Annals of Botany **100**: 1403–1411, 2007
doi:10.1093/aob/mcm227, available online at www.aob.oxfordjournals.org

OXFORD JOURNALS
OXFORD UNIVERSITY PRESS

Nodulation of *Cyclopia* spp. (Leguminosae, Papilionoideae) by *Burkholderia tuberum*

GEOFFREY N. ELLIOTT[1], WEN-MING CHEN[2], CYRIL BONTEMPS[3], JUI-HSING CHOU[2], J. PETER W. YOUNG[3], JANET I. SPRENT[1] and EUAN K. JAMES[1,*]

[1]*College of Life Sciences, University of Dundee, Dundee DD1 5EH, UK,* [2]*Laboratory of Microbiology, Department of Seafood Science, National Kaohsiung Marine University, Kaohsiung City 811, Taiwan and* [3]*Department of Biology 3, University of York, PO Box 373, York YO10 5YW, UK*

Received: 17 May 2007 Returned for revision: 25 June 2007 Accepted: 30 July 2007 Published electronically: 19 September 2007

• *Background and Aims* Species of the genus *Burkholderia*, from the Betaproteobacteria, have been isolated from legume nodules, but so far they have only been shown to form symbioses with species of *Mimosa*, sub-family Mimosoideae. This work investigates whether *Burkholderia tuberum* strains STM678 (isolated from *Aspalathus carnosa*) and DUS833 (from *Aspalathus callosa*) can nodulate species of the South African endemic papilionoid genera *Cyclopia* (tribe Podalyrieae) and *Aspalathus* (Crotalarieae) as well as the promiscuous legume *Macroptilium atropurpureum* (Phaseoleae).
• *Methods* Bacterial strains and the phylogeny of their symbiosis-related (*nod*) genes were examined via 16S rRNA gene sequencing. Seedlings were grown in liquid culture and inoculated with one of the two strains of *B. tuberum* or with *Sinorhizobium* strain NGR 234 (from *Lablab purpureus*), *Mesorhizobium* strain DUS835 (from *Aspalathus linearis*) or *Methylobacterium nodulans* (from *Crotalaria podocarpa*). Some nodules, inoculated with green fluorescence protein (GFP)-tagged strains, were examined by light and electron microscopy coupled with immunogold labelling with a *Burkholderia*-specific antibody. The presence of active nitrogenase was checked by immunolabelling of nitrogenase and by the acetylene reduction assay. *B. tuberum* STM678 was also tested on a wide range of legumes from all three sub-families.
• *Key Results* Nodules were not formed on any of the *Aspalathus* spp. Only *B. tuberum* nodulated *Cyclopia falcata*, *C. galioides*, *C. genistoides*, *C. intermedia* and *C. pubescens*. It also effectively nodulated *M. atropurpureum* but no other species tested. GFP-expressing inoculant strains were located inside infected cells of *C. genistoides*, and bacteroids in both *Cyclopia* spp. and *M. atropurpureum* were immunogold-labelled with antibodies against *Burkholderia* and nitrogenase. Nitrogenase activity was also shown using the acetylene reduction assay. This is the first demonstration that a β-rhizobial strain can effectively nodulate papilioinoid legumes.
• *Conclusions* Papilionoid legumes from widely different tribes can be nodulated by β-rhizobia, forming both indeterminate (*Cyclopia*) and determinate (*Macroptilium*) nodules.

Key words: β-rhizobia, nitrogen fixation, fynbos, *Macroptilium atropurpureum*, 'Siratro', *Mimosa*, *Aspalathus*.

INTRODUCTION

Several species of bacteria in the genus *Burkholderia* are known to be diazotrophs, particularly those that are isolated from the rhizosphere and endorhizosphere of gramineous plants in the tropics and sub-tropics (Gillis *et al.*, 1995; Estrada de Los Santos *et al.*, 2001; Reis *et al.*, 2004; Perin *et al.*, 2006). *Burkholderia* strains that contain *nodA* as well as *nifH* genes have been isolated from legume nodules (Moulin *et al.*, 2001; Vandamme *et al.*, 2002), and it is now well established that so-called 'β-rhizobia' in the genus *Burkholderia* can nodulate and form effective N_2-fixing symbioses with legumes, most particularly those in the large mimosoid genus *Mimosa* (Chen *et al.*, 2003*a*, 2005*a*, *b*; Barrett and Parker, 2005, 2006; Elliott *et al.*, 2007). *Burkholderia* strains have also been isolated from nodules on papilionoid legumes (Moulin *et al.*, 2001; Vandamme *et al.*, 2002; Rasolomampianina *et al.*, 2005), as well as from other mimosoid legumes (*Abarema macradenia*, *Pithecellobium hymenaeafolium*; Barrett and Parker, 2005), but there has been no evidence presented as yet that they can nodulate the plants from which they were isolated. Indeed, in the case of *Burkholderia phymatum* STM815, which was originally isolated from nodules on the papilionoid species *Machaerium lunatum*, Elliott *et al.* (2007) failed to nodulate *Machaerium* spp. with this strain, but it was found to be a highly effective nodulator of several *Mimosa* spp., thus suggesting that this isolate was at most a 'passenger' in the *Machaerium* nodule it was isolated from, and most likely originated instead from a *Mimosa* nodule in the vicinity. This contention is supported by the *nodA* gene sequence from *B. phymatum*, which has high similarity to other *Mimosa*-nodulating *Burkholderia* strains (Chen *et al.*, 2005*a*, *b*), and also by the subsequent identification of another *B. phymatum* strain, NGR195A, originally isolated from *Mimosa* nodules in Papua New Guinea (Elliott *et al.*, 2007). Together with the recent description of *Burkholderia mimosarum* and *Burkholderia nodosa* (Chen *et al.*, 2006, 2007), this brings the number of formally described *Mimosa*-nodulating *Burkholderia* strains to three, but are there any *Burkholderia* spp. that can effectively nodulate papilionoid legumes?

* For correspondence. Email e.k.james@dundee.ac.uk

The *nodA* gene of *Burkholderia tuberum* STM678 is distinct in phylogenetic terms from the *Mimosa*-nodulating β-rhizobia, including *B. mimosarum*, *B. nodosa*, *B. phymatum*, and *Cupriavidus* (*Ralstonia*) *taiwanensis* (Chen *et al.*, 2003*a*, 2005*a*, *b*), being more similar to that of the alphaproteobacterium *Methylobacterium nodulans* (isolated from nodules on *Crotalaria* spp.; Sy *et al.*, 2001*a*, *b*) and to *Bradyrhizobium* (Chen *et al.*, 2003*a*, 2005*a*, *b*). *B. tuberum* STM678 was originally isolated from the papilionoid legume *Aspalathus carnosa*, which is native to the seasonally burnt and acid soils supporting the fynbos vegetation of South Africa (Deschodt and Strijdom, 1976; Dakora, 1998; Muofhe and Dakora, 1999; Cocks and Stock, 2001). The present paper describes another strain of *B. tuberum*, DUS833, which was also isolated from *Aspalathus* nodules, and tests various *Aspalathus* spp. for nodulation with both STM678 and DUS833. In view of reports of *B. tuberum*-like strains isolated from *Cyclopia* nodules (Kock, 2004; Kock and Steyn, 2004), *B. tuberum* STM678 and DUS833 were also tested for nodulation on species of this papilionoid genus, which is also endemic to the South African cape region (Spriggs *et al.*, 2003; Spriggs and Dakora, 2007), as well as on *Macroptilium atropurpureum*, a papilionoid legume well known to be highly promiscuous with regard to microsymbionts (Trinick *et al.*, 1991). Nodulation of *Aspalathus* and *Cyclopia* spp. by the two *B. tuberum* strains was compared with that of *Mesorhizobium* sp. strain DUS835, *Methylobacterium nodulans* strain ORS2060 (Sy *et al.*, 2001*a*) and the broad host-range *Sinorhizobium* (*Ensifer*) strain NGR234 (Trinick, 1980; Pueppke and Broughton, 1999).

MATERIALS AND METHODS

Bacterial strains and culture conditions

All bacterial strains used in this study are listed in Table 1. In addition to these wild-type strains, *B. tuberum* STM678 was transformed using green fluorescent protein (TnGFP) to form STM678GFP as described in Chen *et al.* (2003*b*). STM678 was also transformed using the *nodD*GUS-containing plasmid pRG960SD-32 (Van den Eede *et al.*, 1992) to form STM678*nodD*GUS using the same method, except that a spontaneous chloramphenicol-resistant STM678 mutant was developed and used as the recipient, with chloramphenicol (34 $\mu g\ mL^{-1}$) and streptomycin (20 $\mu g\ mL^{-1}$) used for final transformant selection.

Further identification of strains and phylogeny of symbiosis-related genes

The nearly full-length 16S rRNA gene from DUS833 and DUS835 was amplified and sequenced using primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) (Weisburg *et al.*, 1991). Accession numbers for the 16S rRNA gene sequence of DUS833 and DUS835 are EF566975 and EF566978, respectively. For DUS833 and DUS835, a 501-bp fragment of the *nodA* gene was amplified and sequenced with primers 5′-TGCRGTGGARDCTRYGCTGGGAAA-3′ and 5′-GNC CGTCRTCRAASGTCARGTA-3′ and a 585-bp *nifH* gene fragment with primers 5′-CGCIWTYTACGGIAARGG IGG-3′ and 5′-GGIKCRTAYTSGATIACIGTCAT-3′ (Chen *et al.*, 2003*a*). For DUS833 a 461-bp *nodC* gene fragment was amplified with the reverse primer 5′-CTCAATGTACA CARNGCRTA-3′ and the tagged primer 5′-H1-GAYATGG ARTAYTGGYT-3′ and sequenced with the tag H1 (5′-GG TTCCACGTAAGCTTCC-3′). Accession numbers for partial *nodA* and *nodC* gene sequences of DUS833 are EF566976 and EF566977, respectively, and EF566979 for the *nodA* gene sequence of DUS835.

Plant species, germination and inoculation

Seeds of five species each of *Aspalathus (A. chortophila*, *A. linearis*, *A. nivea*, *A. subtingens*, *A. teres*) and *Cyclopia* spp. (as listed in Table 2) were obtained from Silver Hills Seeds (Capetown, South Africa) or from B and T World Seeds (Paguignan, France). *Macroptilium atropurpureum* 'Siratro' seeds were obtained from the Australian Tropical Crops and Forages Collection, Biloela, Queensland, Australia. All seeds were germinated by immersion in concentrated H_2SO_4 for 10 min, then washed five times in sterile distilled H_2O and placed onto 1 % water agar and left at room temperature in the dark. Resulting seedlings were grown under sterile conditions in 30-mL tubes filled with modified Jensens N-free plant nutrient medium (Somasegaran and Hoben, 1994), and were inoculated with the appropriate bacterial inoculum as previously described (Chen *et al.*, 2003*b*). Plants of all species were inoculated with one each of the five wild-type strains (Table 1), with additional plants

TABLE 1. *Bacterial strains used in this study*

Strain	Identification from 16S rRNA gene sequence	Original host	Geographical origin	Source/Reference
STM678	*Burkholderia tuberum*	*Aspalathus carnosa*	South Africa	Moulin *et al.* (2001), Vandamme *et al.*, (2002)
DUS833	*Burkholderia tuberum**	*Aspalathus callosa*	South Africa	Muofhe and Dakora (1998)
DUS835	*Mesorhizobium* sp.*	*Aspalathus linearis*	South Africa	Muofhe and Dakora (1998)
ORS2060	*Methylobacterium nodulans*	*Crotalaria podocarpa*	Senegal	Sy *et al.* (2001)
NGR234	*Sinorhizobium* sp.	*Lablab purpureus*	Papua New Guinea	Trinick *et al.* (1980)

*Previously named *Bradyrhizobium aspalati* (Boone *et al.*, 1999).

TABLE 2. *Effect of inoculation with* Burkholderia tuberum *strains STM678 and DUS833 on nodulation of* Cyclopia *spp.*

Host plant species	Inoculant strain	Mean (±s.d.) number of nodules
Cyclopia falcata	STM678	3·3 (±0·9)
	DUS833	0·5 (±0·4)
Cyclopia galioides	STM678	5·2 (±1·0)
	DUS833	8·0 (±5·6)
Cyclopia genistoides	STM678	5·3 (±0·9)
	DUS833	5·5 (±3·9)
Cyclopia intermedia	STM678	1·5 (±0·5)
	DUS833	No nodulation
Cyclopia pubescens	STM678	3·8 (±0·8)
	DUS833	No nodulation

All data were collected at 60 d after initial inoculation.

inoculated with STM678GFP and STM678*nodD*GUS for microscopical studies. All plants were inoculated (with controls) in quadruplicate on two or more separate occasions, and were grown at 26 °C under Triplus T5 triphosphor plant growth lamps at 339·5 (± 28·2) μE m^{-2} s^{-1} with a 12-h daylength.

A separate inoculation experiment was performed in Taiwan on a wide range of plants from all three sub-families of the Leguminosae as follows: *Canavalia gladiata*, *C. rosea*, *Chamaecrista mimosoides*, *Glycine max*, *Macroptilium atropurpureum*, *Medicago polymorpha*, *Mimosa diplotricha*, *M. pigra*, *M. pudica*, *Phaseolus vulgaris*, *Prosopis africana*, *Vigna angularis*, *V. radiata* and *Sesbania cannabina*.

Plant assessment and harvest

Plants were harvested at 40 or 60 d after inoculation (dai). Acetylene reduction assays (ARAs) were conducted on intact plants according to Chen *et al.* (2003*b*). Nodules were then harvested for light microscopy, transmission electron microscopy (TEM) and immunogold labelling with antibodies raised against the genus *Burkholderia* or against the nifH (Fe-) protein according to Chen *et al.* (2003*b*, 2005*b*). A separate experiment was also conducted to examine the infection of *Cyclopia genistoides* and *C. pubescens* by *B. tuberum* STM678GFP and STM678*nodD*GUS; this involved harvesting the plants at 7 and 15 dai and examining them for signs of infection using light and epifluorescence microscopy, as well as for more detailed studies using confocal laser scanning microscopy (CLSM) and TEM according to Chen *et al.* (2003*b*).

RESULTS

The 16S rRNA, *nodA* and *nodC* gene sequences of strain DUS833 were identical to *Burkholderia tuberum* STM678 (100 % homology). The 16S rRNA gene sequence of DUS835 showed highest sequence similarity (>99·9 %) to *Mesorhizobium* spp. The *nodA* gene sequence of DUS835 shared 73·8 % DNA identity with those of DUS833, with the most similar sequences within sequence databanks belonging to species of the genera *Sinorhizobium* and *Mesorhizobium*.

Burkholderia tuberum STM678 formed nodules with all five species of *Cyclopia* (Table 2, Fig. 1A–C), whereas DUS833 formed nodules on *C. falcata*, *C. galioides* and *C. genistoides* (Table 2). No nodules formed on any of the five *Aspalathus* species inoculated with either *B. tuberum* strain, and all plants subsequently died from apparent N-limitation. None of the other three inoculant strains (DUS835, NGR234, ORS2060) formed nodules on any of the *Cyclopia* or *Aspalathus* spp. In the case of *C. genistoides*, the inoculated plants were green (Fig. 1A) and gave significant ARA activity with both strains of *B. tuberum* (146·2 ± 72·1 and 140·7 ± 33·8 nmol C_2H_4 $plant^{-1}$ h^{-1} for STM678 and DUS833, respectively). Nitrogenase activity and immunogold labelling of bacteroids of STM678 were also observed in *C. galioides* and *C. pubescens*, but the number of effectively nodulated plants was very low, and these had lower ARA values (8·4 and 11·4 nmol C_2H_4 $plant^{-1}$ h^{-1}, respectively), and no ARA activity was detected with *C. falcata* and *C. intermedia*. Therefore, as nodulation and nitrogen fixation by *C. genistoides* was more consistent compared with the other *Cyclopia* species, it was selected for further studies with GFP- and *nodD*GUS-marked variants of STM678, and the nodules formed by these (Fig. 1C) showed GFP (Fig. 1D, E) and GUS (β-glucuronidase) activity (Fig. 1F), respectively. *Macroptilium atropurpureum* plants were also green and healthy compared with uninoculated control plants after they had been inoculated with either of the *B. tuberum* strains, and were clearly nodulated (Fig. 1G), but the plants inoculated with *Mesorhizobium* sp. strain DUS835 were similar in appearance to the uninoculated controls, even though they were also nodulated (Fig. 1G).

Light and electron microscopy coupled with immunogold labelling with antibodies against *Burkholderia* (Chen *et al.*, 2005*b*) confirmed that the bacteroids in the host cells of *C. genistoides* nodules formed by either *B. tuberum* strain were *Burkholderia* (Fig. 2A–D). Negative control sections incubated in non-immune serum gave little or no signal (Fig. 2E, F). Nodules formed by either *B. tuberum* STM678 or DUS833 on *C. genistoides* were effective in appearance and were typically indeterminate with a meristem, invasion zone and nitrogen-fixing zone (Fig. 3A–D), and hence were generally as described by Vasse *et al.* (1990) for alfalfa (*Medicago sativa*) nodules. Nodules formed on *C. galioides* (Figs 1B and 3E), *C. pubescens* (Fig. 3F) and *C. falcata* (not shown) by *B. tuberum* were essentially similar to those formed on *C. genistoides*. Immunogold labelling with antibodies against the nitrogenase Fe-protein (nifH protein) confirmed that the bacteroids in the host cells of *Cyclopia* nodules formed by the *B. tuberum* strains expressed the nitrogenase enzyme complex, for example in *C. genistoides* (Fig. 3D).

Macroptilium atropurpureum nodulated by the wild-type, GFP- and GUS-marked variant strains of *B. tuberum* STM678, and by *B. tuberum* DUS833, had significant (albeit highly variable) acetylene reduction activities (Table 3), with some of the plants inoculated with the GFP-marked strain having particularly high activity (up to 1181 nmol C_2H_4 $plant^{-1}$ h^{-1}). None of the plants nodulated by *Mesorhizobium* sp. DUS835 had any

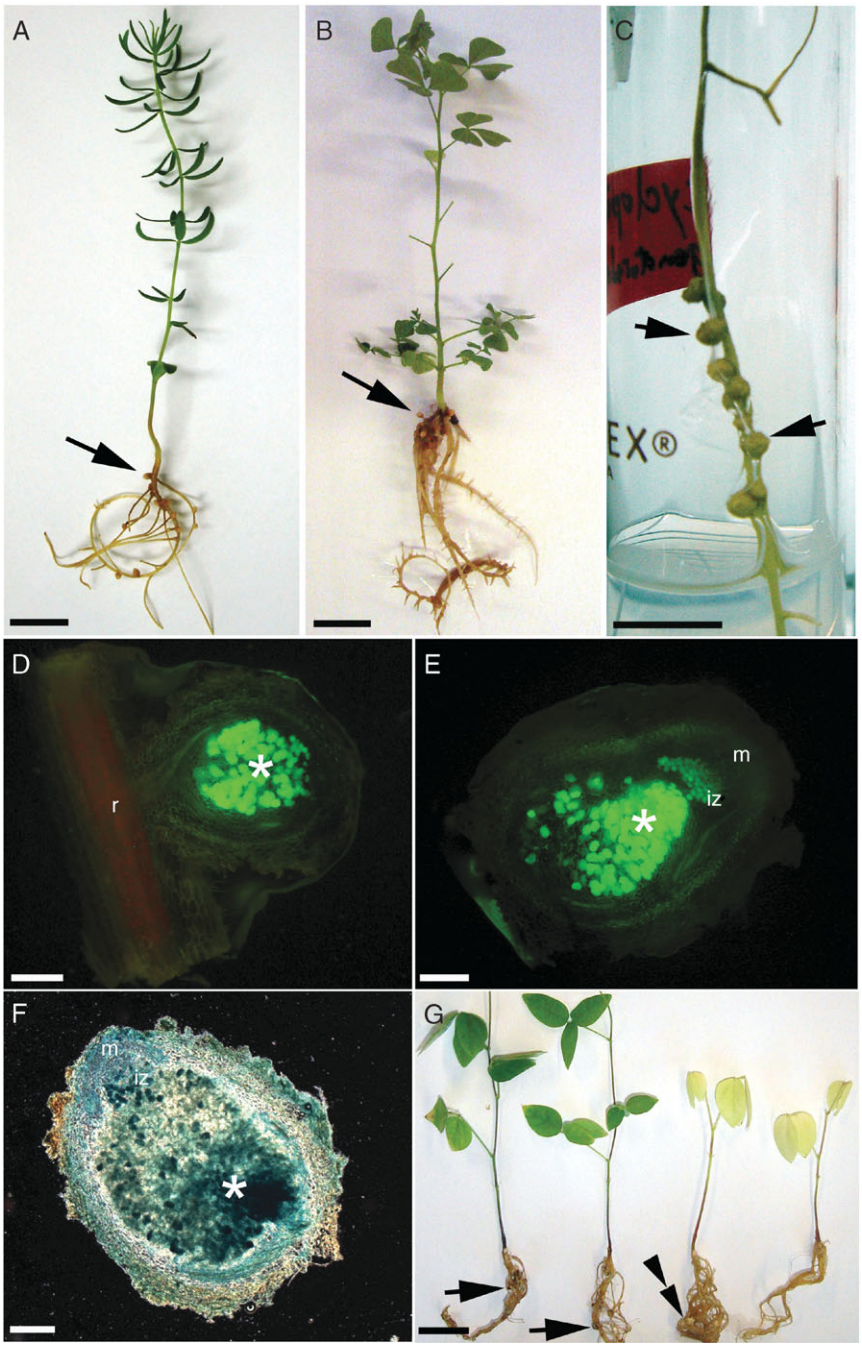

FIG. 1. (A) *Cyclopia genistoides* inoculated with *Burkholderia tuberum* STM678 and (B) *C. galioides* inoculated with *B. tuberum* DUS833. Nodules are indicated by arrows. Nodules (arrows) on a root of *C. genistoides* 50 d after inoculation with *B. tuberum* STM678GFP (C). Sections of (D) young and (E) mature nodules of *C. genistoides* 20 and 50 d after inoculation with *B. tuberum* STM678GFP viewed under epifluorescence. The central infected tissue of the nodules fluoresces green (*) indicating the presence of the GFP-expressing *B. tuberum* cells, but the vascular cylinder of the root (r) subtending the young nodule fluoresces red. (E) The mature *C. genistoides* nodule has a pronounced meristem, thus indicating that it is indeterminate. (F) Section of a mature *C. genistoides* nodule at 50 d after inoculation with *B. tuberum* STM678nodDGUS showing high expression of glucuronidase in the infected cells (*), and in the invasion zone (iz) close to the meristem (m). (G) *Macroptilium atropurpureum* 'Siratro' plants which were either inoculated with *B. tuberum* STM678 (first left), *B. tuberum* DUS833 (second left), *Mesorhizobium* sp. DUS835 (third left) or left uninoculated (right). Nodules on the *B. tuberum*-inoculated plants are indicated by arrows, and the white nodules on the *Mesorhizobium*-inoculated plant by a double arrowhead. Scale bars = 1 cm (A–C), 100 μm (D), 200 μm (E, F), 2 cm (G).

activity. The *M. atropurpureum* nodules formed by the *B. tuberum* strains were effective in appearance and typically determinate (Fig. 4A), with the bacteroids immunogold-labelled with antibodies against *Burkholderia* (Fig. 4B) and the nitrogenase Fe- (nifH) protein (Fig. 4C). Bacteroids incubated in non-immune serum gave a negligible immunogold signal (Fig. 4D). In contrast to the effective *B. tuberum*-induced nodules, the ineffective nodules formed by *Mesorhizobium* sp.

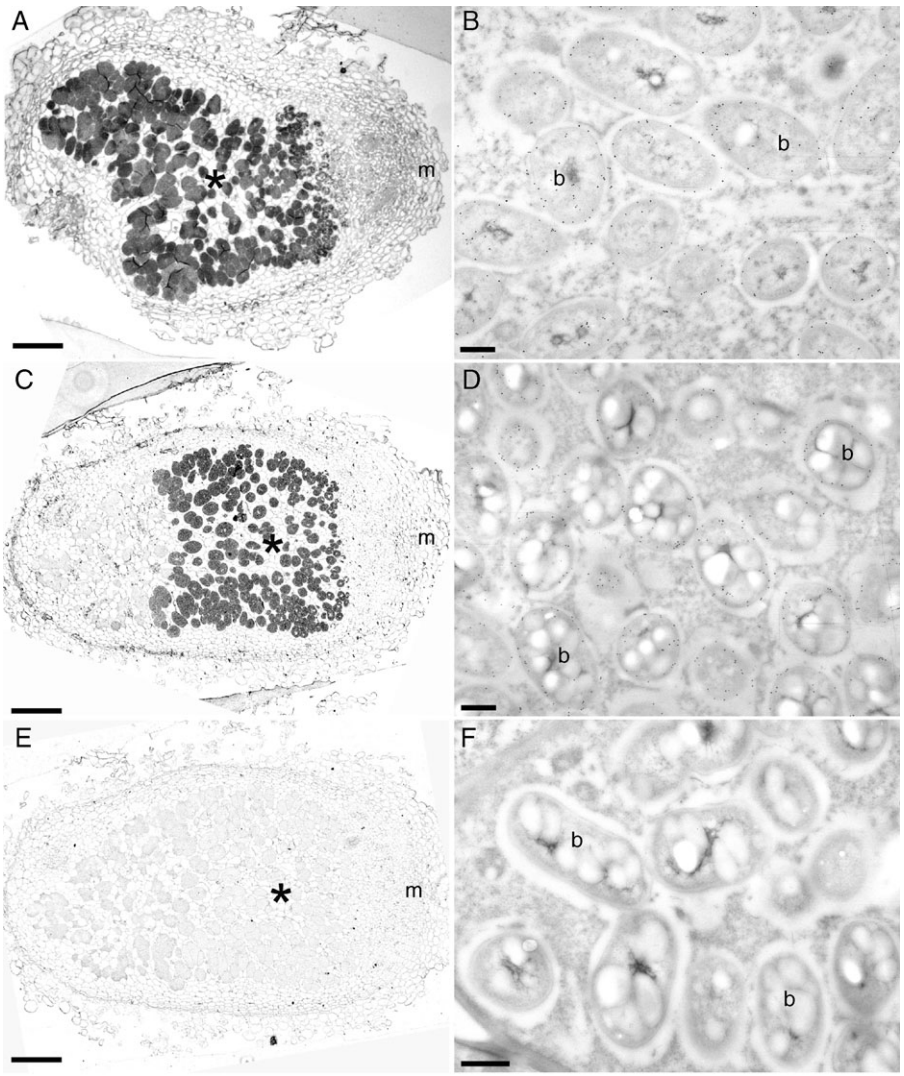

FIG. 2. Light microscopy (A, C, E) and transmission electron microscopy (TEM) (B, D, F) of sections of *Cyclopia genistoides* nodules infected with *B. tuberum* STM678 (A, B) or DUS833 (C–F). The sections were immunogold labelled with either an antibody raised against *B. phymatum* STM815 (A–D), or with pre-immune serum (E, F). The infected zone (*) is clearly labelled in A and C, but there is no signal in E. The meristem (m) is indicated at the right-hand side of each of A, C and E. Similarly, bacteroids (b) in B and D are labelled with gold whereas those in F are not. Scale bars = 100 μm (A, C, E), 500 nm (B, D, F).

DUS835 on *M. atropurpureum* were white in colour (Fig. 1F) and disorganized in structure (not shown).

B. tuberum STM678 failed to nodulate a wider range of legumes, including *Canavalia gladiata*, *C. rosea*, *Chamaecrista mimosoides*, *Glycine max*, *Medicago polymorpha*, *Mimosa diplotricha*, *M. pigra*, *M. pudica*, *Prosopis africana* and *Sesbania cannabina*, but it could ineffectively nodulate *Phaseolus vulgaris* and *Vigna angularis* (Table 3).

DISCUSSION

The 16S rRNA gene sequence of strain DUS833 clearly places it in the species *Burkholderia tuberum*, thereby doubling the number of strains recorded in this species. Interestingly, several 16S rRNA gene sequences from rhizobia isolated from *Cyclopia* spp. and deposited by Kock (2004) and Kock and Steyn (2004) also have a very high similarity to *B. tuberum* STM678 (as well as other *Burkholderia* spp.) and so there is probably a much larger number of strains of this species, particularly in nodules of *Cyclopia* spp. native to the fynbos vegetation of South Africa. The *nodA* and *nodC* genes of DUS833 were also very similar to those of *B. tuberum* STM678, as are those of the strains identified by Kock (2004), and the most similar α-rhizobia (in terms of *nodA* gene sequence) are *Methylobacterium nodulans* ORS2060, with the next closest being members of the large genus *Bradyrhizobium* (Chen *et al.*, 2003*a*, 2005*a*, *b*). The 16S rRNA gene sequence of the other DUS isolate, DUS835, was most similar to those of members of the genus *Mesorhizobium*. Note that both of these strains were originally named as representing '*Bradyrhizobium aspalati*' (Boone *et al.*, 1999) yet neither isolate proved to be a bradyrhizobium.

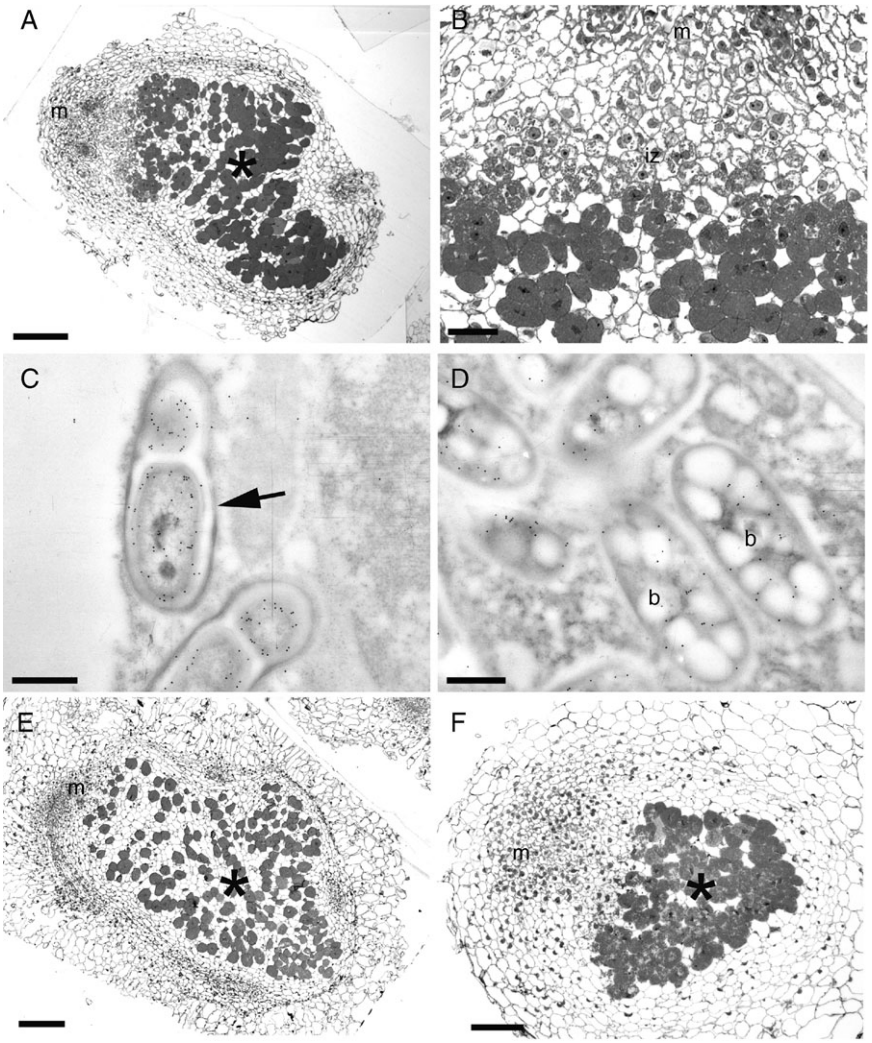

FIG. 3. Light microscopy (A, B, D, E) and TEM (C, F) of sections of nodules of *Cyclopia genistoides* (A–D), *C. galioides* (E) and *C. pubescens* (F) infected with *B. tuberum* STM678. *Cyclopia* nodules are typically indeterminate with a distinct apical meristem (m), an invasion zone (iz) of newly divided cells being penetrated by infection threads containing rhizobia [which in this case have been immunogold labelled with an antibody raised against *B. phymatum* STM815 (arrow in C)], and a central zone of infected cells (*) containing bacteroids (b) that express the enzyme nitrogenase, as indicated in D by immunogold labelling with an antibody against the nifH protein. Scale bars = 100 μm (A, E, F), 25 μm (B), 500 nm (C, D).

It is assumed that this nomenclature was adopted in the absence of any identifying sequence data on the preposition that these isolates were similar to those identified using now somewhat outdated methodology and published some years previously (Dakora, 1998).

There is evidence provided by the sequences deposited by Kock (2004) that *B. tuberum* could nodulate *Cyclopia*. For example, many of the *Cyclopia* strains that were shown to be similar to *B. tuberum* STM678 had been authenticated as being capable of nodulating *Cyclopia* by an earlier study by A. C. Spriggs and F. D. Dakora (unpubl. res.). However, the present study is the first to show conclusively via ASAs and microscopy that two *B. tuberum* strains (STM678 and DUS833) can nodulate *Cyclopia* species effectively, and is the first study to show that *B. tuberum* can effectively nodulate any legume. Nodules on this genus have previously been reported to be indeterminate (Sprent, 2001), but there has been no report of their structure. Taxonomically the tribe Podalyrieae is considered to be close to the tribes Genisteae and Crotalarieae, both of which appear to have nodules that are not formed following root hair infection and where the infected tissue contains no uninfected cells (Sprent, 2007; Sprent and James, 2007). The internal structure of *Cyclopia* nodules is common to many legumes, but not in this part of legume phylogeny (van Wyk, 2005). However, arguably the most interesting aspect of nodulation by *Cyclopia* is that it seems to be so specific in its preference for *B. tuberum* as a symbiont. For example, even though *Sinorhizobium* (*Ensifer*) sp. NGR234 can nodulate a huge range of legumes, including those in the same tribe as *Cyclopia* (the Podylareae) (Pueppke and Broughton, 1999), it failed to nodulate any of the *Cyclopia* spp. Small bumps were formed by *Methylobacterium nodulans* on *C. galioides*, but they were symbiotically ineffective and disorganized structures (G. N. Elliott, J. I. James and E. K. Sprent, unpubl. res.), and so at present it must be concluded from the results of the present study and that of Kock

TABLE 3. *Nodulation and nitrogenase (acetylene reduction) activities of* Macroptilium atropurpureum *inoculated with wild-type and genetically modified variant strains of* Burkholderia tuberum *STM678,* B. tuberum *DUS833 and* Mesorhizobium *sp. DUS835, and nodulation and nitrogenase activities of* Phaseolus vulgaris *and* Vigna angularis *inoculated with* B. tuberum *STM678*

Strain	Number of nodules	Nitrogenase activity (nmol C_2H_4 $plant^{-1}$ h^{-1})
M. atropurpureum		
STM678	18·9 ± 7·0	125·6 ± 41·5
STM678gfp	17·6 ± 4·8	444·4 ± 461·8
STM678(pSD32)	11·6 ± 5·4	60·4 ± 73·0
DUS833	12·8 ± 3·0	45·3 ± 52·4
DUS835	9·8 ± 3·4	0
Control	0	0
P. vulgaris	14·6 ± 4·6	0
V. angularis	17·6 ± 5·5	0

Values are given as mean ± s.d. All data were collected at 40 d after initial inoculation.

(2004) that *B. tuberum* is the dominant symbiont of this genus. Finally with regard to *Cyclopia*, Sprent (2001) lists only four out of the nine *Cyclopia* species as nodulated, including *C. genistoides*, so the reports from the present study of nodulation of *C. galioides*, *C. intermedia* and *C. pubescens* are new, and, together with the recent report of nodulation of *C. falcata* (syn. *C. subternata*) by Spriggs and Dakora (2007), brings the total number of confirmed nodulated *Cyclopia* spp. to eight.

Although Moulin *et al.* (2001) and Vandamme *et al.* (2002) have described *B. tuberum* STM678 as a symbiont of *Aspalathus carnosa*, the present study failed to demonstrate nodulation of any of the five *Aspalathus* spp. tested under the growth conditions that allowed for nodulation of *Cyclopia* spp. and *M. atropurpureum*. It is possible that these conditions were simply not conducive to nodulation of *Aspalathus* spp., although the plants grew well until they succumbed to N-limitation, so the conditions were not considered as inherently bad for plant growth. Unfortunately, seeds of *A. carnosa*, the plant from which STM678 is reported to have been isolated, could not be obtained and so it could not

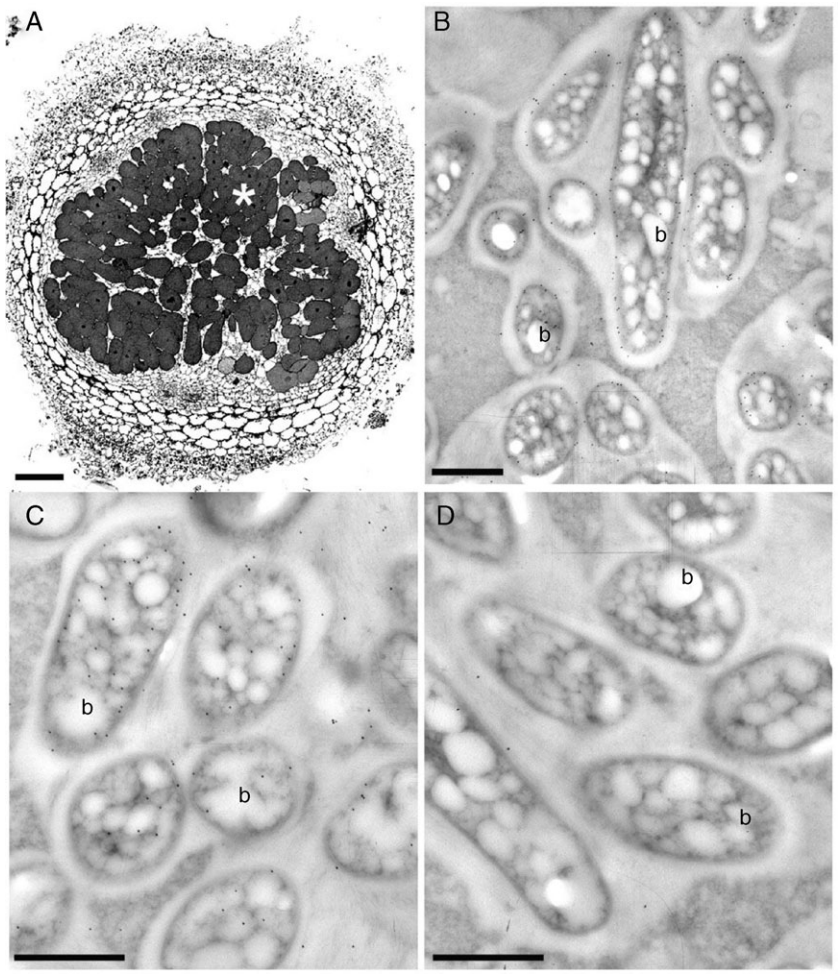

FIG. 4. Sections of *Macroptilium atropurpureum* 'Siratro' nodules after inoculation with *B. tuberum* STM678 viewed under the light microscope (A) or the TEM (B–D). The mature nodule (A) is effective in appearance with densely stained infected cells (*), but it is spherical and without a meristem, and hence is typically determinate. Bacteroids (b) immunogold-labelled with antibodies against either *B. phymatum* STM815 (B) or the nifH protein (C). Bacteroids in serial sections from the same nodules incubated in pre-immune serum had little or no gold labelling (D). Scale bars = 200 μm (A), 1 μm (B–D).

be confirmed if this particular species could be nodulated under the conditions used in the present study. Therefore, the only conclusions that can be reached at present with regard to nodulation of *Aspalathus* spp. by *B. tuberum* or by any rhizobial strain, including NGR234 and the DUS strains, is that these conditions may not allow for it. Indeed, the question as to whether *B. tuberum* can nodulate *A. carnosa* will not be resolved until it is tested on this species, but such tests will need to be done under sterile conditions similar to those used in the present study, and in comparison with positive control strains known to nodulate *Aspalathus* spp. Definitive *Aspalathus*-nodulating rhizobia are uncommon in the literature even though the genus is large (245 species), and it contains 57 known nodulated species (Sprent, 2001), which appear to make a positive contribution to the post-fire N-cycle of the fynbos (Cocks and Stock, 2001). This makes it all the more surprising that, apart from *B. tuberum* STM678, most isolates from *Aspalathus* spp. either remain unidentified (Deschodt and Strijdom, 1976; Allen and Allen, 1981) or have been only provisionally described as *Bradyrhizobium* spp. based solely on their slow growth rates many years previously (Dakora, 1998; Muofhe and Dakora, 1998; Boone *et al.*, 1999).

Unlike the other nodulating species of β-rhizobia so far described (Chen *et al.*, 2001, 2006, 2007; Vandamme *et al.*, 2002), *B. tuberum* STM678 failed to nodulate any of the three invasive *Mimosa* spp. This was expected, as nodulation gene sequences were dissimilar from the known *Mimosa*-nodulators (see Introduction), although experiments are currently underway to determine if selected *Mimosa*-nodulating β-rhizobia can nodulate *Cyclopia* spp. (G. N. Elliott, unpubl. res.). *B. tuberum* STM678 also failed to nodulate a range of legumes in all three subfamilies, but could ineffectively nodulate the promiscuous papilionoid legumes *Phaseolus vulgaris* and *Vigna angularis*, and effectively nodulate *Macroptilium atropurpureum*. Indeed, both strains of *B. tuberum* showed a particular ability to nodulate *M. atropurpureum* effectively, a species known to be very promiscuous with regard to symbionts (Trinick *et al.*, 1991), but which in spite of its promiscuity could not be nodulated effectively by other β-rhizobia, such as *Cupriavidus taiwanensis* or *B. phymatum* (Moulin *et al.*, 2001; Elliott *et al.*, 2007). However, the nitrogenase activity of *M. atropurpureum* nodulated by the *B. tuberum* strains was highly variable, ranging from undetectable to over 1000 nmol C_2H_4 $plant^{-1}$ h^{-1} in the case of the GFP-marked STM678, but only up to just over 100 nmol C_2H_4 $plant^{-1}$ h^{-1} for the wild-type and GUS-marked strains. There is no clear explanation for either the variability of the nitrogenase activity by plants inoculated by wild-type STM678 and its genetically modified variants, or the particularly high activity by some of the plants nodulated with the GFP-marked strain compared with those nodulated by the wild-type and GUS-marked strains, but TnGFP insertion position effects on symbiotic nitrogen fixation in the *Burkholderia* genome are currently being investigated (W-M. Chen, unpubl. res.). However, the high variability in nitrogenase activity of *M. atropurpureum* nodulated by *B. tuberum* (by both wild-type and genetically modified strains) may help to explain why the present study demonstrated an effective symbiois between this plant and strain STM678 whereas the earlier study of Moulin *et al.* (2001) showed an ineffective one.

ACKNOWLEDGEMENTS

Work in Dundee and York was funded by NERC, grant references NE/B505038/1 and NE/B505046/1. Work in Taiwan was funded by the National Science Council, Taipei, Taiwan, Republic of China (NSC 95-2313-B-022-001 and 95-2320-B-022-001-MY2). We thank Alan Prescott and Sam Swift for assistance with confocal microscopy, and Pete Rowell for use of his gas chromatograph. We also thank Bill Broughton, Lionel Moulin and Herman Spaink for bacterial strains.

LITERATURE CITED

Allen ON, Allen EK. 1981. The Leguminosae. A source book of characteristics, uses and nodulation. Madison, WI: The University of Wisconsin Press.

Barrett CF, Parker MA. 2005. Prevalence of *Burkholderia* sp. nodule symbionts on four mimosoid legumes from Barro Colorado Island, Panama. *Systematic and Applied Microbiology* **28**: 57–65.

Barrett CF, Parker MA. 2006. Coexistence of *Burkholderia*, *Cupriavidus*, and *Rhizobium* sp. nodule bacteria on two *Mimosa* spp. in Costa Rica. *Applied and Environmental Microbiology* **72**: 1198–1206.

Boone CM, Olsthoorn MMA, Dakora FD, Spaink HP, Thomas-Oates JE. 1999. Structural characterisation of lipo-chitin oligosaccharides isolated from *Bradyrhizobium aspalati*, microsymbionts of commercially important South African legumes. *Carbohydrates Research* **317**: 155–163.

Chen W-M, Laevens S, Lee TM, Coenye T, de Vos P, Mergeay M, Vandamme P. 2001. *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. *International Journal of Systematic and Evolutionary Microbiology* **51**: 1729–1735.

Chen W-M, Moulin L, Bontemps C, Vandamme P, Béna G, Boivin-Masson C. 2003*a*. Legume symbiotic nitrogen fixation by β-Proteobacteria is widespread in nature. *Journal of Bacteriology* **185**: 7266–7272.

Chen W-M, James EK, Prescott AR, Kierans M, Sprent JI. 2003*b*. Nodulation of *Mimosa* spp. by the β-proteobacterium *Ralstonia taiwanensis*. *Molecular Plant–Microbe Interactions* **16**: 1051–1061.

Chen W-M, de Faria SM, Straliotto R, Pitard RM, Simões-Araùjo JL, Chou Y-J, *et al*. 2005*a*. Proof that *Burkholderia* forms effective symbioses with legumes: a study of novel *Mimosa*-nodulating strains from South America. *Applied and Environmental Microbiology* **71**: 7461–7471.

Chen W-M, James EK, Chou J-H, Sheu S-Y, Yang S-Z, Sprent JI. 2005*b*. Beta-rhizobia from *Mimosa pigra*, a newly-discovered invasive plant in Taiwan. *New Phytologist* **168**: 661–675.

Chen W-M, James EK, Coenye T, Chou J-H, Barrios E, de Faria SM, *et al*. 2006. *Burkholderia mimosarum* sp. nov., isolated from root nodules of *Mimosa* spp. from Taiwan and South America. *International Journal of Systematic and Evolutionary Microbiology* **56**: 1847–1851.

Chen W-M, de Faria SM, James EK, Elliott GN, Lin K-Y, Chou J-H, *et al*. 2007. *Burkholderia nodosa* sp. nov., isolated from root nodules of the woody Brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*. *International Journal of Systematic and Evolutionary Microbiology* **57**: 1055–1059.

Cocks MP, Stock WD. 2001. Field patterns of nodulation in fifteen *Aspalathus* species and their ecological role in the fynbos vegetation of southern Africa. *Basic Applied Ecology* **2**: 115–125.

Dakora FD. 1998. Nodulation specificity of *Aspalathus linearis* subsp. *linearis*, a shrub tea legume indigenous to the Western Cape.

In: Elmerich C, Kondorosi A, Newton WE, eds. *Biological nitrogen fixation for the 21st century.* Dordrecht: Kluwer, 671–672.

Deschodt CC, Strijdom BW. 1976. Effective nodulation of *Aspalathus linearis* by rhizobia from other *Aspalathus* species. *Phytophylactica* **8**: 103–104.

Elliott GN, 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. and fixes nitrogen *ex planta. New Phytologist* **173**: 168–180.

Estrada de Los Santos P, Bustillos-Cristales R, Caballero-Mellado J. 2001. *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Applied and Environmental Microbiology* **67**: 2790–2798.

Gillis M, Tran Van V, Bardin R, Goor M, Hebbar P, Willems A, *et al*. 1995. Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N_2-fixing isolates from rice in Vietnam. *International Journal of Systematic Bacteriology* **45**: 274–289.

Kock M. 2004. *Diversity of root nodule bacteria associated with* Cyclopia *species.* PhD thesis, University of Pretoria, South Africa.

Kock M, Steyn PL. 2004. Determination of the diversity of root-nodulating bacteria associated with *Cyclopia* spp. *In: Proceedings of the 6th European Nitrogen Fixation Conference*, Toulouse, France, Abstract P4·3, 60.

Moulin L, Munive A, Dreyfus B, Boivin-Masson C. 2001. Nodulation of legumes by members of the β-subclass of Proteobacteria. *Nature* **411**: 948–950.

Muofhe ML, Dakora FD. 1998. *Bradyrhizobium* species isolated from indigenous legumes of the Western Cape exhibit high tolerance of low pH. In: Elmerich C, Kondorosi A, Newton WE, eds. *Biological nitrogen fixation for the 21st century.* Dordrecht: Kluwer, 519.

Muofhe ML, Dakora FD. 1999. Nitrogen nutrition in nodulated field plants of the shrub tea legume *Aspalathus linearis* assessed using ^{15}N natural abundance. *Plant and Soil* **209**: 181–186.

Perin L, Martínez-Aguilar L, Castro-González R, Estrada-de los Santos P, Cabellos-Avelar T, Guedes HV, *et al*. 2006. Diazotrophic *Burkholderia* species associated with field-grown maize and sugarcane. *Applied and Environmental Microbiology* **72**: 3103–3110.

Pueppke SG, Broughton WJ. 1999. *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. *Molecular Plant–Microbe Interactions* **12**: 293–318.

Rasolomampianina R, Bailly X, Fetiarison R, Rabevohitra R, Béna G, Ramaroson L, *et al*. 2005. Nitrogen-fixing nodules from rose wood legume trees (*Dalbergia* spp.) endemic to Madagascar host seven different genera belonging to α- and β-Proteobacteria. *Molecular Ecology* **14**: 4135–4146.

Reis VM, Estrada-de los Santos P, Tenorio-Salgado S, Vogel J, Stoffels M, Guyon S, *et al*. 2004. *Burkholderia tropica* sp. nov., a novel nitrogen-fixing, plant-associated bacterium. *International Journal of Systematic and Evolutionary Microbiology* **54**: 2155–2162.

Somasegaran P, Hoben HJ. 1994. Handbook for rhizobia: methods in legume–rhizobium technology. New York: Springer.

Sprent JI. 2001. *Nodulation in legumes.* Kew: Royal Botanic Gardens.

Sprent JI. 2007. Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation. *New Phytologist* **174**: 11–25.

Sprent JI, James EK. 2007. Legume evolution: where do nodules and mycorrhizas fit in? *Plant Physiology* **144**: 575–581.

Spriggs AC, Dakora FD. 2007. Competitive ability of selected *Cyclopia* Vent. rhizobia under glasshouse and field conditions. *Soil Biology Biochemistry* **39**: 58–67.

Spriggs AC, Stock WD, Dakora FD. 2003. Influence of mycorrhizal associations on foliar $\delta^{15}N$ values of legume and non-legume shrubs and trees in the fynbos of South Africa: implications for estimating N_2 fixation using the ^{15}N natural abundance method. *Plant and Soil* **255**: 495–502.

Sy A, Giraud E, Jourand P, Garcia N, Willems A, de Lajudie P, *et al*. 2001*a*. Methylotrophic *Methylobacterium* bacteria nodulate fix nitrogen in symbiosis with legumes *Journal of Bacteriology* **183**: 214–220.

Sy A, Giraud E, Samba R, de Lajudie P, Gillis M, Dreyfus B. 2001*b*. Certaines légumineuses du genre *Crotalaria* sont spécifiquement nodulées par une nouvelle espèce de *Methylobacterium. Canadian Journal of Microbiology* **47**: 503–508.

Trinick MJ. 1980. Relationships amongst the fast-growing rhizobia of *Lablab purpureus*, *Leucaena leucocephala*, *Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. *Journal of Applied Bacteriology* **49**: 39–53.

Trinick MJ, Miller M, Hadobas PA. 1991. Formation and structure of root nodules induced on *Macroptilium atropurpureum* inoculated with various species of *Rhizobium*. *Canadian Journal of Botany* **69**: 1520–1532.

Vandamme P, Goris J, Chen WM, de Vos P, Willems A. 2002. *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. *Systematic and Applied Microbiology* **25**: 507–512.

Van den Eede G, Deblaere R, Goethals K, Van Montagu M, Holsters M. 1992. Broad host range and promoter selection vectors for bacteria that interact with plants. *Molecular Plant–Microbe Interactions* **5**: 228–234.

Vasse J, de Billy F, Camut S, Truchet G. 1990. Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *Journal of Bacteriology* **172**: 4295–4306.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**: 697–703.

van Wyk B-E. 2005. Podalyrieae. In: Lewis GP, Schrire B, Mackinder B, Lock M, eds. *Legumes of the World.* Kew: Royal Botanic Gardens, 267–271.