International Journal of Systematic Bacteriology (1998), 48, 349–356 Printed in Great Britain

Phylogeny of *Rhizobium galegae* with respect to other rhizobia and agrobacteria

Zewdu Terefework,[1] Giselle Nick,[1] Sini Suomalainen,[2] Lars Paulin[2] and Kristina Lindström[1]

Author for correspondence: Zewdu Terefework. Tel: +358 9 7085 9281. Fax: +358 9 7085 9322. e-mail: zewdu.terefework@helsinki.fi

Department of Applied Chemistry and Microbiology[1] and Institute of Biotechnology[2], Biocenter 1, PO Box 56, FIN-00014 University of Helsinki, Finland

PCR-RFLP with nine restriction enzymes was applied to the 16S and 23S rRNA genes of 42 rhizobial and agrobacterial strains to determine the phylogenetic position of *Rhizobium galegae* and increase the understanding of the evolution of ribosomal operons. The strains were selected based on previous phylogenetic studies. PCR primers were designed so that they amplified a 2·3 kb fragment of the 23S rRNA gene (excluding the B8 loop). Universal primers rD1 and fD1 were used to amplify the full-length 16S rRNA. The RFLP analysis resulted in 27 and 32 different restriction patterns for 16S and 23S, respectively. The RFLP patterns were transformed to genetic distances and dendrograms were constructed from the data using the unweighted pair group method with averages. The shapes of the dendrograms derived from the analysis of the 16S and 23S rRNA genes correlated well, with only a few strains having different positions. The 23S tree generally had deeper branching than the 16S tree, allowing better discrimination between species and strains. The combined data from the two analyses described 36 genotypes. The eight *R. galegae* strains formed a homogeneous cluster in all dendrograms. The RFLP analysis was confirmed by partial sequence analysis of the 16S rRNA gene (the first 800 bp), which correlated well with full-length 16S rRNA sequence analysis. The 16S data placed *R. galegae* near the genus *Agrobacterium* with *Agrobacterium vitis* as its nearest neighbour, whereas in the 23S and the combined dendrograms it showed closer affinity to the genus *Rhizobium*.

Keywords: rhizobia, *Rhizobium galegae*, PCR-RFLP, 16S/23S rDNA, phylogeny

INTRODUCTION

Rhizobia are root- and stem-nodulating bacteria belonging to the five genera: *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* in the alpha subdivision of the proteobacteria. The stem-nodulating *Azorhizobium* genus and the slow-growing *Bradyrhizobium* genus are phylogenetically divergent from the other, fast-growing, genera of which *Mesorhizobium* is clearly distinct from the others. *Rhizobium* and *Sinorhizobium* are phylogenetically fairly closely related to each other and they are intermixed with plant-pathogenic and non-symbiotic bacteria belonging to the genus *Agrobacterium*. In addition, there are phenotypically diverse bacterial genera and species dispersed between the rhizobial and agrobacterial genera. The need for continued taxonomic revision of this group of bacteria, based on polyphasic approaches, is evident (3, 16, 33).

The genus *Rhizobium* presently contains the species *Rhizobium leguminosarum* (the type species of the genus), *Rhizobium etli*, *Rhizobium tropici* and *Rhizobium galegae*. The plant pathogen *Agrobacterium rhizogenes* is on the same phylogenetic branch, taxonomically close to *R. tropici* (33). The species *Rhizobium galegae* (15), which forms a specific nitrogen-fixing symbiosis with the perennial forage legumes *Galega orientalis* and *Galega officinalis*, is phylogenetically distant from all the other root-nodule bacteria, with the exception of *Rhizobium* sp. strain OK55, isolated from *Sesbania aculeata* (24). Analysis of full-length 16S rRNA gene sequences places *R. galegae* on the same phylogenetic branch as the *Agrobacterium* species, *Agrobacterium tumefaciens*, *Agrobacterium rubi*, *Agrobacterium radiobacter* and

Abbreviations: IGS, intergenic spacer region; LSU, large subunit; SSU, small subunit; UPGMA, unweighted pair group method with averages.

The EMBL/GenBank/DDBJ accession numbers for the sequences reported in this paper are Y12350–Y12362.

Table 1. List of strains used in this study

Strain	Host plant	Geographical origin	Accession no.	16S rDNA genotype	23S rDNA genotype
Rhizobium galegae					
HAMBI 540^T	*Galega orientalis*	Finland	Y12355*	9	21
HAMBI 1428	*Galega orientalis*	Russia	Y12356*	9	22
HAMBI 1461	*Galega orientalis*	Russia	Y12357*	10	21
CIAM 0707	*Galega orientalis*	Russia	Y12358*	9	21
HAMBI 490	*Galega officinalis*	New Zealand	Y12359*	9	20
HAMBI 1141	*Galega officinalis*	USA	Y12360*	9	21
BG 5	*Galega officinalis*	Bulgaria	Y12362*	9	20
BG 1	*Galega officinalis*	Bulgaria	Y12361*	9	20
Rhizobium leguminosarum					
ATCC 10004^T		UK	U29386†	18	18
Rhizobium tropici					
CIAT 899^T (HAMBI 1163)	*Phaseolus vulgaris*	Mexico	X67233†	17	11
Agrobacterium tumefaciens					
B6^T (HAMBI 1811)			X67223†	6	8
C58 (HAMBI 1217)				5	2
Agrobacterium radiobacter					
ATCC 4718 (HAMBI 105)				4	7
ATCC 19358^T (HAMBI 1814)				4	10
Agrobacterium rhizogenes					
ATCC 11325^T (HAMBI 1816)			X67224†	17	12
Agrobacterium rubi					
ATCC 13335^T (HAMBI 1812)			X67228†	5	5
Agrobacterium vitis					
LMG 8750^T (HAMBI 1817)			X67225†	7	13
AG 61 (HAMBI 1815)				8	14
Mesorhizobium loti					
NZP 2213^T (HAMBI 1129)	*Lotus tenuis*	New Zealand	X67229†	24	31
NZP 2037 (HAMBI 1338)	*Lotus pedunculatus*	New Zealand		25	30
Mesorhizobium ciceri					
UPM-Ca7^T (HAMBI 1750)	*Cicer arietinum*	France	U07934†	26	28
Mesorhizobium huakuii					
CCBAU 2609^T (HAMBI 1674)	*Astragalus sinicus*	China	D12797†	23	29
Sinorhizobium meliloti					
NZP 4027^T (LMG 6133)	*Medicago sativa*		X67222†		
Balsac (HAMBI 1167)	*Medicago sativa*	Uruguay		15	19
Sinorhizobium terangae					
ORS 1009^T (HAMBI 220)	*Acacia laeta*	Senegal	X68388†	16	16
***Rhizobium* sp.**					
HAMBI 1625	*Acacia senegal*	Sudan		6	4
HAMBI 1932	*Acacia senegal*	Sudan		3	1
HAMBI 249	*Acacia senegal*	Sudan		6	4
HAMBI 1705	*Acacia senegal*	Sudan		19	17
HAMBI 1604	*Acacia senegal*	Sudan		6	4
JL 84 (HAMBI 1835)	*Astragalus complanatus*	China	Y12353*	13	25
DUS 551 (HAMBI 1785)	*Astragalus sinicus*	China		6	3
MPI 3004 (HAMBI 1620)	*Brownea ariza*	Singapore		1	4
SH215214 (HAMBI 1831)	*Caragana microphylla*	China	Y12350*	22	27
SH21321 (HAMBI 1832)	*Caragana pruinosa*	China	Y12351*	21	24
MPI 3008 (HAMBI 1621)	*Centrosema plumieri*	Malaysia		2	4
SH19312 (HAMBI 1829)	*Glycyrrhiza pallidiflora*	China		6	7
SH19352 (HAMBI 1830)	*Glycyrrhiza pallidiflora*	China		6	6
IMAP 835 (HAMBI 1713)	*Hedysarum coronarium*	Italy	Y12354*	12	23
MPI 3031 (HAMBI 1614)	*Leucaena leucocephala*	Singapore		14	26
INPA 45R (HAMBI 1818)	*Leucaena* sp.	China		20	9
SH1124 (HAMBI 1840)	*Sophora viicifolia*	China	Y12352*	11	27
ORS 1001 Cluster U	*Acacia senegal*	Senegal		27	32

* EMBL/GenBank/DDBJ accession number of sequences from this study.

† EMBL/GenBank/DDBJ accession number of retrieved sequences.

Agrobacterium vitis, the last being its closest neighbour. RFLP analysis of 16S rDNA of over 100 rhizobia and agrobacteria also placed *R. galegae* on the *Agrobacterium* phylogenetic branch (20). On the other hand, partial sequencing of 16S gene regions (260 bp of the first variable region) placed *R. galegae* close to *Mesorhizobium loti* (33), and so did the PCR-RFLP analysis of the 16S-IGS (IGS, intergenic spacer region) region performed by Nour *et al.* (21).

The aim of this work was to resolve the contradictions in the previous studies and clarify the phylogenetic position of the *R. galegae* species. *R. galegae* strains, which represented all the taxonomic variations found within the species, and reference strains from the four phylogenetic branches suggested so far among rhizobia and agrobacteria (*Agrobacterium*, *Mesorhizobium*, *Rhizobium*, *Sinorhizobium*) were selected. We also included strains that in taxonomic studies had shown some affinity to *R. galegae*, with the hope of finding putative new species with the same phylogeny, as well as newly discovered rhizobia that had not been given names or classified (20).

In addition to the well-established methods of sequence and PCR-RFLP analysis of the 16S ribosomal genes, we decided to make use of the phylogenetic information available in the 23S ribosomal genes. The 23S rRNA gene, which encodes the large subunit (LSU) of the ribosomes, has the virtues of the 16S rRNA gene, which encodes the small subunit (SSU), but because of its larger size it contains more information. Ludwig *et al.* (17) evaluated its use as a phylogenetic marker and obtained results consistent with those observed for SSU. Finally, we performed experiments with the IGS region between the two genes to test its use as a taxonomic tool when PCR-RFLP is employed for analysis.

METHODS

Bacterial strains and culture conditions. A complete list of the strains used in this study, their geographical origin and sources is contained in Table 1. All of the strains were grown in 5 ml yeast extract mannitol broth (14) for 2–3 d at 28 °C.

DNA isolation. Total DNA was isolated from $2 \times 1{\cdot}5$ ml cultures using the procedure described by Ausubel *et al.* (1). The concentration of DNA was determined by comparison with known concentrations of λ DNA in agarose gel electrophoresis.

PCR amplification and oligonucleotide primers. PCR was performed in a final volume of 100 µl, with the following reaction components: 25 ng template DNA, 2 mM dNTP (Promega), 1 µM each primer pair, 0·5 U Redhot DNA polymerase, 10 × reaction buffer, 2·5 mM $MgCl_2$ (Advanced Biotechnologies UK). Primers fD1 and rD1 described by Weisburg *et al.* (28) were used to amplify 16S rRNA. For 23S rRNA amplification the following primers were used. Primer 3, 5′-CCG TGA GGG AAA GGT GAA AAG TAC C-3′, which corresponds to the sequence from 461 to 485 bp in the *Escherichia coli* nomenclature, was designed during this work. It is a slight modification of primer 3415 (26). Primer 4, 5′-CCC GCT TAG ATG CTT TCA GC-3′, which corresponds to sequence from 2744 to 2763 bp in the *E. coli* nomenclature, was described by Van Camp *et al.* (7) as 97ar. For IGS amplification we used primers FGPS1490-72 and FGPL132′ described by Normand *et al.* (22). DNA amplification was done in a PTC-200 Peltier thermal cycler (MJ Research). The cycling profiles were an initial denaturation at 95 °C for 3 min; 30 cycles of denaturation (1 min at 94 °C), annealing (1 min at 55 °C), extension (2 min at 72 °C), and final extension at 72 °C for 3 min (13). The size of the amplification products was verified by electrophoresis in 1 % agarose gel.

Restriction fragment analysis. The restriction enzymes used were *Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Hin*fI, *Mbo*I, *Mse*I, *Msp*I and *Rsa*I (13). Aliquots of 8–12 µl PCR products were digested with these restriction endonucleases (1·5 U) at 37 °C for at least 1 h. The restricted DNA was separated on 5 % agarose (Promega) gel containing ethidium bromide and photographed under UV illumination with Polaroid type 55 instant film. Restriction profiles for every enzyme were resolved and the fragments in each pattern that were clearly visible were compared in a pairwise manner for all strain combinations. However, separation of fragments by agarose gel electrophoresis removed the smaller fragments (100 bp or less), so these were not used in RFLP analysis. The sequence divergence between 16S rRNA and 23S rRNA gene regions for pairs of strains was estimated from the proportion of common restriction fragments and expressed as a genetic distance according to the method described by Nei & Li (18). The distance matrix thus obtained was analysed by the unweighted pair group method with averages (UPGMA) in the SPSS for Windows program release 6.1.3 (1995) and dendrograms were constructed from the results.

DNA sequencing and analysis. Based on the RFLP results of the 16S and 23S rRNA genes eight *R. galegae* and six other rhizobial strains were chosen for partial sequencing (800 bp) of the 16S rRNA gene. Sequencing was done directly from PCR products that were amplified by using a combination of primers: pA 5′-AGA GTT TGA TCC TGG CTC AG-3′ and pE′, 5′-CCG TCA ATT CCT TTG AGT TT-3′ (23). Amplification was performed as described earlier by using 1 U Dynazyme DNA polymerase (Finnzymes) and the same cycling conditions as used for amplification of the full-length 16S rDNA. The amplified PCR product was sequenced by the solid-phase method using an ALF DNA sequencer (Pharmacia) (23). The quality of the sequences was verified by sequencing both strands. These sequences were added to the EMBL database (Table 1). 16S rRNA sequences from the type strains of the different genera used in this study were retrieved from the EMBL database and used for cladistic analysis (Table 1). The edited sequences were aligned using PILEUP from the GCG package (version 8, September 1994) and a phylogenetic tree was constructed using CLUSTAL W (27) (Fig. 2). To verify that we were dealing with the 23S gene, the 23S rDNA fragment of *R. galegae* (HAMBI 540) was amplified using the same primers as previously described. Segments of DNA from different regions of this fragment were sequenced and compared with 23S rDNA sequences from the EMBL database.

RESULTS

The PCR amplification of the 16S rDNA produced a 1·5 kb fragment from all samples. The sum of the estimated sizes of the digested fragments of the

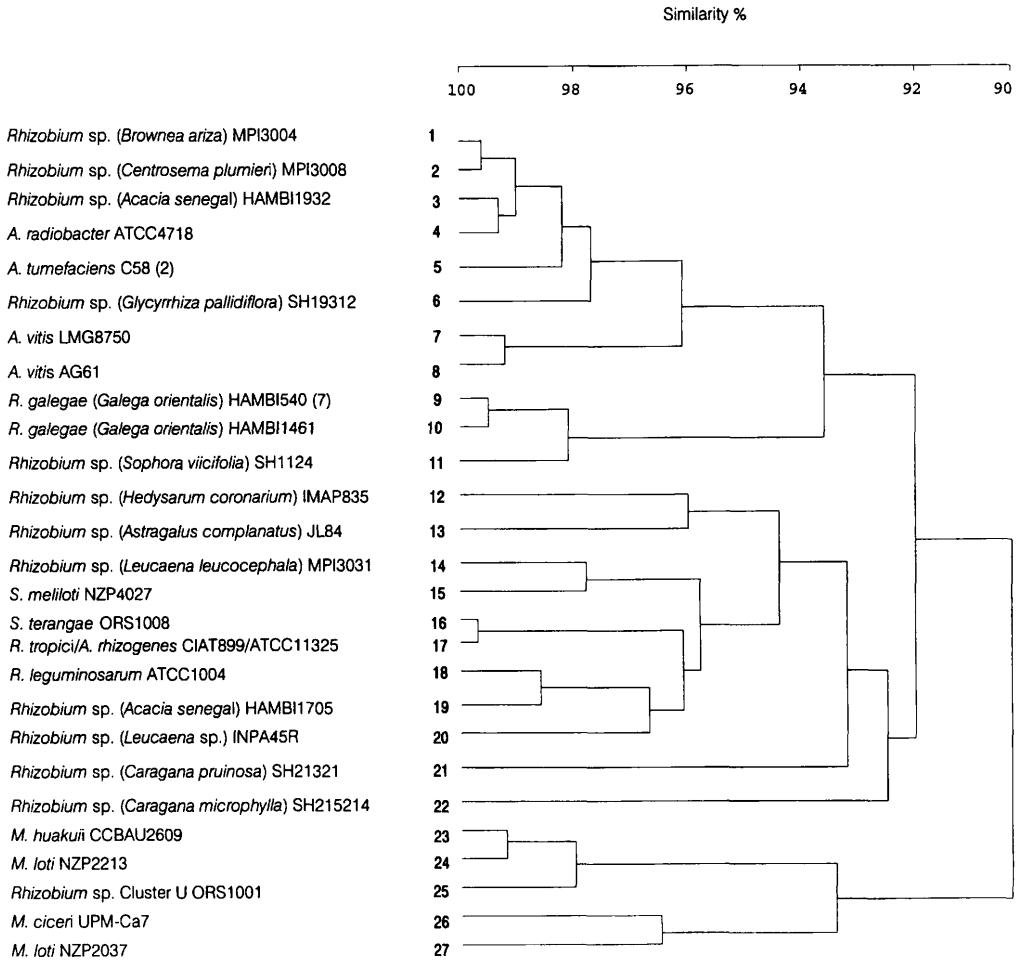

Fig. 1. UPGMA dendrogram generated from RFLP analysis of PCR-amplified 16S rDNA. The distinct genotypes are numbered in order of their appearance. Numbers in parentheses indicate the number of strains that showed the same restriction pattern (genotype).

amplified products was close to that of the full size. From restriction patterns of the 16S gene we identified 27 different genotypes (Table 1). The clustering of the strains in the dendrogram constructed from RFLP analysis of the 16S rRNA (Fig. 1) was in agreement with previously reported trees based on whole 16S sequences (29, 33). *R. galegae* (genotypes 9 and 10) formed its own group together with strain SH1124 (genotype 11). The group closest to *R. galegae* and SH1124 consisted of *Agrobacterium* and some unclassified strains. The dendrogram confirmed that the *Mesorhizobium* strains are quite distinct from the others. The strains that were isolated from *Caragana* sp. (genotypes 21 and 22), *Hedysarum coronarium* (genotype 12) and *Astragalus complanatus* (genotype 13) grouped with the *Rhizobium* and *Sinorhizobium* strains, but might represent new species.

Our results from partial (800 bp) sequence analysis of the 16S rRNA gene (Fig. 2) were in agreement with those obtained by PCR-RFLP. The taxonomically unclassified strain from *Caragana pruinosa* grouped with *Sinorhizobium*, and the strain from *Caragana microphylla* was by itself. Strain SH1124 from *Sophora viicifolia* grouped with *R. galegae* as expected.

The SSU gene has turned out to be a very good tool for the assessment of organismal phylogenies down to the genus level. In this study we also wanted to evaluate the use of LSU gene analysis for phylogenetic studies and to challenge the SSU phylogenies by using another phylogenetic marker gene for genus assessment. Since there is still very little sequence data available for LSU genes, we decided to apply PCR-RFLP analysis to the LSU genes of *R. galegae* and reference organisms. Because the 5′ end of the 23S rRNA genes contains highly variable extra stem–loop structures in which cleavage and fragmentation of 23S rRNA occur (2, 5, 6, 8), often referred to as IVSs (intervening sequences) or B8 loop structures, we designed the PCR primers for LSU so that this hypervariable region was excluded from the amplified fragment. The 23S rDNA amplification produced a 2·3 kb fragment and no polymorphism was observed. The sum of the sizes of the digested fragments of the amplified products was close to the full size. Out of all the 23S gene sequences

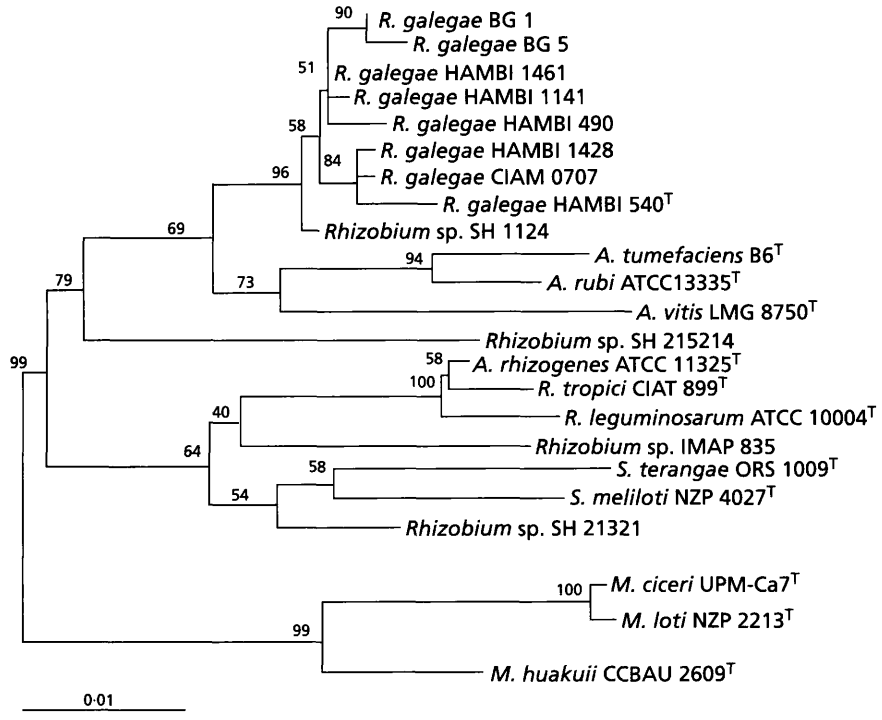

Fig. 2. Phylogenetic tree of rhizobia based on partial sequence analysis of the 16S rRNA gene, constructed using the neighbour-joining method in CLUSTAL W.

retrieved from the EMBL database, the 23S sequence of *Agrobacterium vitis* was aligned with 95% similarity to *R. galegae* HAMBI 540 sequence (data not presented). This confirmed that we had been using the 23S gene. A total of 32 genotypes were obtained from the RFLP analysis of the 23S gene, and the dendrogram constructed from RFLP analysis of this gene also placed *R. galegae* (genotypes 20, 21 and 22) in its own group (Fig. 3), some unclassified strains and the other rhizobia being its closest relatives. In the tree constructed from the 23S rRNA gene data, however, the recognized rhizobial species were further apart from the agrobacteria. It also shows increased separation between the mesorhizobia and the other groups when compared with the 16S rDNA value. The 23S dendrogram showed deeper branching than the 16S dendrogram, and more genotypes were resolved, although in some cases the sequence divergence is not particularly high. *R. tropici* and *Agrobacterium rhizogenes*, which were identical in the 16S analysis, were resolved in the 23S analysis, and a 2% sequence divergence was observed. Thus, the arrangement of the strains into clusters or groups in the two dendrograms were partly in good agreement with each other. The *R. galegae* strains, for instance, form their own group in both cases. However, some strains have changed their positions. For instance, the distance between *Rhizobium* sp. strain SH1124 (genotype 11 in the 16S dendrogram) and the *R. galegae* group was larger, although it is still in the same cluster as *R. galegae* (genotype 27 in the 23S dendrogram).

When the distance matrices for both genes were combined, 36 genotypes were identified and the relationships shown in Fig. 4 were observed. The *R. galegae* strains were placed in their own group with *Rhizobium* sp. SH1124 as their closest relative. The mesorhizobia maintained their own distinct group. The *Sinorhizobium* group included the unnamed rhizobial strains: MPI3031 from *Leucaena leucocephala* (genotype 24), IMAP835 from *H. coronarium* (genotype 26), JL84 from *Astragalus complanatus* (genotype 27), SH21321 from *Caragana pruinosa* (genotype 28) and INPA 45R from *Leucaena* sp. (genotype 30). SH215214 from *Caragana microphylla* (genotype 31) was again only distantly related to the other strains. The agrobacteria, the unclassified or unnamed strains and some rhizobia formed an intermingled cluster. *R. tropici* (genotype 15), *Agrobacterium rhizogenes* (genotype 16) and an unclassified *Rhizobium* sp. HAMBI 1705 from *Acacia senegal* (genotype 18) grouped with *R. leguminosarum* (genotype 17).

The amplified fragments of the IGS varied in size as expected, but in some strains multiple PCR products of varying size were observed, and thus were not used for further analysis.

DISCUSSION

Using PCR-RFLP analysis of 16S and 23S ribosomal genes and partial sequencing of the 16S gene, we were able to show that *R. galegae* is phylogenetically distinct from other rhizobia and agrobacteria. The relatedness of strain SH1124 from *Sophora viciifolia* to *R. galegae* indicates that relatives of this species exist in addition to *Rhizobium* sp. strain OK55, a strain which is not available from the authors, an isolate from *Sesbania aculeata* reported by Sawada *et al.* (24). Additional

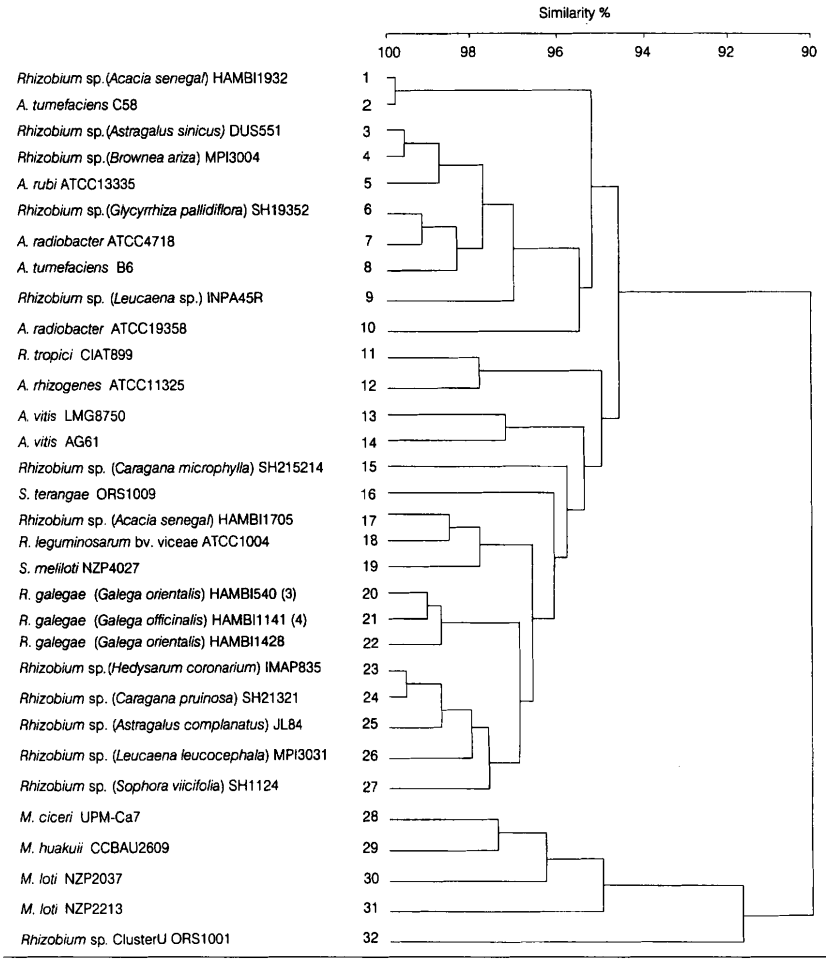

Fig. 3. UPGMA dendrogram generated from RFLP analysis of PCR-amplified 23S rDNA. The distinct genotypes are numbered in order of their appearance. Numbers in parentheses indicate the number of strains with the same genotype.

related strains might be recovered in the future, when more rhizobial biodiversity resources are explored.

Our results contradict those of Young *et al.* (32) and Nour *et al.* (21), who sequenced 260 bp of the 16S gene and analysed the 16S-IGS region, respectively, and claimed that *R. galegae* is closely related to mesorhizobia. Our 16S data confirms previous phylogenies based on sequencing of the full length of the 16S gene (29, 33). We conclude that the stretch of 800 bp used by us is sufficient to cover the variation observed within groups of root-nodule bacteria, whereas 260 bp is not. The longer fragment also contains enough conserved regions to avoid errors introduced by random noise. An interesting fact remains though, that part of the 16S gene and the IGS region of *R. galegae* have features in common with the corresponding regions of mesorhizobial ribosomal operons (4, 21, 32). A recombinational event during the evolution of these genera cannot be excluded. By comparing patterns of 16S nucleotide sequence polymorphism in members of both *Aeromonadaceae* and the *Rhizobiaceae*, Eardly *et al.* (4) have shown results that demonstrate lateral transfer and recombination of these genes among species of the same genus. Another reason for the discrepancy between the 16S and 16S-IGS results might be explained by our observation that in many strains the IGS existed in multiple, variable copies, which rendered this region unsuitable for PCR-RFLP analysis. Amplification of rDNA spacer regions from conserved sequences in the flanking 16S and 23S genetic loci not only generates double-stranded intergenic spacers but also results in the appearance of both single-stranded structures and heteroduplex structures containing substantial single-stranded regions(11).

By including eight strains of *R. galegae* instead of just one, which has been the case in most phylogenetic studies, we believe that we have obtained more robust, reliable and representative results than previous studies. According to Young & Haukka (33), *R. galegae* is not closely related to typical members of the genus *Rhizobium*, but not close enough to *Agrobacterium* to be transferred to that genus, either, and our 16S data support this view. A complementary approach was taken by Jarvis *et al.* (9), who analysed total cellular fatty acids from rhizobial and agrobacterial species. The *R. galegae* strains used formed a coherent group, which was neither *Rhizobium* nor *Agrobacterium* but close to both. By analysing the 23S gene we were hoping to shed more light on this problem. Ludwig *et al.* (17) claimed that phylogenies based on 16S and 23S genes are virtually the same, at least when comparing distantly related genera, even

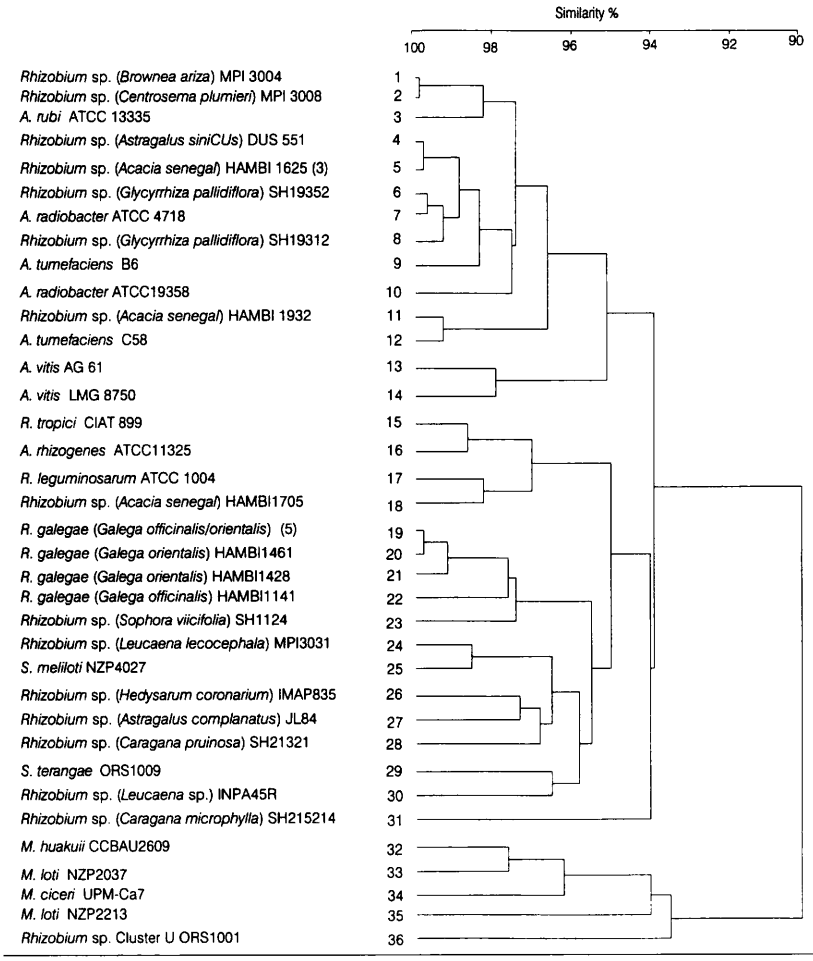

Fig. 4. UPGMA dendrogram constructed from the combined distance matrices obtained from RFLP analyses of 16S and 23S rDNAs. The distinct genotypes are numbered in order of their appearance. Numbers in parentheses indicate the number of strains that show the same restriction pattern for both genes.

though the 23S gene contains more information and thus might give deeper branching and better resolution. Our study applied 23S rDNA analysis to a large group of phylogenetically closely related bacteria. The results largely confirmed the 16S results, but there were some significant differences. Whereas the *Mesorhizobium* phylogenetic branch remained distinct from the *Agrobacterium*, *Rhizobium* and *Sinorhizobium* branches, the latter three were less distinct from each other than in the 16S analysis. Some strains switched branch between the two dendrograms, indicating that their ribosomal regions have a mosaic structure. This suggests that recombination has taken place during their evolution and supports the view of Eardly *et al.* (4) that gene exchange has shaped the ribosomal genes in rhizobia. Also, the phylogenetic position of the *R. galegae* cluster is affected by the type of gene under study. In 16S analysis, *R. galegae* formed a sub-group on the *Agrobacterium* branch, but in the 23S analysis they are part of the *Rhizobium* branch. The dendrogram obtained by combining data from the two data sets gives the mean of the two and might serve as the basis for further discussions on rhizobial and agrobacterial genera. As more sequence data for the 23S gene becomes available, our RFLP results can be verified by full-length sequence analysis.

Because of the mechanisms of evolution, the 16S paradigm might not always apply to closely related species, and the use of additional marker genes will then be useful for genus assignment. Therefore, until more information is available, we propose that *R. galegae* remains in the genus *Rhizobium*.

ACKNOWLEDGEMENTS

This work was funded by a grant from the University of Helsinki to K.L. and from the Maj and Tor Nessling foundation to S.S. We thank Wenxin Chen, Entao Wang, Philippe de Lajudie, Monique Gillis and Fatima Moreira for providing strains.

REFERENCES

1. **Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987).** *Current Protocols in Molecular Biology*. New York: Wiley.
2. **Burgin, A. B., Parodos, K., Lane, D. J. & Pace, N. R. (1990).** The excision of intervening sequences from *Salmonella* 23S ribosomal RNA. *Cell* **60**, 405–414.
3. **de Lajudie, P., Willems, A., Pot, B., Dewettinck, M. D., Dreyfus, B., Kersters, K. & Gillis, M. (1994).** Polyphasic taxonomy of rhizobia: emendation of the genus *Sino-*

rhizobium and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. *Int J Syst Bacteriol* **44**, 715.

4. **Eardly, B. D., Wang, F.-S. & van Berkum, P. (1996).** Corresponding 16S rRNA segments in *Rhizobiaceae* and *Aeromonas* yield discordant phylogenies. *Plant Soil* **186**, 69–74.
5. **Euvgenieva-Hackelberg, E. & Selenska-Pobell, S. (1995).** Variability of the 5′ end of the large subunit rDNA and the presence of a new short class of rRNA in the family of *Rhizobiaceae*. *Lett Appl Microbiol* **21**, 405–406.
6. **Gutell, R. R., Gray, M. W. & Schnare, M. (1993).** A compilation of large subunit (23S and 23S-like) ribosomal RNA structures. *Nucleic Acids Res* **21**, 3055–3074.
7. **Van Camp, G., Chapelle, S. & De Wachter, R. (1993).** Amplification and sequencing of variable regions in bacterial 23S ribosomal RNA genes with conserved primer sequences. *Curr Microbiol* **27**, 147–151.
8. **Hsu, D., Zee, Y. C., Ingraham, J. & Shih, L.-M. (1992).** Diversity of cleavage patterns of *Salmonella* 23S rRNA. *J Gen Microbiol* **138**, 199–203.
9. **Jarvis, B. D., Downer, H. L. & Young, J. P. W. (1992).** Phylogeny of fast-growing soybean- nodulating rhizobia supports synonymy of *Sinorhizobium* and *Rhizobium* and assignment to *Rhizobium fredii*. *Int J Syst Bacteriol* **42**, 93–96.
10. **Jarvis, B. D. W., Sivakumaran, S., Tighe, S. W. & Gillis, M. (1996).** Identification of *Agrobacterium* and *Rhizobium* species based on cellular fatty acid composition. *Plant Soil* **184**, 143–158.
11. **Jensen, M. A. & Straus, N. (1993).** Effect of PCR conditions on the formation of heteroduplex and single-stranded DNA products in the amplification of ribosomal DNA spacer regions. *PCR Methods Appl* **3**, 186–194.
12. **Jensen, M. A., Webster, J. A. & Straus, N. (1993).** Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl Environ Microbiol* **59**, 945–952.
13. **Laguerre, G., Allard, M. R., Revoy, F. & Amarger, N. (1994).** Rapid identification of rhizobia by restriction fragment polymorphism analysis of PCR-amplified 16S rRNA genes. *Appl Environ Microbiol* **60**, 56–63.
14. **Lindström, K., Sarsa, L. M., Polkunen, J. & Kansanen, P. (1985).** Symbiotic nitrogen fixation of *Rhizobium* sp. (*Galega*) in acid soils, and its survival in soil under acid and cold stress. *Plant Soil* **87**, 293–302.
15. **Lindström, K. (1989).** *Rhizobium galegae*, a new species of legume root nodule bacteria. *Int J Syst Bacteriol* **39**, 365–367.
16. **Lindström, K., Van Berkum, P., Gillis, M., Martinez, E., Novikova, N. & Jarvis, B. (1995).** Report from the roundtable on *Rhizobium* taxonomy. In *Nitrogen Fixation: Fundamentals and Applications*, pp. 807–810. Edited by I. A. Tikhonovich, N. A. Provorov, V. I. Romanov & W. E. Newton. Dordrecht, The Netherlands: Kluwer.
17. **Ludwig, W. & Schleifer, K. H. (1994).** Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiol Rev* **15**, 155–173.
18. **Nei, M. & Li, W. H. (1979).** Mathematical model for studying genetic variations in terms of restriction endonucleases. *Proc Natl Acad Sci USA* **76**, 5269– 5273.
19. **Nick, G. & Lindström, K. (1994).** Use of repetitive sequences and the polymerase chain reaction to fingerprint the genomic DNA of *Rhizobium galegae* strains and to identify the DNA obtained by sonicating the liquid cultures and root nodules. *Syst Appl Microbiol* **17**, 265–273.
20. **Nick, G. (1997).** Unpublished.
21. **Nour, S. M., Fernandez, M. P., Normand, P. & Clayet-Marel, J.-C. (1994).** *Rhizobium ciceri* sp. nov., consisting strains that nodulate chickpeas (*Cicer artenium* L.). *Int J Syst Bacteriol* **44**, 511–522.
22. **Normand, P., Cournoyer, B., Nazaret, S. & Simonet, P. (1992).** Analysis of a ribosomal operon in the actinomycete *Frankia*. *Gene* **111**, 119–124.
23. **Ridell, J., Siitonen, A., Paulin, L., Lindroos, O., Korkeala, H. & Albert, J. M. (1995).** Characterization of *Hafnia alvei* by biochemical tests, random amplified polymorphic DNA PCR, and partial sequencing of 16S rRNA gene. *J Clin Microbiol* **33**, 2372–2376.
24. **Sawada, H., Oyaizu, H. & Matsumoto, S. (1993).** Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. *Int J Syst Bacteriol* **43**, 694–702.
25. **Selenska-Pobell, S., Gigova, L. & Petrova, N. (1995).** Strain-specific fingerprints of *Rhizobium galegae* generated by PCR with arbitrary and repetitive primers. *J Appl Bacteriol* **79**, 425–431.
26. **Stone, B. B., Wietupski, R. M., Breton, G. L. & Wiesburg, W. G. (1995).** Comparison of *Mycobacterium* 23S rRNA sequences by high-temperature reverse transcription and PCR. *Int J Syst Bacteriol* **45**, 811–819.
27. **Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
28. **Wiesburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991).** 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.
29. **Willems, A. & Collins, M. D. (1993).** Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. *Int J Syst Bacteriol* **43**, 305–313.
30. **Woese, C. R. (1987).** Bacterial evolution. *Microbiol Rev* **51**, 221–271.
31. **Yanagi, M. & Yamasato, K. (1993).** Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16S rRNA genes using PCR and DNA sequencer. *FEMS Microbiol Lett* **107**, 115–120.
32. **Young, J. P. W., Downer, L. H. & Eardly, B. D. (1991).** Phylogeny of the phototropic *Rhizobium* strain BTAil by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. *J Bacteriol* **173**, 2271–2277.
33. **Young, J. P. W. & Haukka, K. (1996).** Diversity and phylogeny of rhizobia. *New Phytol* **133**, 87–94.