Nodulation of *Sesbania* species by *Rhizobium* (*Agrobacterium*) strain IRBG74 and other rhizobia

Stephen P. Cummings,1† Prasad Gyaneshwar,2† Pablo Vinuesa,3 Frank T. Farruggia,4 Mitchell Andrews,5 David Humphry,6 Geoffrey N. Elliott,2 Andrew Nelson,1 Caroline Orr,1 Deborah Pettitt,1 Gopit R. Shah,2 Scott R. Santos,8 Hari B. Krishnan,9 David Odee,10 Fatima M. S. Moreira,11 Janet I. Sprent,12 J. Peter W. Young6 and Euan K. James13*

1School of Applied Sciences, Ellison Building, University of Northumbria, Newcastle-upon-Tyne NE1 8ST, UK.
2Biological Sciences, University of Wisconsin Milwaukee, 3209 N Maryland Ave, Milwaukee, WI 53211, USA.
3Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, AP 565 A, Cuernavaca, Morelos, México.
4School of Life Sciences, Arizona State University, PO Box 874601, Tempe, AZ 85287-4601, USA.
5School of Sciences, University of Sunderland, Sunderland SR1 3SD, UK.
6Department of Biology, University of York, PO Box 373, York YO10 5YW, UK.
7Macaulay Institute, Craigiebuckler, Aberdeen, AB15 8QH, UK.
8Department of Biological Sciences and Cell and Molecular Biosciences Peak Program, Auburn University, 101 Life Science Building, Auburn, AL 36849, USA.
9Plant Genetics Research Unit, USDA-ARS, 108W Curtis Hall, University of Missouri, Columbia, MO 65211, USA.
10Kenya Forestry Research Institute, PO Box 20412–00200, Nairobi, Kenya.
11Departamento de Ciência do Solo, Universidade Federal de Lavras, Caixa Postal 3037, Lavras, MG, CEP 37 200-000, Brazil.
12College of Life Sciences, University of Dundee, Dundee DD1 SEH, UK.
13Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK.

Summary

Concatenated sequence analysis with 16S rRNA, *rpoB* and *fusA* genes identified a bacterial strain (IRBG74) isolated from root nodules of the aquatic legume *Sesbania cannabina* as a close relative of the plant pathogen *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*). However, DNA:DNA hybridization with *R. radiobacter*, *R. rubi*, *R. vitis* and *R. huautlense* gave only 44%, 5%, 8% and 8% similarity respectively, suggesting that IRBG74 is potentially a new species. Additionally, it contained no *vir* genes and lacked tumour-forming ability, but harboured a *sym*-plasmid containing *nifH* and *nodA* genes similar to those in other *Sesbania* symbionts. Indeed, IRBG74 effectively nodulated *S. cannabina* and seven other *Sesbania* spp. that nodulate with *Ensifer* (*Sinorhizobium*)/*Rhizobium* strains with similar *nodA* genes to IRBG74, but not species that nodulate with *Azorhizobium* or *Mesorhizobium*. Light and electron microscopy revealed that IRBG74 infected *Sesbania* spp. via lateral root junctions under flooded conditions, but via root hairs under non-flooded conditions. Thus, IRBG74 is the first confirmed legume-nodulating symbiont from the *Rhizobium* (*Agrobacterium*) clade. Cross-inoculation studies with various *Sesbania* symbionts showed that *S. cannabina* could form fully effective symbioses with strains in the genera *Rhizobium* and *Ensifer*, only ineffective ones with *Azorhizobium* strains, and either partially effective (*Mesorhizobium huakii*) or ineffective (*Mesorhizobium plurifarium*) symbioses with *Mesorhizobium*. These data are discussed in terms of the molecular phylogeny of *Sesbania* and its symbionts.

Introduction

*Sesbania* is a genus of approximately 60 species of tropical legume of which 40 have so far been reported to nodulate (Sprent, 2001). Many species occur naturally in wet or flooded soils and these have considerable potential as green manure in wetland rice production due to their ability to fix large quantities of N₂ (James et al., 2001 and references therein). *Sesbania* nodules may be induced by a variety of rhizobia, including *Azorhizobium* spp. (Dreyfus et al., 1988; Gonçalves and Moreira, 2004;
common bean. Tan and colleagues (2001) detected a
nifH gene in
Ensifer spp. (De Lajudie et al., 1994; 1998; Boivin et al., 1997; Chen and Lee, 2001; Sharma et al., 2005), Mesorhizobium spp. (McInroy et al., 1999; Bala et al., 2002; Odee et al., 2002; Vinuesa et al., 2005) and Rhizobium spp. (Rana and Krishnan, 1995; Wang et al., 1998; Wang and Martinez-Romero, 2000; Chen and Lee, 2001; Bala et al., 2002; Sharma et al., 2005; Vinuesa et al., 2005). In addition to the ‘standard’ rhizobial types, strains of the genus Agrobacterium (which are now, rather controversially, included in the genus Rhizobium; Young et al., 2001; Farrand et al., 2003) have also been isolated frequently from Sesbania nodules (Tan et al., 2001; Bala et al., 2002; Odee et al., 2002). The present study concerns one of these, Rhizobium strain IRBG74, isolated from root nodules of Sesbania cannabina (Retz.) Pers. (Biswas et al., 2000). This strain has been used to promote the growth of rice, as one of a group of plant growth-promoting rhizobacteria (Biswas et al., 2000). On the basis of its 16S rRNA gene sequence, it is most closely related to the plant pathogen Rhizobium radiobacter (syn Agrobacterium tumefaciens) (Tan et al., 2001). Biswas and colleagues (2000) tagged IRBG74, isolated from rice roots, with a gusA reporter gene and found that it not only expressed gus activity on plates containing X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid), but could also nodulate S. cannabina. Although plant growth was strongly promoted, both acetylene reduction and 15N isotope dilution assays found no activity associated with rice roots (Biswas et al., 2000). On the other hand, using both PCR and Southern hybridization, Tan and colleagues (2001) detected a nifH gene in IRBG74, one of the components required for nitrogenase production. Although some rhizobia occasionally express nitrogenase activity in culture, only the genus Azorhizobium (Dreyfus et al., 1988; Moreira et al., 2006) and some nodulating species of Burkholderia (Elliott et al., 2007) can grow on the products of nitrogen fixed ex planta. There are many reports of isolation of agrobacteria from nodules, but few have established whether or not these strains are capable of inducing nodules, and even if they can, whether or not the nodules are effective in fixing nitrogen.

The species R. radiobacter (Young et al., 2001) now includes the two former agrobacteria, Agrobacterium radiobacter (avirulent) and A. tumefaciens (tumour forming) (Willems, 2006). Although in the laboratory sym genes have been mobilized into R. radiobacter and the recipient strain was able to form N2-fixing nodules on common bean (Phaseolus vulgaris L) (Martinez et al., 1987), there is little published evidence that any Agrobacterium or Agrobacterium-like strains can fix nitrogen in the wild, even if they induce nodulation (De Lajudie et al., 1999; Mhamdi et al., 2005; Wang et al., 2006). Arguably the best evidence prior to the present study comes from recent work on two strains of the causal agent of hairy root disease, Rhizobium rhizogenes (syn. Agrobacterium rhizogenes), ATCC11325 and 163C, isolated from tumours on apple (Malus domestica Borkh.) and Prunus persica (L) Batsch respectively. These strains have been shown to harbour both sym and vir genes (Velázquez et al., 2005). Moreover, the sym plasmid in both cases had nodD and nifH genes that were phylogenetically close to known bean symbionts, and the strains were able to produce tumours/hairy roots on tomato or nodules on common bean. However, although the bean plants accumulated significantly more N than uninoculated control plants without added N, the total N accumulated was half that of plants inoculated with Rhizobium etli CFN42, and there was no confirmation via either acetylene reduction assays (ARA) of nitrogenase activity or 15N-based techniques that symbiotic N2 fixation was responsible for this N accumulation (Velázquez et al., 2005).

In this context, the aims of the present study were to clarify the phylogenetic position of strain IRBG74 within the genus Rhizobium and to obtain further evidence on its symbiotic properties with S. cannabina and other hydrophytic Sesbania spp. Because IRBG74 is only one of many bacteria that can nodulate species of Sesbania, we also compared its nifH and nodA genes with those of the four rhizobial genera known to nodulate Sesbania spp. from Africa, Asia and South America [i.e. Azorhizobium, Ensifer (Sinorhizobium), Mesorhizobium and Rhizobium]. Finally, we compared the ability of all these bacteria to nodulate S. cannabina with that of IRBG74. The symbiotic properties (i.e. host range and nodA sequences) of IRBG74 and the other Sesbania symbionts are discussed in the context of a molecular phylogeny of Sesbania.

Results

Phylogenetic analysis of strain IRBG74 and the other Sesbania-nodulating strains

The 16S rRNA gene sequence was used in initial phylogenetic analyses to compare the four available sequences of IRBG74 with the most similar homologues derived from the NCBI database attributable to validly described species. The inferred tree showed that all four IRBG74 sequences grouped with a low bootstrap value (61%) in a clade with R. radiobacter NCIMB 9042 and NCIMB 13307 (Fig. 1), the type strains of Agrobacterium radiobacter and A. tumefaciens respectively. To explore these phylogenetic relationships further, two housekeeping genes, fusA and rpoB, were included in a concatenated analysis of nine rhizobial strains for which the three sequences (i.e. 16S rRNA, fusA, rpoB) were available. These nine strains included three of R. radiobacter
From the inferred tree, IRBG74 clustered with a high bootstrap value (89%) with these three strains (Fig. S1), congruent with the 16S rRNA sequence analysis. The similarity between the 16S rRNA gene sequence of IRBG74 and that of the *R. radiobacter* strains was 99%, suggesting that they may be conspecific. To resolve this, DNA:DNA hybridization studies were carried out against strains of *R. radiobacter* (the type strain, NCIMB 9042, NCIMB 13307 and NCIMB 4034) plus the type strains of closely related organisms identified by the analysis using the three housekeeping genes (16S rRNA, *fusA*, *rpoB*).

The degree of hybridization of IRBG74 to the *R. radiobacter* strains NCIMB 13307 and NCIMB 4034 was 57% and 44% respectively. Against type strains of *R. rubi*, *R. vitis* and *R. huautlense*, hybridizations were much lower (8%, 5% and 8% respectively). None of these values approached the upper threshold value (70%) for the definition of a bacterial species (Wayne et al., 1987). Moreover, strain IRBG74 did not form tumours on tobacco, and no *virD2* or *ipt* gene homologues could be amplified by PCR, thus indicating the absence of the Ti plasmid. Therefore, these data support IRBG74 being placed within a species distinct from the other former *Agrobacterium* species now housed in the genus *Rhizobium* (Young et al., 2001).

During the course of this work we isolated an additional strain, DUS1110, from *Sesbania exasperata* Kunth nodules collected during the study of James and colleagues (2001) (Table 1), and its 16S rRNA sequence (not shown) suggested that it was potentially related to *R. huautlense*, a 'water Rhizobium' species (Wang and Martínez-Romero, 2000) that has frequently been isolated from *Sesbania* nodules. It is represented in the present study by strains Ss121 and Se127 isolated, respectively, from nodules of *Sesbania sericea* (Willd.) Link and *S. exasperata* growing in seasonally flooded regions of Venezuela (Vinuesa et al., 2005). The 16S rRNA sequence from another *Rhizobium* strain, SIN-1, originally isolated from *S. bispinosa* (Jacq.) W.F. Wight nodules by Rana and Krishnan (1995), suggested that it was also closely related to *R. huautlense* (Fig. 1). All the
other strains used in the following sections have been previously characterized using their 16S rRNA sequences and, in some cases, via sequences of other genes (see references in Table 1).

### Characterization of symbiosis-related genes of IRBG74 and other Sesbania microsymbionts

The presence of the nifH and nodA genes within the genome of IRBG74 was confirmed using specific PCR primers. Attempts were made to amplify the nifH and nodA genes of all the strains listed in Table 1, except for A. caulinodans ORS571 and Rhizobium sp. SIN-1, which were already available in the GenBank database. With the exception of the nifH gene of R. huautlense Se127 and the nodA gene from Mesorhizobium huakuii KFR647, amplified products of both genes were obtained for all strains. Amplicons were cloned, sequenced and utilized in GenBank database searches via BLASTN.

The phylogenetic analysis of the nifH sequence showed that IRBG74 nested in a cluster, with high bootstrap support (97%), containing a number of *Ensifer* isolates, but was most similar to *Ensifer saheli* ORS609\(^\text{L}\) (Fig. S2A), which was also originally isolated from *S. cannabina* (Boivin et al., 1997). Also in this cluster were a number of other recognized type species including *Ensifer kostiense* and *Ensifer teranga*, as well as a sequence derived from the *Sesbania* strains DUS1110, Ss121 and SIN-1, all putatively identified as *Rhizobium* spp. (Vinuesa et al., 2005; this study). In contrast, strains KFR647 and Sp45, previously identified as *Mesorhizobium* spp. by Ode and colleagues (2002) and Vinuesa and colleagues (2005) respectively, produced sequences that clustered with a number of *Mesorhizobium* sequences (Fig. S2A) and were clearly distinct from the *Ensifer* cluster. The nifH sequences of the two *Azorhizobium* strains, *A. caulinodans* ORS571 (Lee et al., 2008) and *A. doebereinerae* Br5401 (accession number FJ223129), were 95% similar to each other, but they did not cluster with the other *Sesbania*-nodulating rhizobial strains (not shown).

The nodA sequences of IRBG74 and the *Sesbania*-nodulating *Rhizobium* strains Ss121, SIN-1, DUS1110 and Se127 clustered together, consistent with the nifH phylogeny, and also formed a group with a high bootstrap support (100%) with *E. saheli* ORS609 and several other nodA sequences derived from *Ensifer* strains isolated from *Sesbania* (Fig. S2B). The nodA sequence of Sp45 clustered with other mesorhizobial sequences (data not shown), reinforcing the findings from the nifH analysis that this organism and its sym genes are mesorhizobial in origin. *Mesorhizobium huakuii* strain KFR647, however, did not produce a PCR product for nodA despite nodulating *S. cannabina* and its original host, *S. sesban* (L) Merr. (Table 3, Fig. S4D). It did, however, produce a PCR product for nodD (GenBank accession number FJ514244), which was closest to other mesorhizobial strains, particularly *Mesorhizobium loti*. In the case of the azorhizobia, the nodA sequence of *A. doebereinerae* Br5401 (GenBank accession number FJ223128) was 95% similar to that of *A. caulinodans* ORS571 (Lee et al., 2008), and neither of the *Azorhizobium* sequences clustered with any nodA sequences from the other known rhizobial genera.

### Plasmid profiles

The plasmid profile of IRBG74 (Fig. S3) was compared with that of the type strains of *E. saheli* ORS609, *R. radiobacter* NCIMB 13307 (formerly the type strain of *A. tumefaciens*), *R. radiobacter* NCIMB 9042 (current type strain of this species) and *Rhizobium* sp. SIN-1. Strain IRBG74, like *R. radiobacter* NCIMB 13307, had a single plasmid of approximately 700 kb. The nodA gene of IRBG74 hybridized with this plasmid and also with the plasmids derived from *E. saheli* ORS609 and *Rhizobium* sp. SIN-1, thus indicating that in IRBG74 it was, indeed, a symbiotic plasmid, and also that the nodA gene from this strain was very similar to that found in the other *Sesbania* symbionts, *E. saheli* ORS609 and *Rhizobium* sp. SIN-1.

---

Table 1. Rhizobial strains used in this study.

| Strain | Original host | Country of origin | Reference |
|--------|---------------|-------------------|-----------|
| Rhizobium (Agrobacterium) sp. IRBG74 | *S. cannabina* | Philippines | Tan et al. (2001) |
| Rhizobium (Agrobacterium) sp. IRBG74GUS | *S. cannabina* | Philippines | This study |
| Rhizobium sp. SIN-1 | *S. bispinosa* | India | Rana and Krishnan (1995) |
| Rhizobium sp. DUS1110 | *S. exasperata* | Brazil | This study |
| Rhizobium huautlense Se127 | *S. exasperata* | Venezuela | Vinuesa et al. (2005) |
| Rhizobium huautlense Ss121 | *S. sericea* | Venezuela | Vinuesa et al. (2005) |
| Azorhizobium caulifodans ORS571\(^\text{L}\) | *S. rostrata* | Senegal | Dreyfus et al. (1988) |
| Azorhizobium doebereinerae Br5401\(^{T}\) | *S. virgata* | Brazil | Moreira et al. (2006) |
| Mesorhizobium huakuii KFR647 | *S. sesban* | Kenya | McInroy et al. (1999) |
| Mesorhizobium plurifarium Sp45 | *S. punicea* | Venezuela | Vinuesa et al. (2005) |
| *Ensifer saheli* ORS609\(^{L}\) | *S. cannabina* | Senegal | Boivin et al. (1997) |

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*, 11, 2510–2525
Nodulation of Sesbania spp. and other legumes by IRBG74

Effective nodules on the roots were indicated by plants being green and healthy at 60 days after inoculation (dai), by significant acetylene reduction activity, and by microscopic examination of nodules. On this basis, strain IRBG74 effectively nodulated eight of the 13 Sesbania species tested (S. bispinosa, S. cannabina, S. exasperata, S. formosa (F. Muell.) N.T. Burb., S. grandiflora (L.) Pers., S. macrantha Welw. ex Phillips and Hutch., S. madagascariensis Du Puy and Labat and S. pachycarpa DC.), formed ineffective nodules on S. herbacea (Mill.) McVaugh, small, ineffective ‘bumps’ on the roots of S. rostrata Beremek. and Oberm., S. sesban and S. virgata (Cav.) Pers., and no outgrowths on S. punicea (Cav.) Benth. (Table S2, Fig. 2). No nodules were formed on either of the ‘promiscuous’ legumes, Macroptilium atropurpureum (Moc. and Sessé ex DC.) Urb. or Phaseolus vulgaris (Table S2). Effective nodules were large (up to...
and 15 dai and stained with X-Gluc to detect the location of the bacteria on the roots. All four species examined, i.e. *S. cannabina* (Fig. 3A), *S. bispinosa* (Fig. 3B), *S. rostrata* (Fig. 3C) and *S. sesban* (Fig. 3D), showed clear evidence of root colonization by IRBG74GUS by 7 dai with particularly intense staining at lateral root junctions (e.g. Fig. 3A, C and D). Staining was also intense in the root hair zone close to root tips, even on *S. bispinosa* (Fig. 3B), which is known to be infected via cracks at lateral root junctions (Rana and Krishnan, 1995). Both WT and GUS-tagged strains induced visible nodules on *S. cannabina* by 15 dai (Fig. 3E and F); the WT (control) strain showed no blue staining after treatment with X-Gluc (Fig. 3F). Functional nodules were also formed on *S. bispinosa* by IRBG74GUS (not shown), but only small ‘bumps’ were formed on *S. rostrata* and *S. sesban* by 15 dai (as described previously for the WT strain; Fig. 2G and H). Under non-flooded conditions, IRBG74 infected *S. cannabina* via root hairs (Fig. 4A and B), and nodule development thereafter was as described previously for other *Sesbania* spp., i.e. with the development of an apical meristem containing newly divided cells being penetrated by infection threads which ‘released’ bacteria into symbiosomes (Fig. 4C). These bacteria subsequently developed into bacteroids as the host cells expanded (Fig. 4C and D). Using an antibody raised against pea Lb (Fig. 4D), which had previously been shown to recognize Lb in *N.-fixing* tissue was, indeed, *Rhizobium* (*Agrobacterium*) sp. IRBG74 (e.g. *S. cannabina*; Fig. 2A and B).

Nodulation by IRBG74 on its original host, *S. cannabina*, grown under flooded conditions to simulate its natural wetland environment, was studied in more detail. Flooding greatly enhanced growth, nodulation and nitrogenase activity, giving two to three-fold increases in all parameters compared with non-flooded conditions (Table 2). Flooding also increased the nodulation and nitrogenase activity of *S. bispinosa*, but not its overall growth (as determined by plant dry weight). Indeed, *S. cannabina* was generally a much more robust plant than *S. bispinosa* under both flooded and non-flooded growth conditions (Table 2).

A more detailed study of the interaction between four *Sesbania* spp. and *Rhizobium* (*Agrobacterium*) sp. IRBG74 wild-type (WT) and glucuronidase (GUS)-tagged strains was carried out under both flooded (Fig. 3) and non-flooded conditions (Fig. 4). Plants were harvested at 7

### Table 2. Effect of flooding on growth, nodulation and nitrogenase (acetylene reduction activity, ARA) of *Sesbania cannabina* and *S. bispinosa* (syn. *S. aculeata*) at 30 days after inoculation with *Rhizobium* (*Agrobacterium*) sp. strain IRBG74.

|                | Plant dry weight (mg) | Nodule No. | Nodule dry weight (mg) | ARA (nmol C₂H₄ per plant h⁻¹) |
|----------------|-----------------------|------------|------------------------|-------------------------------|
| **S. cannabina** |                       |            |                        |                               |
| Non-flooded    | 81.4 ± 7.5            | 9 ± 2      | 4.6 ± 0.4              | 247.8 ± 51.2                  |
| Flooded        | 187.6 ± 9.2           | 24 ± 3     | 26.3 ± 1.7             | 755.6 ± 89.9                  |
| **S. bispinosa** |                       |            |                        |                               |
| Non-flooded    | 16.0 ± 2.3            | 4 ± 1      | 0.7 ± 0.1              | 41.2 ± 11.0                   |
| Flooded        | 18.8 ± 3.3            | 12 ± 1     | 2.9 ± 0.3              | 84.4 ± 11.2                   |

n = 6. Values are means ± SE.
4E and F). The two *Mesorhizobium* strains differed in their ability to nodulate *S. cannabina; M. huakuii* KFR647, which formed fully effective nodules on its original host, *S. sesban* (Fig. S4C), formed partially effective nodules on *S. cannabina* (Fig. S4D), while *Mesorhizobium plurifarium* Sp45, which can effectively nodulate *S. punicea* (Vinuesa et al., 2005), only formed ineffective nodules (small bumps similar to Fig. S4B). In contrast to the *Azorhizobium* and *Mesorhizobium* strains, all of the *Rhizobium-Agrobacterium-Sinorhizobium* strains produced effective nodules on *S. cannabina*, regardless of their geographical origins. They included *Rhizobium* sp. SIN-1 (Fig. S4E), which was isolated from *S. bispinosa* in India (and confirmed to nodulate it by Rana and Krishnan, 1995), and the two strains isolated from the South American wetland species, *S. exasperata, Rhizobium* sp. DUS1110 from Brazil (this study) and *R. huaetlense* Se127 from Venezuela (Vinuesa et al., 2005), both of which could also effectively nodulate their original host (e.g. DUS1110; Fig. S4F). The same was also true of the other *R. huaetlense* strain from Venezuela, Ss121 (not shown), which was originally isolated from *S. sericea* by Vinuesa and colleagues (2005). *Ensifer* (*Sinorhizobium*) saheli ORS609 from Senegal also effectively nodulated *S. cannabina*, as expected, because it was originally isolated from it (Boivin et al., 1997).

Using *S. cannabina* as a test host, a more detailed comparison of strain IRBG74 with other *Sesbania*-nodulating strains was performed. The bacteria selected were *A. doebereinerae* Br5401, *M. huakuii* KFR647, *Rhizobium* sp. DUS1110 and *E. saheli* ORS609T. IRBG74 was found to be as effective in nodulation, N2 fixation and plant growth promotion (dry weight accumulation) as the other *S. cannabina* strain, ORS609, but was slightly less effective in promoting the growth of *S. cannabina* than *Rhizobium* sp. DUS1110 (which, interestingly, had much lower nitrogenase activity than either IRBG74 or ORS609) (Table 3). Surprisingly, however, was the fact that *A. doebereinerae* strain Br5401, although it could not form effective N2-fixing nodules on *S. cannabina*, was capable of promoting dry matter accumulation to a level equal to that of the symbiotically effective strains (Table 3), thus suggesting that it has plant growth-promoting rhizobacterium
(PGPR) properties, although, it should be noted that at the time of harvest (30 days) the *S. cannabina* plants were showing symptoms of N-deficiency (i.e. yellowing of the leaves). The plants inoculated with *M. huakuii* KFR647 showed highly variable nodulation ranging from no nodules through root bumps to partially effective nodules (Fig. S4D), and the mean dry weight accumulation was not significantly different to uninoculated *S. cannabina* (Table 3).

**Discussion**

**Phylogeny of the core genome of Rhizobium (Agrobacterium) sp. IRBG74**

The primary objectives of this study were (1) to understand the phylogenetic relationship of the rice growth-promoting strain IRBG74 to validly published species of *Rhizobium* and *Agrobacterium* and (2) to confirm whether or not it is able to nodulate and fix N\(_2\) in association with

**Table 3.** Nodulation, nitrogenase activity and dry weight accumulation of *Sesbania cannabina* at 30 days after inoculation with rhizobial strains isolated from various *Sesbania* spp.

| Strain                | Original host | Number of nodules | ARA (μmol C\(_2\)H\(_4\) per plant h\(^{-1}\)) | Total dry weight (mg) |
|-----------------------|---------------|-------------------|-----------------------------------------------|-----------------------|
| *Rhizobium* (Agrobacterium) IRBG74 | *S. cannabina* | 43 ± 6            | 18.57 ± 6.77                                 | 293 ± 59*             |
| *Azorhizobium doebereinereae* Br5401\(^T\) | *S. virgata* | Several small bumps | 0                                              | 354 ± 32*             |
| *Mesorhizobium huakuii* KFR647 | *S. sesban*   | 7 ± 2             | 2.87 ± 1.23                                  | 153 ± 19              |
| *Rhizobium* sp. DUS1110 | *S. exasperata* | 55 ± 5       | 3.55 ± 0.32                                  | 441 ± 27*             |
| *Ensifer saheli* ORS609T | *S. cannabina* | 52 ± 5            | 16.14 ± 1.27                                 | 360 ± 32*             |
| Uninoculated           |               | 0                 | 0                                             | 130 ± 11              |

Values are means ± SE (n = 6) and those marked with * are significantly greater than the uninoculated plants at P < 0.01 using analysis of variance.

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*, 11, 2510–2525
Sesbania spp. Strain IRBG74 was originally isolated from nodules of the aquatic legume S. cannabina and classified on the basis of 16S rRNA sequence analysis as a member of the revised Rhizobium genus (Young et al., 2001), and the closest organisms on the basis of the 16S rRNA gene similarities were R. radiobacter strains NCIMB9402 and 13307 (Tan et al., 2001). Here, we repeated and extended the analyses of Tan and colleagues (2001) to include additional data (Fig. S1). A concatenated approach was deployed using two universally conserved protein-coding genes, rpoB and fusA, which are useful alternatives to 16S rRNA sequences in determining the relationship between both divergent and highly related lineages (Santos and Ochman, 2004). The phylogenetic tree derived from the concatenated sequence matrix under a maximum likelihood approach offered the opportunity to infer a phylogeny based on the total evidence, from which the underlying species tree could be estimated. This tree supports the phylogeny estimated from the 16S rRNA sequence, namely that strain IRBG74 clusters, with high bootstrap support (89%), with three validly described strains of R. radiobacter, including the type strain NCIMB9042. However, the inferred tree does not identify whether IRBG74 is a strain of R. radiobacter or a unique species. To accomplish this, DNA–DNA hybridization, which remains the ‘gold standard’ to differentiate between these alternatives, was used. Our data demonstrated that against closely related strains of the Rhizobium genus, the DNA–DNA similarity was greatest against two strains of R. radiobacter, NCIMB9042, and NCIMB4034 (a recently described strain that has been used as a PGPR in agricultural systems; Humphry et al., 2007), although neither of these strains is diazotrophic. Nevertheless, the similarity was significantly lower than the 70% threshold (Wayne et al., 1987) required for designating IRBG74 as a strain of R. radiobacter or, indeed, any of the other species examined. These observations therefore support the inclusion of IRBG74 as a novel species within the R. radiobacter–R. rubi clade, and, to our knowledge, is the first N2-fixing bacterium to be identified from among these species. However, these data should be interpreted with caution, because there are potential issues (which have yet to be resolved) in using DNA hybridization to circumscribe bacterial species in isolates with large accessory genomes (Young et al., 2006). Moreover, this study was on a single strain (i.e. IRBG74), and several strains should be studied before a robust species description can be developed.

Phylogeny of symbiosis-related genes and host range
Analyses of two genes essential for symbiotic N2 fixation, nirH and nodA, indicated that they had a distinct evolutionary history to the conserved 16S rRNA, fusA and rpoB ‘housekeeping’ loci. Alignment of the nirH and nodA sequences against homologous genes in GenBank revealed that both were most closely related to homologues present in members of the genus Ensifer, particularly those that had been isolated from Sesbania spp. The nirH gene of IRBG74 showed highest similarity to homologues from E. saheli strain ORS609 (also isolated from S. cannabina; De Lajudie et al., 1994; Boivin et al., 1997), whereas the nodA gene sequence was most similar to those of E. saheli bv. sessaniae strain ORS611 and E. terrangae bv. sessaniae (although it did also hybridize with the nodA gene from E. saheli ORS609). The other Rhizobium strains isolated from Sesbania nodules (Ss121, Se127, SIN-1 and DUS1110) also contained nirH and nodA genes that were very similar to those of the Ensifer symbionts, and a phylogenetic relationship between the nirH genes of Rhizobium symbionts of Sesbania and those of E. saheli was previously noted by Vinuesa and colleagues (2005). It therefore appears that although the core genome of IRBG74 is essentially ‘agrobacterial’, its accessory symbiosis-related genome is similar to that of a wide range of Rhizobium/Ensifer Sesbania symbionts.

Distinct and divergent evolutionary histories between symbiotic and housekeeping genes have been reported in other rhizobia (Vinuesa et al., 2005). This phenomenon is most likely explained by the high mobility of the plasmid-borne genes in Rhizobium and close relatives such as Ensifer (Turner et al., 2002; Baillie et al., 2007). In the case of Agrobacterium, this mobility has been exemplified in laboratory experiments in which strains have had rhizobial sym plasmids mobilized into them, and the genetically modified Agrobacterium strains thus formed have consequently shown a limited ability to nodulate legumes (Hirsch et al., 1985; Martínez et al., 1987; Van de Wiel et al., 1988). Interestingly, and in spite of the apparent ease with which Agrobacterium can acquire sym genes in the laboratory, fully effective N2-fixing agrobacterial symbioses with legumes had not been demonstrated and/or discovered (Mhamdi et al., 2005; Velázquez et al., 2005; Wang et al., 2006) until the present study of strain IRBG74, which is therefore the first confirmed naturally occurring agrobacterial symbiont of legumes to be fully described in both genetic and symbiotic terms.

Rhizobium strain IRBG74 showed a range of symbiotic phenotypes on the different Sesbania species tested, from no nodulation on S. punicea, through ineffective nodulation on S. herbacea, S. rostrata, S. sesban and S. virgata to effective nodulation on S. bispinosa, S. cannabina, S. exasperata, S. formosa, S. grandiflora, S. madagascariensis, S. macrantha and S. pachycarpa. There appears to be no obvious geographical link between those species that do nodulate and those that do not, other than that all of the Asian Sesbania spp. nodulated effectively. Indeed, our data suggest that symbioti-
cally effective N₂ fixation is observed only with those species that can also nodulate with *Rhizobium or Ensifer*. Host range in rhizobia is determined by 'decorations' on the structure of the lipoprotein oligosaccharide 'nod factors' transcribed by the nodulation genes, such as *nodA* (Sprent, 2001; Kobayashi and Broughton, 2008), and so it is likely that the host range of IRBG74 (and the other symbionts in this study) is reflected more in its *nodA* than its core genome phylogeny. The *nodA* phylogenies suggest that the *Rhizobium* isolates studied here have acquired their symbiosis-related genes by lateral gene transfer from an *Ensifer* sp. (Fig. 3B), with a potential candidate being *E. saheli*. In the case of IRBG74, this is further supported by the fact that the type strain of *E. saheli*, ORS609, which was originally isolated from *S. cannabina* (De Lajudie *et al.*, 1994), has a similar host range (e.g. it is capable of effectively nodulating many of the *Sesbania* species tested positive in the present study for symbiotic nodulation with IRBG74, such as *S. bispinosa*, *S. formosa*, *S. grandiflora* and *S. pachycarpa*; Boivin *et al.*, 1997). On the other hand, unlike ORS609 (Boivin *et al.*, 1997), strain IRBG74 was unable to form effective nodules with those *Sesbania* spp. that establish symbiotic partnerships with azorhizobia (*S. rostrata*, *S. virgata*; Table 3) and mesorhizobia (*S. punicea*, *S. sesban*), thus suggesting that although they share similarities in their *nodA* sequences, the *nod* genes of IRBG74 are not identical to those of ORS609.

On the plant side, the host of IRBG74, *S. cannabina*, has shown a clear preference for symbionts with *nodA* sequences in the *Ensifer-Rhizobium* clade, but it also has the ability to nodulate (albeit, only partially effectively) with mesorhizobial strains from *Sesbania*, such as *M. huakuii* KFR647. Unfortunately, the sequence of *nodA* from KFR647 could not be obtained, but its *nodD* gene sequence was different from the aforementioned *Ensifer-Rhizobium* clade, and the *nodA* sequence of *M. plurifarium* Sp45, which only forms ineffective nodules on *S. cannabina*, was also distant from this clade, thus lending support to the suggestion that *S. cannabina* has a preference for *Ensifer-Rhizobium* symbionts with plasmidborne symbiosys-related genes. On the other hand, *S. cannabina* may not be typical in this respect, as other studies of *Sesbania* have demonstrated that both mesorhizobial and (sino)rhizobial symbionts are found within the same species, e.g. in *S. sericea* (Vinuesa *et al.*, 2005) and *S. sesban* (Bala *et al.*, 2002; Sharma *et al.*, 2005). Of the two species that are known to nodulate with Azorhizobium, *S. rostrata* appears to be more capable of forming effective symbioses with other rhizobia (such as *E. saheli* and *E. terangae*; De Lajudie *et al.*, 1994; Boivin *et al.*, 1997), than *S. virgata*, which seems to be capable of forming effective symbioses only with *A. doebereineriae* (Gonçalves and Moreira, 2004; this study). *Sesbania punicea* also appears to be highly conservative in its choice of symbiont, as evidenced by the lack of nodulation with IRBG74 (this study) or even with the other mesorhizobial strain from *S. sesban*, *M. huakuii* KFR647 (E.K. James, unpubl. data), and the data so far obtained about this species suggest that it can nodulate only with *M. plurifarium* (Vinuesa *et al.*, 2005). Interestingly, the highly selective nature of *S. virgata* and *S. punicea* with regard to symbionts in comparison with other *Sesbania* spp. was illustrated by a rhizobial soil 'trapping' study in Brazil in which they were the only *Sesbania* spp. that failed to nodulate in uninoculated soils (Veasey *et al.*, 1997). In contrast, good nodulation was obtained with the relatively more promiscuous *S. exasperata*, *S. sesban* and *S. tetrapetra* Hochst. ex Baker.

**Infection and nodulation of *S. cannabina* and other *Sesbania* spp.**

Stem nodulation, or stem-borne lateral root base nodulation [as it should more correctly be called (Den Herder *et al.*, 2006)], in the genus *Sesbania* has so far only been confirmed in *S. rostrata* (Dreyfus and Dommergues, 1981). However, the crack entry infection process via adventitious roots that leads to the initial formation of aerial stem nodules on *S. rostrata* has also been observed in flooded roots of this species (Ndoye *et al.*, 1994; Goormachtig *et al.*, 2004) and *S. bispinosa* (Rana and Krishnan, 1995), and it also probably occurs on the other hydrophytic *Sesbania* spp. regardless of the type of rhizobial symbiont. The present study of *Rhizobium (Agrobacterium)* sp. IRBG74 suggests that a crack entry infection probably occurs with *S. cannabina* under flooded conditions, whereas under non-flooded conditions, as in *S. rostrata* (Goormachtig *et al.*, 2004), it nodulates via a 'standard' root hair infection pathway. It remains to be seen if the other *Sesbania* spp. in this study also switch from crack entry under flooded conditions to root hair infection under the non-flooded conditions described by Goormachtig and colleagues (2004) for *S. rostrata*. However, given that the subsequent nodule development and structure of N₂-fixing *Sesbania* nodules is so distinctive and uniform across the genus (this study, Harris *et al.*, 1949; Dreyfus and Dommergues, 1981; Ndoye *et al.*, 1994; Boivin *et al.*, 1997; James *et al.*, 2001), it is possible that all the hydrophytic species share common strategies in terms of rhizobial infection.

**Concluding remarks: is there a plant phylogenetical component to nodulation of *Sesbania* by different symbionts?**

This study confirms the earlier observations of Tan and colleagues (2001), using a number of techniques, that a
bacterium (IRBG74) isolated from nodules on the wetland legume *S. cannabina* is phylogenetically a strain of ‘Agrobacterium’, that it is not a phytopathogen, but a plant growth-promoting diazotroph. Although this is by no means the first report of a non-pathogenic *Agrobacterium* strain being isolated from nodules (e.g. Mhamdi et al., 2005; Wang et al., 2006), the fact that IRBG74 possesses a *sym*-plasmid with symbiosis-specific genes (e.g. *nodA*) and can effectively nodulate its original host and fix N2 to the benefit of the growth of the plant to the same degree as ‘conventional’ rhizobia, is a novel observation. Indeed, it could be argued that despite its core genome being that of *Agrobacterium* (Tan et al., 2001; this study), IRBG74 behaves in all respects (including its infection processes) like typical legume-nodulating rhizobia, and thus supports the recent decision to incorporate all *Agrobacterium* strains into the genus *Rhizobium* (Young et al., 2001). It is likely that as further rhizobia are isolated from more legumes, particularly those in the tropics, the incidence of potentially genuine symbiotic ‘Agrobacterium’ isolates will increase (e.g. see Bala et al., 2002).

This is also the first study to compare a nodulation gene (in this case, *nodA*) within a range of *Sesbania* symbionts. The *nodA* gene phylogeny, which groups the bacteria into three distinct clades (i.e. *Azorhizobium*, *Mesorhizobium* and *Rhizobium* [Agrobacterium]/*E. saheli*), also appears linked to these microsymbionts’ host range. Indeed, the present study, together with data from previous published work, have indicated three definable ‘groups’ of *Sesbania* spp. in terms of their propensity to nodulate with symbionts harbouring particular *nodA* gene types. Groups 1 and 2 consist of only one species each, i.e. the two South American species, *S. punicae* and *S. virgata*, which both have a high specificity for a very narrow range of symbionts (*M. plurifarium* and *A. dobereinerae* respectively) harbouring *nodA* sequences that are very different from each other and from the other *Sesbania* symbionts examined in the present study. Group 3, on the other hand, is a large group that contains several non-selective (‘promiscuous’) species that can nodulate with a wide range of symbionts in the *Rhizobium* [Agrobacterium]/*E. saheli* clade harbouring similar *nodA* genes (as well as in, some cases, with *M. huakuii* and *A. caulindan*). The species in Group 3 include *S. cannabina* and *S. sesban*, and given their ability to nodulate effectively with strain IRBG74 (this study) and/or *S. saheli* ORS609 (Bovin et al., 1997), probably also include *S. exasperata*, *S. formosa*, *S. grandiflora*, *S. macrantha* and *S. pachycarpa*. This group also includes *S. herbacea*, the original source of *R. huautlense* (Wang et al., 1998), but which can also nodulate with *Mesorhizobium* strains and IRBG74 (Wang and Martinez-Romero, 2000; this study), and *S. sericea*, which, along with *S. cannabina* and *S. exasperata*, nodulates with *R. huautlense* strains (Vinuesa et al., 2005). Although both the Group 1 and 2 species are South American in origin, the three nodulation groups appear to be independent of geography, as other South America spp. are present in Group 3 (e.g. *S. exasperata*) and, indeed, the Group 3 species come from all parts of the tropical world. This therefore leaves open the possibility that the nodulation preferences (and *nodA* types) are actually linked to plant phylogeny, and this is demonstrated by a tree inferred from rRNA Internal Transcribed Spacer 1 and 2 sequences of several *Sesbania* spp., including all those used in the present study (Fig. 5). This preliminary study has shown that although the genus is monophyletic (F.T. Farruggia, unpublished), it contains two distinct clades that appear to match the symbiont preference/nodA groups described above. For example, both the selective species, *S. punicae* and *S. virgata*, are present in one of the clades, whereas all the promiscuous species and/or those species that can nodulate with IRBG74 and other members of the *Rhizobium* [Agrobacterium]/*E. saheli nodA* group of *Sesbania* symbionts are in the other clade. Further studies of *nod* genes of symbionts from other members of the 60 plus species in the genus should confirm if the heterogeneity in symbiont preference is, indeed, linked to the molecular phylogeny of *Sesbania*.

**Experimental procedures**

**Culture conditions and DNA–DNA hybridizations**

All strains used in this study (Table 1) were routinely grown in yeast mannitol broth (YMB; Vincent, 1970). DNA–DNA hybridizations of strain IRBG74 against *R. radiobacter* NCIMB 9042, *R. vitis* LMG8750, *R. rubi* LMG 17935 and *R. huautlense* LMG 18254 were carried out by the identification service of DSMZ (Braunschweig, Germany) as described by De Ley and colleagues (1970), with the modification described by Huss and colleagues (1983) and Escara and Hutton (1980) using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program by Jahnke (1992).

The amplification, cloning and sequencing of the 16S rRNA, housekeeping and symbiotic genes

The 16S rRNA genes of IRBG74, SIN-1 and DUS1110 were amplified by PCR using recombinant *Taq* polymerase (Life Technologies) and a pair of primers designed from *Escherichia coli* rRNA positions, 8-27F and 1509-1491R (Weisburg et al., 1991). Reaction and PCR conditions were as described by Humphry and colleagues (2001). Amplification of the housekeeping genes from strain IRBG74 and the Agrobacterium type strains shown in Fig. 1 was performed using the protocol described by Santos and Ochman (2004). The primers employed were rpoBBDUP1, rpoBBDUP4, rpoBBJDN2 and rpoBBDJDN4 for the *rpoB* genes and fusAF and fusAR for the *fusA* sequence (Santos and Ochman, 2004).
The PCR protocols to amplify \textit{nifH} products from all the strains except \textit{A. doebereinerae} Br5401 were performed according to the method of Poly and colleagues (2001) with primer pair PolF and PolR. The amplification of \textit{nodA} sequences (except that of \textit{A. doebereinerae} Br5401) was performed using the methods described by Haukka and colleagues (1998), with primers nodA-1 and nodA-2. The primers used in this study are detailed in Table S1. Amplification products were visualized using electrophoresis in a 1% agarose gel and stained with SYBR safe (Invitrogen).

For each 16S rRNA, \textit{fusA}, \textit{rpoB}, \textit{nifH} and \textit{nodA} reaction, the amplified products from multiple independent inserts were cloned into pGEM-T ‘easy’ plasmid (Promega) and sequenced (Lark Technologies).

To obtain \textit{A. caulinodans} Br5401 \textit{nifH} and \textit{nodA} sequences, cells were grown in YM broth and genomic DNA was isolated as described by Wheatcroft and Watson (1998). Two microlitres of genomic DNA was used as a template in a 25 ml reaction volume containing 25 mM TAPS-HCl, 50 mM KCl, 2 mM MgCl$_2$, 1 mM $\beta$-mercaptoethanol, 0.2 mM of each of dATP, dCTP, dGTP and dTTP, 0.5 mM of each primer (Table S1) and 1 U of Phusion Taq DNA polymerase (New England Biolabs). The PCR conditions employed were initial denaturation at 95°C for 4 min followed by 35 cycles of 95°C for 45 s, 51°C for 45 s, 68°C for 1 min and a final extension at 72°C for 7 min. The amplified products were purified and sequenced directly using either the \textit{nifH} or \textit{nodA} primers.

The DNA sequence from 16S rRNA, \textit{rpoB}, \textit{fusA}, \textit{nifH} and \textit{nodA} genes from strain IRBG74 were aligned with sequences obtained from GenBank using CLUSTAL W (Benson et al., 1998). The 16S rRNA sequences were checked for intragenic recombination using Chimerae as implemented in RDP2. The 16S rRNA, \textit{fusA} and \textit{rpoB} sequences were concatenated with BIOEDIT (Hall, 1999) and a phylogenetic tree inferred using the maximum likelihood approach with PhyML 3.0 (Guindon et al., 2005). The appropriate nucleotide substitution model, GTR plus gamma, was selected using FindModel (Posada and Crandall, 2001). The robustness of the ML topology was inferred by non-parametric bootstrap tests with 100 pseudoreplicates using PhyML. The Neighbour-joining phylogenetic trees for \textit{nifH} and \textit{nodA} were performed with the Phylip package. The analysis included SEQBOOT, DNADIST, NEIGHBOR, CONSENSE (PHYLIP 3.5c package; Felsenstein, 1993) and TREEVIEW (Page, 1996) as described by Humphry and colleagues (2001).
Plasmid profiles and Southern blotting of nodA genes

Plasmid profiles were studied on horizontal gels using the modified Ekhardt technique as described by Kuykendall and colleagues (1996). Gels were blotted onto nylon membranes and DNA fixed by UV cross-linking. Southern blotting was carried out using a nodA probe of IRBG74 prepared using the PCR amplified gene products described above and labelled by random priming using the Dig-High Prime system (Roche). Hybridization conditions were as described by Turner and colleagues (2002). Hybridization was detected using the antidigoxigenin kit with the chemiluminescent substrate CSPD (Roche) according to the manufacturer’s instructions.

Phytopathogenic testing

The ability of IRBG74 to form crown galls on young tobacco (Nicotiana tabacum) plants was tested by wounding stems and inoculating the wounds as described by Moore and colleagues (2001). The PCR methods used to amplify the characteristic VirD2 and ipt gene portions found in functional ‘Agrobacterial’ Ti and Ri plasmids were those described by Haas and colleagues (1995).

Nodulation of Sesbania spp. by IRBG74

Seeds of Sesbania spp. and M. atropurpureum cv. Siratro (Table 3) were surface sterilized and their dormancy broken by treating them with concentrated sulphuric acid for 20 min (Elliott et al., 2007). Phaseolus vulgaris cv. Contender seeds were surface sterilized by immersion in 70% ethanol for 10 min and germinated by placing them in the dark on wet paper towels. The seedlings were grown in pots with a 1:1 mixture of vermiculite and perlite in a greenhouse according to Elliott and colleagues (2007). The plants were watered with either N-free nutrient solution or tap water so that the potting mixture of vermiculite and perlite was also flooded so that the whole of the developing root system was submerged. The plants were harvested at 30 dai, with IRBG74 to nodulate and fix N2 under flooded conditions. The plants were harvested at 7 and 15 dai for staining to detect GUS activity according to Gyaneshwar and colleagues (2001). Another experiment was set up in parallel, but in this case the seedlings were grown under non-flooded conditions in pots filled with vermiculite/perlite (see above for details), and were inoculated with WT IRBG74 5 days after sowing. At harvesting (7 and 15 dai), the Sesbania roots (and nodules, if present) were examined by light and transmission electron microscopy according to James and colleagues (2001) and Elliott and colleagues (2007). Sections were immunogold labelled according to James and colleagues (1996), either with a polyclonal antibody raised against IRBG74 (diluted 1:500) or with a polyclonal antibody (diluted 1:100) raised against Lb purified from pea (Pisum sativum) nodules (Van de Wiel et al., 1988). The IRBG74 antibody was tested for specificity via an enzyme-linked immunosorbent assay (ELISA) with a range of common soil and plant-associated bacteria according to Gyaneshwar and colleagues (2001), and it was also tested via immunogold labelling of sections of nodules formed on Sesbania spp. by all the bacteria listed in Table 1. No significant ELISA or immunogold reaction was obtained with any bacterium except for IRBG74 and its derivative strain, IRBG74GUS.

Nodulation of S. cannabina by other Sesbania-nodulating rhizobia

All the WT strains listed in Table 1 were inoculated on to seedlings of S. cannabina. Although all the strains, with the exception of DUS1110 (S. exasperata) and KFR647 (S. sesban), are known to be symbionts of their original hosts (see references cited in Table 1), they were also inoculated on to their original hosts (depending on availability of seeds) to confirm their symbiotic effectiveness. The plants were grown under sterile flooded conditions in glass tubes (as for the experiment using the GUS-tagged IRBG74 strain; see above). The plants were harvested at 60 dai, and were scored for presence of nodules, plant health (i.e. green shoots), ARA and nodule structure. From the results of this initial screening process, a more extensive experiment was set up to compare the symbiotic performance (growth, nodulation and nitrogenase activity) of Rhizobium (Agrobacterium) sp. IRBG74 on S. cannabina with representative strains from each of the four different genera of rhizobia known to
nodule Sesbania spp. (i.e. Rhizobium sp. DUS1110, A. doebereinerae Br5401, Mesorhizobium huakii KFR647 and Sinorhizobium (Ensifer) saheli ORS609; Table 1). Plants 'inoculated' with sterile YMB alone served as controls. The plants were grown under controlled environmental growth conditions for 30 days in pots filled with flooded vermiculite/perlite under a 12-h day, at a day/night temperature of 28/21°C and an irradiance of 1500 μE m⁻² s⁻¹. At harvest, nitrogenase activity (ARA) was measured, nodules were counted, and total plant dry weights were determined.

Acknowledgements

E.K.J. and G.N.E. were funded by the Natural Environment Research Council, grant reference NE/B505038/1. We thank Philippe de Lajudie for strain ORS609, Pete Rowell for use of his gas chromatograph, and Marty Wojciechowski for helpful discussions.

References

Bailly, X., Olivier, I., Brunel, B., Cleyet-Marechal, J.-C., and Béna, G. (2007) Horizontal gene transfer and homologous recombination drive the evolution of the nitrogen-fixing symbionts of Medicago species. J Bacteriol 189: 5223–5236.

Bala, A., Murphy, P., and Giller, K.E. (2002) Occurrence and genetic diversity of rhizobia nodulating Sesbania sesban in African soils. Soil Biol Biochem 34: 1759–1768.

Benson, D.A., Boguski, M.S., Lipman, D.J., Ostell, J., and Ouellette, B.F.F. (1998) GenBank. Nucleic Acids Res 26: 1–7.

Biswas, J.C., Ladha, J.K., Dazzo, F.B., Yanni, Y.G., and Chen, W.-M., and Lee, T.-M. (2001) Genetic and phenotypic

Boivin, C., Ndoye, I., Lortet, G., Ndiaye, A., de Lajudie, P., Bala, A., Murphy, P., and Giller, K.E. (2002) Occurrence and genetic diversity of rhizobia nodulating Sesbania sesban in African soils. Soil Biol Biochem 34: 1759–1768.

De Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestro-juan, G., Neyra, M., et al. (1994) Polyphasic taxonomy of rhizobia. Emendation of the genus Sinorhizobium and description of Sinorhizobium meliloti comb. nov., Sinorhizobium saheli sp. nov. & Sinorhizobium teranga sp. nov. Int J Syst Bacteriol 44: 715–733.

De Lajudie, P., Willems, A., Hoste, B., et al. (1998) Characterization of tropical tree rhizobia and description of Mesorhizobium plurifarium sp. nov. Int J Syst Bacteriol 48: 369–382.

De Lajudie, P., Willems, A., Hoste, B., et al. (1998) Characterization of tropical tree rhizobia and description of Mesorhizobium plurifarium sp. nov. Int J Syst Bacteriol 48: 369–382.

De Lajudie, P., Willems, A., Nick, G., Mohamed, S.H., Torck, U., Coopman, R., et al. (1999) Agrobacterium bv. 1 strains isolated from nodules of tropical legumes. Syst Appl Microbiol 22: 119–132.

De Ley, J., Cattoir, H., and Reynaerts, A. (1970) The quantitative measurement of DNA hybridisation from renaturation rates. Eur J Biochem 12: 133–142.

Den Herder, G., Schroeyers, K., Holsters, M., and Goormachtig, S. (2006) Signaling and gene expression for water-tolerant legume nodule. Crit Rev Plant Sci 25: 367–380.

Dreyfus, B., and Dommergues, Y.R. (1981) Nitrogen-fixing nodules induced by Rhizobium on the stem of the tropical legume Sesbania rostrata. FEMS Microbiol Lett 10: 313–317.

Dreyfus, B., Garcia, J.L., and Gillis, M. (1988) Characterization of Azorhizobium caulinodans gen. nov. sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from Sesbania rostrata. Int J Syst Bacteriol 38: 89–98.

Elliott, G.N., Chen, W.-M., Chou, J.-H., Wang, H.-C., Sheu, S.-Y., Perin, L., et al. (2007) Burkholderia phymatum is a highly effective nitrogen-fixing symbiont of Mimosa spp. & fixes nitrogen ex planta. New Phytol 173: 168–180.

Escara, J.F., and Hutton, J.R. (1980) Thermal stability and renaturation of DNA in dimethylsulphoxide solutions: acceleration of renaturation rate. Biopolymers 19: 1315–1327.

Farrand, S.K., van Berkum, P.B., and Oger, P. (2003) Agrobacterium is a definable genus of the family Rhizobiaceae. Int J Syst Evol Microbiol 53: 1681–1687.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Felsenstein, J. (1993) PHYLIP (Phylogenetic Inference Package), Version 3.5.1. Distributed by the author. Seattle, WA, USA: Department of Genetics. University of Washington.

Gonçalves, M., and Moreira, F.M.S. (2004) Specificity of the symbionts of Sesbania spp. & Azorhizobium johannae with other legume hosts and rhizobia. I. Symbiosis 36: 57–68.

Goormachtig, S., Capoen, W., James, E.K., and Holsters, M. (2004) Switch from intracellular to intercellular invasion during water stress-tolerant legume nodule. Proc Natl Acad Sci 101: 6303–6308.

Guindon, S., Lethiec, F., Duroux, P., and Gascuel, O. (2005) PHYML Online – a web server for fast maximum likelihood-based phylogenetic inference. Nucleic Acids Res 33: W557–W559.

Gyaneshwar, P., James, E.K., Mathan, N., Reddy, P.M., Reinhold-Hurek, B., and Ladha, J.K. (2001) Endophytic colonization of rice by a diazotrophic strain of Serratia marcescens. J Bacteriol 183: 2634–2645.

Haas, J.H., Moore, L.W., Ream, W., and Manulis, S. (1995) Universal PCR primers for detection of phytopathogenic Agrobacterium strains. Appl Environ Microbiol 61: 2879–2884.

Hall, T.A. (1999) BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows95/98/NT. Nucleic Acids Symp Series 41: 95–98.

Harris, J.O., Allen, E.K., and Allen, O.N. (1949) Morphological development of nodules on Sesbania grandiflora poir., with reference to the origin of nodule rootlets. Am J Bot 36: 651–661.

Haukka, K., Lindström, K., and Young, J.P.W. (1998) Three phylogenetic groups of nodA and nifH genes in Sinorhizobium and Mesorhizobium isolates from leguminous trees growing in Africa and Latin America. Appl Environ Microbiol 64: 419–426.

Hirsch, A.M., Drake, D., Jacobs, T.W., and Long, S.R. (1985)
Nodules are induced on alfalfa roots by *Agrobacterium tumefaciens* and *Rhizobium trifolii* containing small segments of *Rhizobium melliloti* nodule region. *J Bacteriol* 161: 223–230.

Humphry, D.R., George, A., Black, G.W., and Cummings, S.P. (2001) *Flavobacterium frigadarium* sp. nov., an aerobic, psychrophilic, xylanolytic and laminarinolytic bacterium from Antarctica. *Int J Syst Evol Microbiol* 51: 1235–1243.

Humphry, D.R., Andrews, M., Santos, S.R., James, E.K., Vinogradova, L.V., Perin, L., et al. (2007) Phylogenetic assignment and mechanism of action of a crop growth promoting *Rhizobium radiobacter* strain used as a biofertiliser on gramineous crops in Russia. *Ant V Leeuwen Int J Gen Mol Microbiol* 101: 105–113.

Huss, V.A.R., Festl, H., and Schleifer, K.H. (1983) Studies on the spectroscopic determination of DNA hybridisation from renaturation rates. *J Syst Appl Microbiol* 4: 184–192.

Jahneke, K.D. (1992) Basic computer program for evaluation of spectroscopic DNA renaturation data from GILDORD System 2600 spectrometer on a PC/XT/AT type personal computer. *J Microbiol Methods* 15: 61–73.

James, E.K., Iannetta, P.P.M., Nixon, P.J., Whiston, A.J., Peat, L., Crawford, R.R.M., et al. (1996) Photosystem II and oxygen regulation in *Sesbania rostrata* stem nodules. *Plant Cell Environ* 19: 895–910.

James, E.K., Loureiro, M.F., Pott, A., Pott, V.J., Martins, C.M., Franco, A.A., and Sprent, J.I. (2001) Flooding-tolerant legume symbioses from the Brazilian Pantanal. *New Phytol* 150: 723–738.

Kobayashi, H., and Broughton, W.J. (2008) Fine-tuning of symbiotic genes in rhizobia: flavonoid signal transduction cascades. In *Nitrogen-Fixing Legume Symbioses*. Dilworth, M.J., James, E.K., Sprent, J.I., and Newton, W.E. (eds). Dordrecht, The Netherlands: Springer, pp. 117–152.

Kuykendall, L.D., Swelim, D.M., Hashem, F.M., Abdel Wahab, S.M., and Hegazi, N.I. (1996) Symbiotic competence, genetic diversity and plasmid profiles of Egyptian isolates of a *Rhizobium* species from *Leucaena leucocephala* (Lam) Dewit. *Letts Appl Microbiol* 22: 347–352.

Lee, K.-B., De Backer, P., Aono, T., Liu, C.-T., Suzuki, S., Suzuki, T., et al. (2008) The genome of the versatile nitrogen fixer *Azorhizobium caulinodans* ORS571. *BMC Genomics* 9: 271. doi: 10.1186/1471-2164-9-271.

McInroy, S.G., Campbell, C.D., Haukka, K.E., Odee, D.W., Sprent, J.I., Wang, W.-J., et al. (1999) Characterisation of rhizobia from African acacias and other tropical woody legumes using Biolog and partial 16S rRNA sequencing. *FEBS Microbiol Lett* 170: 111–117.

Martinez, E., Palacios, R., and Sanchez, F. (1987) Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harboring *Rhizobium phaseoli* plasmids. *J Bacteriol* 169: 2828–2834.

Mhamdi, R., Rabiet, M., Laguerre, G., Tiwari, R., and Aouani, M.E. (2005) Colonisation of *Phaseolus vulgaris* nodules by *Agrobacterium*-like strains. *Can J Microbiol* 51: 105–111.

Moore, I.W., Bouzar, H., and Burr, T. (2001) *Agrobacterium*. In *Laboratory Guide for Identification of Plant Pathogenic Bacteria*. Schaad, N.W., Jones, J.B., and Chun, W. (eds). St. Paul, MN, USA: American Phytopathology Society, pp. 17–35.

Moreira, F.M.S., Cruz, L., de Faria, S.M., Marsh, T., Martinez-Romero, E., Pedrosa, F.O., et al. (2006) *Azorhizobium doebereinerae* sp. nov. microsymbiont of *Sesbania virgata* (Caz.) Pers. *Syst Appl Microbiol* 29: 197–206.

Ndoye, I., de Billy, F., Vasse, J., Dreyfus, B., and Truchet, G. (1994) Root nodulation of *Sesbania rostrata*. *J Bact* 176: 1060–1068.

Odee, D.W., Haukka, K., McInroy, S.G., Sprent, J.I., Sutherland, J.M., and Young, J.P.W. (2002) Genetic and symbiotic characterization of rhizobia isolated from tree and herbaceous legumes grown in soils from ecologically diverse sites in Kenya. *Soil Biol Biochem* 34: 801–811.

Page, R.D.M. (1996) Tree View: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* 12: 357–358.

Poly, F., Monrozier, J.L., and Bally, R. (2001) Improvement in RFLP procedure to study the community of nitrogen fixers in soil through the diversity of *nifH* gene. *Res Microbiol* 152: 95–103.

Posada, D., and Crandall, K.A. (2001) Selecting the best-fit model of nucleotide substitution. *Syst Biol* 50: 580–601.

Rana, D., and Krishnan, H.B. (1995) A new root-nodulating symbiont of the tropical legume *Sesbania, Rhizobium* sp. SIN-1, is closely related to *R. galegae*, a species that nodulates temperate legumes. *FEMS Microbiol Lett* 134: 19–25.

Santos, S.R., and Ochman, H. (2004) Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. *Environ Microbiol* 6: 754–759.

Sharma, R.S., Mohmmed, A., Mishra, V., and Babu, C.R. (2005) Diversity in a promiscuous group of rhizobia from three *Sesbania* spp. colonizing ecologically distinct habitats of the semi-arid Delhi region. *Res Microbiol* 156: 57–67.

Sprent, J.I. (2001) *Nodulation in Legumes*. London, UK: Royal Botanic Gardens, Kew.

Swofford, D.L. (2002) *PAUP*. *Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4. Sunderland, MA, USA: Sinauer Associates.

Tan, Z., Hurek, T., Vinuesa, P., Müller, P., Ladha, J.K., and Reinhold-Hurek, B. (2001) Specific detection of *Bradyrhizobium* and *Rhizobium* strains colonizing rice (*Oryza sativa*) roots by 16S–23S ribosomal DNA intergenic spacer-targeted PCR. *Appl Environ Microbiol* 67: 3655–3664.

Turner, S.L., Knight, K.A.L., and Young, J.P.W. (2002) Identification and analysis of rhizobial plasmid origins of transfer. *FEMS Microbiol Ecol* 42: 227–234.

Van de Wiel, C., Nap, J.-P., van Lammeren, A., and Bisseling, T. (1998) Histological evidence that a defence response of the host plant interferes with nodulin gene expression in *Vicia sativa* root nodules induced by an *Agrobacterium* transconjugant. *J Plant Physiol* 132: 446–452.

Veasey, E.A., Ghisi, O.M.A.A., Valarini, M.J., Otsuk, I.P., Cardelli, M.A., Sanchez, M.J.F., and Beisman, D.A. (1997) Early growth and native nodulation of leguminous shrub and tree species in Brazil. *Trop Grassls* 31: 40–48.

Velázquez, E., Peix, A., Zurdo Piñeiro, J.L., Palomo, J.L., Mateos, P.F., Rivas, R., et al. (2005) The coexistence of symbioses and pathogenicity-determining genes in *Rhizobium rhizogenes* strains enables them to induce nodules.
and tumors or hairy roots in plants. Mol Plant-Microbe Interact 18: 1325–1332.

Vincent, J.M. (1970) A Manual for the Practical Study of Root Nodule Bacteria. Oxford, UK: Blackwell Scientific Publications.

Vinuesa, P., Silva, C., Lorite, M.J., Izaguirre-Mayoral, M.L., Bedmar, E.J., and Martinez-Romero, E. (2005) Molecular systematics of rhizobia based on maximum likelihood and Bayesian phylogenies inferred from rrs, atpD, recA and nifH sequences, and their use in the classification of Sesbania microsymbionts from Venezuelan wetlands. Syst Appl Microbiol 28: 702–716.

Wang, E.T., and Martínez-Romero, E. (2000) Sesbania herbacea-Rhizobium huautlense nodulation in flooded soils and comparative characterization of S. herbacea-nodulating rhizobia in different environments. Microb Ecol 40: 25–32.

Wang, E.T., van Berkum, P., Beyene, D., Sui, X.H., Dorado, O., Chen, W.X., and Martinez-Romero, E. (1998) Rhizobium huautlense sp. nov., a symbiont of Sesbania herbacea that has a close phylogenetic relationship with Rhizobium galegae. Int J Syst Bacteriol 48: 687–699.

Wang, L.L., Wang, E.T., Liu, J., Li, Y., and Chen, W.X. (2006) Endophytic occupation of root nodules and roots of Melilotus dentatus by Agrobacterium tumefaciens. Microb Ecol 52: 436–443.

Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., et al. (1987) Report of the ad-hoc Committee on the reconciliation of approaches to bacterial systematics. Int J Syst Bact 37: 463–464.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173: 697–703.

Wheatcroft, R., and Watson, R.J. (1998) A positive strain identification method for Rhizobium melloti. Appl Environ Microbiol 54: 574–576.

Willems, A. (2006) The taxonomy of rhizobia: an overview. Plant Soil 287: 3–14.

Wilson, K., Sessitsch, A., Corbo, J.C., Giller, K.E., Akkermans, A.D.L., and Jefferson, R.A. (1995) β-Glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other Gram-negative bacteria. Microbiology 141: 1691–1705.

Young, J.M., Kuykendall, L.D., Martinez-Romero, E., Kerr, A., Sawada, H. (2001) A revision of Rhizobium Frank 1889, with an emended description of the genus, and the inclusion of all species of Agrobacterium Conn 1942 and Azorhizobium undicola de Lajudie et al., 1998 as new combinations: Rhizobium radiobacter, R. rhizogenes, R. rubi, R. undicola and R. viti. Int J Syst Evol Microbiol 51: 89–103.

Young, J.P.W., Crossman, L.C., Johnston, A.W.B., Thomson, N.R., Ghazoui, Z.F., Hull, K.H., et al. (2006) The genome of Rhizobium leguminosarum has a recognizable core and accessory components. Genome Biol 7: R34.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Maximum likelihood phylogram inferred from concatenated 16S rRNA + rpoB + fusA sequences estimated using the GTR +G substitution model for nine Sesbania isolates. Bootstrap support for 100 pseudoreplicates of the data set are provided at the corresponding nodes. The scale bar represents the number of nucleotide substitutions per site. Numbers in bold are GenBank accession numbers and T denotes the type strain of the species.

Fig. S2. Phylogenetic dendrograms based upon (A) nifH – 561 bp (B) nodA – 525 bp sequences of IRBG74. The Jukes and Cantor algorithm and the Neighbour-joining method were employed; bootstrap confidence percentages were calculated from 1000 replicate trees and shown on the branches if these occurred in more than 50% of the trees. The scale bar represents nucleotide substitutions per site. Numbers in bold are GenBank accession numbers and T denotes the type strain of the species.

Fig. S3. Plasmid profile of Rhizobium sp. IRBG74 compared with bacterial type strains with the most similar chromosomal and symbiotic gene sequences. Lane A – Ensifer saheli ORS 609, lane B – Rhizobium sp. SIN-1, lane C – Rhizobium sp. IRBG74, lane D – R. radiobacter NCIMB 13307, lane E – R. radiobacter NCIMB 9042(T). The size standard was produced using the plasmids of Rhizobium leguminosarum biovar viciae strain 3841, for which the sizes are already known. Arrows indicate the symbiotic plasmids.

Fig. S4. Nodulation of S. cannabina and other Sesbania species by various rhizobial strains. (A) S. virgata + Azorhizobium doebereinerae Br5401. (B) S. cannabina + A. doebereinerae Br5401. (C) S. sesban + Mesorhizobium huakuii KFR647. (D) S. cannabina + M. huakuii KFR647. (E) S. cannabina + Rhizobium sp. SIN-1. (F) S. exasperata + Rhizobium sp. DUS1110. The infected tissue is marked with a white asterisk in the effective, N2-fixing nodules in A, C, D, E, F. The smaller, ineffective nodule shown in B did not have any internal colonization by bacteria (black asterisk), but there was dense colonization of the epidermal tissue (arrow). Note in D that compared with the other effective nodules those formed by M. huakuii KFR647 on S. cannabina are more sparsely populated by infected, N2-fixing cells. The transient meristem in a nodule on S. virgata is indicated by an arrow in A, Bars, 500 μm (A, C–F), 100 μm (B).

Table S1. Primers used in this study.

Table S2. Nodulation of Sesbania spp. and other legumes at 60 days after inoculation with Rhizobium (Agrobacterium) sp. strain IRBG74.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.