniae* **(J1740)**																		100

* Sequence determined in a related study (unpublished results)

DISCUSSION

Rhizobia described thus far belong to three distinct 16S rDNA-based phylogenetic branches within the α- subclass of the *Proteobacteria*. The first main branch comprises *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium* and the plant pathogen *Agrobacterium*. The second branch contains the genus *Bradyrhizobium*, while the third branch contains the stem nodulating *Azorhizobium*. Recently, the polyphyletic origin of the rhizobia within the α-*Proteobacteria* was yet again confirmed when Sy *et al.* (2001) described nodulating *Methylobacterium*, recovered from the root nodules of *Crotalaria* spp.. The genus *Methylobacterium* (Patt, *et al.*, 1976) is a group of strictly aerobic, facultatively methylotrophic, gram-negative bacteria and are usually pink to red due to the presence of carotenoids. They are distributed in a wide range of natural habitats, including soil, dust, air, fresh water and aquatic sediment (Hiraishi, *et al.*, 1995). Regardless of their ubiquitous nature in soil and water reservoirs, the recent description of *Methylobacterium nodulans* provided the first evidence of symbiotic nature of these microorganisms. However, *M. nodulans* did not exhibit the characteristic pink pigment of the methylobacteria.

In South Africa almost 100 different genera of leguminous plants are found, growing under diverse geographical and climatological conditions (Strijdom, 1998). In this study isolates obtained from the root nodules of *Lotononis bainesii* were characterised in terms of phenotypic features as well as 16S rDNA and *nifH* phylogeny. These symbionts were pink-pigmented and 16S rDNA sequencing proved them to be closely related to *M. nodulans*. Their methylotrophic nature was indicated by growth on medium 72 (results not shown), with methanol as sole carbon source. However, the maximum methanol tolerance values were not determined. Methylotrophy is not a common trait among rhizobia since none of the rhizobial reference strains were capable of utilising methanol.

Nitrogen fixing genes (*nif* genes) are found in many bacteria besides rhizobia (Haukka *et al.*, 1998). Although it has been reported that the *nifH* phylogeny closely resembles that of the 16S rRNA gene (Hennecke, *et al.*, 1985; Ueda, *et al.*, 1995), a report by Eardly *et al.* (1992) presented evidence of phylogenetic discordance that could be due to the lateral transfer of *nif* genes. In this report it was difficult to determine the exact phylogenetic position of xct 7 based on *nifH* sequence due to a lack of corresponding sequence data for the

Methylobacterium genus. However, the *nifH* sequence of xct7 was closely related to that of *Azospirillum brasilense*.

Recently, Moulin, *et al.* (2001) described the isolation of a nodulating *Burkholderia* sp. (β-subclass of *Proteobacteria*) from the legume *Aspalathus carnosa*. This finding showed the range of bacteria able to nodulate legumes is more widespread than previously anticipated. Two novel features (nodulation by β-*Proteobacteria* and methylotrophy) of legume symbiosis are now known. It is however, interesting to note that the plant genera (*Crotalaria, Aspalathus* and *Lotononis*) involved have the same tribal affiliation (Tribe Crotalarieae). Further investigations of the symbionts associated with plant species within this tribe and other uninvestigated legumes are therefore warranted. It should now be evident that our understanding of the true bacterial diversity involved in legume symbiosis is very limited and can only increase as more host species are investigated.