CHAPTER 8

Polyphasic evaluation of a selection of the indigenous rhizobia

INTRODUCTION

Polyphasic taxonomy has become a widely applied approach in bacterial classification and has led to numerous revisions of the stem- and root nodulating bacteria (van Berkum & Eardly, 1998). Within such an approach, various traits of the organism are considered in an effort to generate a consensus taxonomy (Colwell, 1970; Murray *et al.*, 1990; Vandamme *et al.*, 1996). Polyphasic taxonomy therefore aims to prevent too much emphasis being placed on one method, which may lead to possible erroneous conclusions of generic relationships. This is amply illustrated by the report of Young *et al.* (2001), which indicated that results obtained by 16S rDNA sequence analyses depended on the chosen algorithm and the selection of the included sequences. Based on these observations and other phenotypic data, Young *et al.* (2001) subsequently proposed the amalgamation of the genera *Agrobacterium, Allorhizobium* and *Rhizobium* into a single genus, *Rhizobium.*

An extensive rhizobial collection has been established by the previous investigations of Dagutat (1995) and Kruger (1998). These rhizobial isolates were characterised phenotypically by comparison of whole cell protein profiles and their ability to utilise different carbon sources. In this study, the genotypic variation of a selection of the indigenous isolates was investigated. From these results it was clear that the indigenous rhizobial microflora of South Africa comprised a heterogeneous group related to the genera *Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium* and *Methylobacterium.* The generic status of some isolates, however, remained uncertain. It should be evident that a substantial amount of information has accumulated on the diversity of the indigenous rhizobia. The aim of this section is therefore to briefly summarise results obtained thus far. Genera such as *Rhizobium* and *Sinorhizobium*, who were represented by only a few isolates, will not be discussed. Furthermore, only isolates for which the full range of both phenotypic and phylogenetic data is available will be discussed. All discussions on 16S rDNA and IGS-RFLP groupings refer to those presented in Figures 5.1, 5.2, 5.4 & 7.2 in Chapters 5 & 7.

The mesorhizobia

Except for one isolate (xhj12s), all isolates obtained from *Aspalathus linearis* and *Aspalathus cordata* formed the major component of isolates related to the genus *Mesorhizobium* (Fig. 5.1 & 5.2). Numerical analysis based on substrate utilisation patterns as well as whole cell protein profiles showed that these isolates were present in a homogeneous cluster with an

overall similarity of almost 80%. The overall 16S rDNA sequence similarity (based on a 1300 bp fragment) among the different *Aspalathus* strains ranged from 96.7% to 99.9%. With the exception of xhj12FR, which shared 98% sequence homology with *M. ciceri,* all the isolates were closely related to *M. amorphae, M. plurifarium M. huakuii* and *M. tianshanense.* Considering the clustering obtained by 16S-23S IGS-RFLP more heterogeneity was evident. A comparative study performed by Willems *et al.* (2001c) indicated that sequence analysis of the IGS region sometimes provided taxonomic information similar to DNA-DNA hybridisation for the bradyrhizobia. Although similar evidence for the genus *Mesorhizobium* is still lacking, this is a more variable region that gives better resolution of strains with closely related 16S rDNA sequences. Isolates xhj7 and xhj8 were found to have almost identical IGS-RFLP patterns and near identical protein profiles and substrate utilisation patterns. These isolates were also distinct from all the recognised *Mesorhizobium* species. Based on IGS variation, isolate xhj26 was closely related to the former two, but differed significantly with regard to its whole cell protein profiles.

Isolates xhj15, xhj18 and xhj23 were indistinguishable when considering their IGS-RFLP and Biolog patterns. The isolates also showed near identical protein profiles (correlation of higher than 80%).

In view of the current available data, it appears possible that there are at least two new *Mesorhizobium* species present within this group. The first is represented by two strains, xhj7 and xhj8, and the second by xhj15, xhj18 and xhj23. Since these isolates demonstrated almost identical 16S rDNA sequences, DNA-DNA homology analyses of representatives of these two groups will be necessary to accurately describe their species status. The IGS-RFLP patterns of xhj13, xhj20, xhj27 and xhm5, showed no close association with any mesorhizobial reference strains or any of the other *Aspalathus linearis* isolates. This divergence might be due to minor nucleotide differences, which might have affected the restriction recognition sites, or they in fact are new species. The heterogeneity of these four isolates and one such as xhj26 can only be suitably resolved when more related strains become available and DNA-DNA homologies are determined.

The bradyrhizobia

The high 16S rDNA sequence similarity among the different bradyrhizobia makes it difficult to adequately describe the relationships within this group. On the basis of 16S rDNA sequences and DNA homology values, *B. elkanii* is clearly a distinct species from *B. japonicum* and *B. liaoningense*. The latter two species are phenotypically distinct, but genotypically closely related (Willems *et al.*, 2001b). The fact that *B. japonicum* is phylogenetically closer related to other genera (such as *Afipia, Agromonas, Blastobacter, Nitrobacter* and *Rhodopseudomonas*) than to *B. elkanii* indicates that the current bradyrhizobial taxonomy is unsatisfactory.

SDS-PAGE of total protein has a limited ability to delineate groups in bradyrhizobia (Doignon-Bourcier *et al.*, 1999). Among the reasons stated for this inability are total protein profiles with fewer bands, variation in band intensities and high background. Moreover generation times of the various bradyrhizobia may vary and the starting material may not be comparable. Grouping of *Bradyrhizobium* obtained from protein profiles should therefore be regarded with reservation. Similarly Kruger (1998) also indicated that the stability of substrate utilisation patterns depended on the standardisation of the length of incubation time. In general the clustering of the indigenous bradyrhizobia by whole cell protein profiles and variation of the 16S rDNA sequences showed little correspondence. In view of the obvious instability of the phenotypic methods in bradyrhizobial taxonomy, only the groupings obtained by 16S rDNA sequencing and 16S-23S IGS-RFLP will be discussed here. Willems *et al.* (2001c) observed that the IGS sequences of *Bradyrhizobium* sometimes give similar taxonomic information as DNA-DNA homology values. Although no IGS sequences are available for the indigenous South African bradyrhizobia, the RFLP patterns of this genomic region will be considered to describe the diversity of this group.

The overall 16S rDNA sequence similarity among the indigenous bradyrhizobial isolates ranged from 94.7 to 100%. Additionally, the indigenous bradyrhizobia also shared high homology to the different bradyrhizobia genospecies as proposed by Lafay & Burdon, (1998) [results not shown]. The variation of the IGS region was, however, suitably able to indicate further variation. Among the 19 indigenous bradyrhizobia, 14 different IGS-RFLP types were observed. These types did not show any correspondence to the IGS-RFLP patterns of the three known *Bradyrhizobium* species. Therefore DNA homology studies should be

performed and differentiating phenotypic data described for representatives of the 14 IGS-RFLP types.

The methylobacteria

Recently, Sy *et al.* (2001) described a group of root nodulating strains from *Crotalaria* which were shown to belong to the *Methylobacterium* genus. These strains were facultative methylotrophs, an uncommon trait among rhizobia, and represented the fourth rhizobial lineage. The genomic and symbiotic properties of these methylotrophic nodulating symbionts were investigated and subsequently assigned to a new species, *Methylobacterium nodulans* (Sy *et al.*, 2001).

A group of pink-pigmented strains was isolated from the root nodules of *Lotononis bainesii*. An investigation of their whole cell protein profiles showed that these isolates were a homogeneous group (correlation of more than 80%). With the exception of isolate, xct9, these isolates also showed very similar substrate utilisation patterns. However, none of these strains showed any relation to the known rhizobial genera. These phenotypic characterisations were performed before the description of *M. nodulans* and their generic status was only resolved once 16S rDNA sequences analyses (Fig. 7.2) of these isolates were expanded to include that of *M. nodulans* and other methylobacteria. Most of the *Lotononis bainesii* isolates shared at least 99% 16S rDNA sequence homology with *M. nodulans*. Pending DNA homology values, it appears that isolates xct7, xct8, xct9, xct10, xct12, xct13, xct14 and xct16 are *M. nodulans* species. On the other hand, the sequence similarity between isolate xct17 and other *Methylobacterium* species, including *M. nodulans*, was around 95%. Based on this low sequence similarity value, it appears likely that xct17, represents an additional *Methylobacterium* nodulating species.

Our phenotypic characterisations did not include *M. nodulans* since it only became available after our phenotypic analyses were completed. It is therefore impossible to relate phenotypic differences between the *Lotononis bainesii* isolates and *M. nodulans*.

CONCLUSION

The rapid and reliable identification of strains remains one of the most important tasks in taxonomy. The range of methods to perform this task has been extended significantly by the introduction of molecular techniques. The extensive analyses of the diversity of the indigenous rhizobia, have indicated possible new species within existing rhizobial genera and strains of known species. DNA-DNA hybridisation levels are still considered to be an important criterion for species delineation (Stackebrandt & Goebel, 1994) and therefore representatives of these possible novel South African rhizobial isolates will have to be evaluated by this procedure. DNA homology studies require good quality DNA, which in the case of *Bradyrhizobium* is not always easily obtained. DNA homology determinations currently fall beyond the scope of the laboratory where this work was performed, but the results obtained should provide a suitable basis for selection of isolates for this procedure.