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Legume Symbiotic Nitrogen Fixation by β-Proteobacteria Is Widespread in Nature

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Following the initial discovery of two legume-nodulating *Burkholderia* strains (L. Moulin, A. Munive, B. Dreyfus, and C. Boivin-Masson, Nature 411:948–950, 2001), we identified as nitrogen-fixing legume symbionts at least 50 different strains of *Burkholderia caribensis* and *Ralstonia taiwanensis*, all belonging to the β-subclass of proteobacteria, thus extending the phylogenetic diversity of the rhizobia. *R. taiwanensis* was found to represent 93% of the *Mimosa* isolates in Taiwan, indicating that β-proteobacteria can be the specific symbionts of a legume. The *nod* genes of rhizobial β-proteobacteria (β-rhizobia) are very similar to those of rhizobia from the α-subclass (α-rhizobia), strongly supporting the hypothesis of the unique origin of common *nod* genes. The β-rhizobial *nod* genes are located on a 0.5-Mb plasmid, together with the *nifH* gene, in *R. taiwanensis* and *Burkholderia phymatum*. Phylogenetic analysis of available *nodA* gene sequences clustered β-rhizobial sequences in two *nodA* lineages intertwined with α-rhizobial sequences. On the other hand, the β-rhizobia were grouped with free-living nitrogen-fixing β-proteobacteria on the basis of the *nifH* phylogenetic tree. These findings suggest that β-rhizobia evolved from diazotrophs through multiple lateral *nod* gene transfers.

Members of the Leguminosae, comprising about 18,000 species, play an important ecological role, with representatives in nearly every type of plant on Earth. Most species are able to form nitrogen-fixing symbioses with specific bacteria known as rhizobia. The recent identification of two β-proteobacterial strains of the genus *Burkholderia* able to nodulate legumes (10) changed the long-held dogma that only bacteria of the α subdivision are able to nodulate legumes (18, 23). These two strains were subsequently described as *Burkholderia tuberum* and *Burkholderia phymatum* (24). In addition, eight strains isolated from root nodules of *Mimosa* spp. were recently described as *Ralstonia taiwanensis*, also classified as β-proteobacteria (1), although their nodulation capacity was not confirmed. The terms α- and β-rhizobia were proposed to distinguish the rhizobial α- and β-proteobacteria, respectively (10). This unexpected discovery raised the question as to whether nodulation by β-proteobacteria is an extremely rare phenomenon or whether it had simply been overlooked until now. Moreover, the fact that the first two nodulating *Burkholderia* strains were isolated from *Aspalathus* and *Machaerium* spp., which are known to be associated with *Bradyrhizobium* (2, 12), may suggest that these β-proteobacteria are not the specific partners of the respective host legumes.

In this article, we confirm the widespread phylogenetic diversity of nitrogen-fixing legume symbionts by identifying as β-rhizobia an additional 2 *Burkholderia* strains from the species *Burkholderia caribensis* and a collection of at least 44 *R. taiwanensis* strains. These data increase to four the number of different β-rhizobial species identified so far, originating from three different continents. Moreover, we show that *R. taiwanensis* is the preferred partner of *Mimosa pudica* and *Mimosa diplotricha* in Taiwan. β-Rhizobia possess *nod* and *nif* genes which are very similar to those of α-rhizobia and which are located on a symbiotic plasmid. Phylogenetic analysis of available *nodA* and *nifH* genes from α- and β-proteobacteria suggests that β-rhizobia have evolved from diazotrophs through multiple lateral gene transfers.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Mimosa* strains used in these studies are listed in Table 1. *B. phymatum* STM815, isolated from *Machaerium lunatum* in French Guiana, was previously described (10, 24). *Mimosa* strains were isolated from root nodules collected at 14 sites in Taiwan (Fig. 1) by using a previously described isolation procedure (1). Strains were maintained and grown on yeast extract-mannitol medium (18) at 28°C.

DNA manipulation. For pulsed-field gel electrophoresis (PFGE) genotyping, agarose plugs containing intact bacterial genomic DNA were digested with *Xba*I (Boehringer Mannheim) and subjected to electrophoresis on 1.2% SeaKem GTG agarose (FMC) gels in 0.5× Tris-borate-EDTA buffer for 24 h at 14°C with a pulse ramp of 5 to 35 s at 200 V (LKB 2015 system; Pharmacia). For PFGE genome organization analysis, intact genomic DNA in agarose plugs was electrophoresed on an 0.8% agarose gel in Tris-agarose-EDTA for 41 h with a pulse time of 500 s at 100 V (CHEF-Mapper XA system; Bio-Rad). PFGE agarose gels were blotted on nylon membranes (Hybond), hybridized with ^{32}P-labeled *nodA*, *nodC*, and *nifH* PCR products for 17 h at 65°C, and washed at 55°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate.

Nearly full-length 16S ribosomal DNA (rDNA) was amplified and sequenced as previously described (1). 16S rDNA PCR-restriction fragment length polymorphism (RFLP) analysis was performed as described previously (1), except

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TABLE 1. Rhizobial strains and relevant characteristics

Strain(s) (no. of isolates)	Host plant	Geographical origin[a]	PCR-RFLP pattern		PFGE pattern	Reference or source
			16S rDNA	*nodA*		
Ralstonia taiwanensis						
LMG 19425	*Mimosa diplotricha*	Pingtung	I	V	E	1
TJ1 to TJ4 (4)	*Mimosa pudica*	Pingtung	I	I	A	This study
TJ12 (1)	*Mimosa pudica*	Taoyuan	I	I	A	This study
TJ13 to TJ18 (6)	*Mimosa pudica*	Miaoli	I	V	A	This study
TJ19 to TJ28 (10)	*Mimosa pudica*	Taichung	I	I	A	This study
TJ29 (1)	*Mimosa pudica*	Nantou	I	II	B	This study
TJ30 to TJ35 (6)	*Mimosa pudica*	Nantou	I	I	A	This study
TJ36 to TJ39 (4)	*Mimosa pudica*	Tainan	I	V	A	This study
TJ41 to TJ48 (8)	*Mimosa pudica*	Kaohsiung	I	I	A	This study
TJ49 to TJ57 (9)	*Mimosa pudica*	Hengchuen	I	V	A	This study
TJ60 and TJ61 (2)	*Mimosa pudica*	Eluanbi	I	V	I	This study
TJ62 to TJ64 (3)	*Mimosa pudica*	Hualien	I	I	A	This study
TJ65 to TJ77 (13)	*Mimosa pudica*	Penghu	I	I	A	This study
TJ78 to TJ86 (9)	*Mimosa diplotricha*	Pingtung	I	I	A	This study
TJ87 (1)	*Mimosa diplotricha*	Pingtung	I	V	E	This study
TJ89 to TJ97 (9)	*Mimosa diplotricha*	Taipei	I	V	A	This study
TJ99 to TJ107 (9)	*Mimosa diplotricha*	Taoyuan	I	V	A	This study
TJ108 to TJ110 (3)	*Mimosa diplotricha*	Taichung	I	III	O	This study
TJ112 to TJ119 (8)	*Mimosa diplotricha*	Taichung	I	IV	P	This study
TJ120 to TJ122 (3)	*Mimosa diplotricha*	Taichung	I	V	N	This study
TJ123 and TJ124 (2)	*Mimosa diplotricha*	Wuchi	I	V	A	This study
TJ125 (1)	*Mimosa diplotricha*	Wuchi	I	V	D	This study
TJ128 to TJ130 (3)	*Mimosa diplotricha*	Nantou	I	IV	D	This study
TJ131 and TJ132 (2)	*Mimosa diplotricha*	Nantou	I	V	E	This study
TJ137 and TJ138 (2)	*Mimosa diplotricha*	Nantou	I	V	A	This study
TJ141 (1)	*Mimosa diplotricha*	Yunlin	I	V	D	This study
TJ142 to TJ144 (3)	*Mimosa diplotricha*	Tainan	I	V	B	This study
TJ145 to TJ153 (9)	*Mimosa diplotricha*	Kaohsiung	I	I	A	This study
TJ154 (1)	*Mimosa diplotricha*	Kaohsiung	I	I	J	This study
TJ160 (1)	*Mimosa diplotricha*	Hengchuen	I	V	L	This study
TJ161 and TJ162 (2)	*Mimosa diplotricha*	Hengchuen	I	V	A	This study
TJ163 and TJ164 (2)	*Mimosa diplotricha*	Hengchuen	I	IV	M	This study
TJ190 to TJ199 (10)	*Mimosa diplotricha*	Penghu	I	I	A	This study
LMG 19426	*Mimosa pudica*	Pingtung	II	IV	K	1
TJ40 (1)	*Mimosa pudica*	Kaohsiung	II	II	K	This study
TJ58 and TJ59 (2)	*Mimosa pudica*	Eluanbi	II	II	K	This study
TJ111 (1)	*Mimosa diplotricha*	Taichung	II	IV	G	This study
TJ126 and TJ127 (2)	*Mimosa diplotricha*	Nantou	II	IV	C	This study
TJ133 and TJ134 (2)	*Mimosa diplotricha*	Nantou	II	IV	F	This study
TJ135 and TJ136 (2)	*Mimosa diplotricha*	Nantou	II	IV	B	This study
TJ139 and TJ140 (2)	*Mimosa diplotricha*	Yunlin	II	IV	G	This study
TJ155 to TJ159 (5)	*Mimosa diplotricha*	Hengchuen	II	IV	K	This study
LMG 19424	*Mimosa pudica*	Pingtung	III	V	H	1
TJ5 to TJ10 (6)	*Mimosa pudica*	Pingtung	III	V	H	This study
TJ98 (1)	*Mimosa diplotricha*	Taipei	III	V	H	This study
LMG 19430	*Mimosa diplotricha*	Pingtung	IV	V	R	1
TJ11 (1)	*Mimosa pudica*	Pingtung	IV	V	Q	This study
TJ88 (1)	*Mimosa diplotricha*	Pingtung	IV	V	R	This study
Rhizobium sp.						
TJ167[b] to TJ169 (3)	*Mimosa diplotricha*	Taoyuan	V	ND	T	This study
TJ173[b] to TJ176 (4)	*Mimosa diplotricha*	Nantou	V	ND	X	This study
TJ171[b] (1)	*Mimosa diplotricha*	Hualien	VI	ND	V	This study
TJ172[b] (1)	*Mimosa diplotricha*	Hualien	VII	ND	W	This study
TJ189 (1)	*Mimosa diplotricha*	Pingtung	VII	ND	W	This study
Burkholderia caribensis						
TJ182[b] (1)	*Mimosa diplotricha*	Pingtung	VIII	ND	Y	This study
TJ183 (1)	*Mimosa pudica*	Pingtung	VIII	ND	Y	This study
Sinorhizobium sp. strain TJ170[b] (1)	*Mimosa pudica*	Penghu	IX	ND	U	This study

[a] Sites were located in Taiwan (Fig. 1).
[b] 16S rDNA of the indicated strain was sequenced as part of this study. ND, not determined.

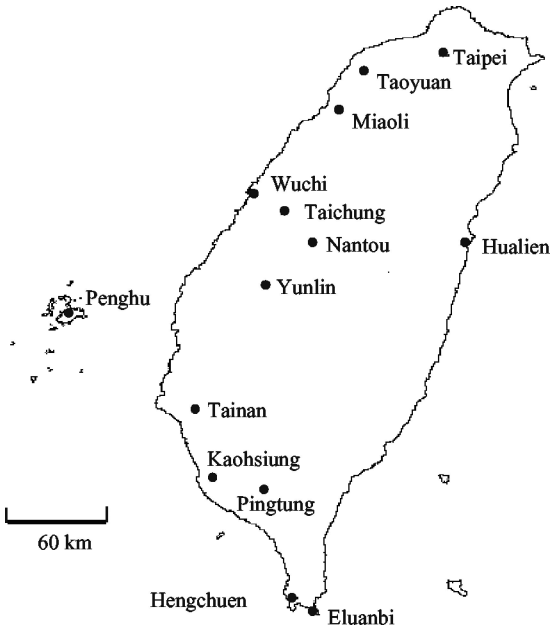

FIG. 1. Sampling sites for *M. pudica* and *M. diplotricha* in Taiwan.

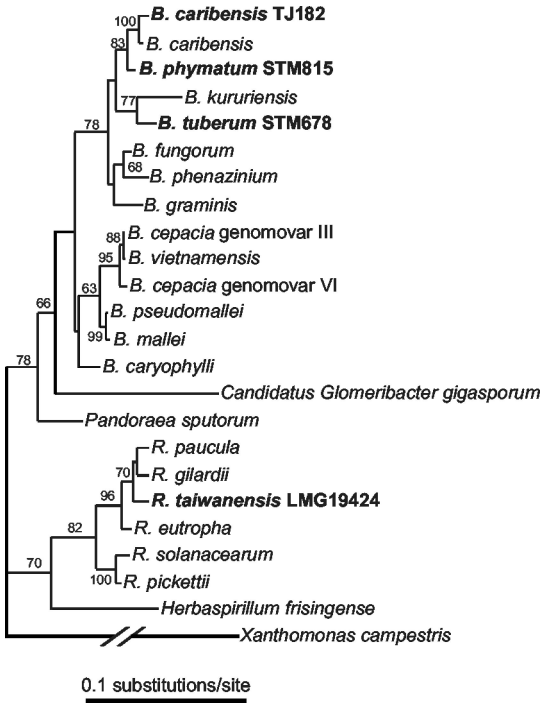

FIG. 2. 16S rDNA tree showing phylogenetic positions of legume-nodulating *Ralstonia* and *Burkholderia* species within the β-proteobacteria. The ML tree (base frequencies estimated, mutation rates drawn from an γ + INV distribution, four classes of mutations) was reconstructed by using PAUP. *Xanthomonas campestris* was used as an outgroup. Legume symbionts are shown in bold type. Nodulating *Burkholderia* strains are named according to Vandamme et al. (24). GenBank/EMBL accession numbers for the 16S rDNA sequences were AF175314 (*B. cepacia* genomovar VI), AF148556 (*B. cepacia* genomovar III), U96928 (*B. vietnamensis*), U91839 (*B. pseudomallei*), AF110188 (*B. mallei*), AB021423 (*B. carophylli*), AJ302312 (*B. phymatum*), AJ505301 (*B. caribensis*), Y17009 (*B. caribensis*), AF215705 (*B. fungorum*), AB021394 (*B. phenazinium*), U96939 (*B. graminis*), AB024310 (*B. kuruiensis*), AJ302311 (*B. tuberum*), AF139176 (*P. sputorum*), AF139176 (*G. gigasporum*), AJ238359 (*H. frisingense*), AL646072 (*R. solanacearum*), AB004790 (*R. pickettii*), AF085226 (*R. paucula*), AF300324 (*R. taiwanensis*), AF076645 (*R. gilardii*), M32021 (*R. eutropha*), and AF188831 (*X. campestris*).

that *Alu*I, *Cfo*I, *Hin*fI, and *Msp*I were used. *nodA* amplification and sequencing were performed with pairs of primers with the sequences 5′-TGGARVBTNYSY TGGGAAA-3′ and 5′-TCAYARYTCNGRNCCRTTYC-3′ (strains LMG 19424, LMG 19425, TJ171, and TJ182), 5′-TGGARVBTNYSYTGGGAAA-3′ and 5′-GGRTKNGGNCCRTCRTCRAANGT-3′ (strains TJ167, TJ172, and TJ173), and 5′-TGCRGTGGAARNTRBVYTGGGAAA-3′ and 5′-TCACARC TCKGGCCCGTTCCG-3′ (strain STM815). *nodA* PCR-RFLP analysis was performed with a 531-bp PCR product obtained with a primer pair with the sequences 5′-ATCTTGAACTCTCCGACC-3′ and 5′-GTTCGATTGTTTCGCC G-3′ and digested with *Alu*I, *Cfo*I, *Hin*fI, *Msp*I, and *Nde*II.

A 520-bp fragment containing part of the *nodH* and *nodA* genes of strain LMG 19424 was amplified and sequenced with primers with the sequences 5′-GCCA TCCACATCATCGATG-3′ and 5′-CGGCTTCGCATTGAAAGGC-3′. A 2.1-kb fragment containing the *nodB* gene and part of the *nodC* gene of strain LMG 19424 was amplified with primers with the sequences 5′-CAGATCNAG DCCBTTGAARCGCA-3′ and 5′-CTNCGNGCCCARCGNAGTTG-3′. A 1.2-kb overlapping fragment containing part of the *nodC* and *nodI* genes was amplified with primers with the sequences 5′-GTATGTTCCTAACGCTATCG CGGC-3′ and 5′-TCTTCCATVAWRTGVGTNGTCA-3′. These fragments were further sequenced with pairs of degenerate primers based on available *nodB*, *nodC*, and *nodI* alignments.

A 640-bp fragment containing part of the *nifH* gene was amplified and sequenced with primers with the sequences 5′-CGCIWTYTACGGIAARGGIG G-3′ and 5′-GGIKCRTAYTSGATIACIGTCAT-3′.

PCR products of 440 to 636 bp, used as probes for PFGE hybridization, were amplified from LMG 19424 and STM815 with primer pairs with the sequences 5′-AARGGNGGNATYGGHAARTC-3′ and 5′-GCRTAVAKNGCCATCATY TC-3′ (for *nifH*), 5′-GGTTCCACGTAAGCTTCCCTCWCCGAYCAYWTSG ARTTGGC-3′ and 5′-GCGATTACCCTGTACACCCACAGSTYKGGYCCC CGTTCCG-3′ (for *nodA*), and 5′-GGTTCCACGTAAGCTTCCCGACATGG AGTACTGGCTCGC-3′) and 5′-GCGATTACCCTGTACACCCGACAGCCA ATCGCTATTTCCG-3′ (for *nodC*).

Phylogenetic analysis. Multiple alignments were performed with CLUSTAL X (19) and manually corrected by using GeneDoc (11). Phylogenetic analysis was carried out with a maximum-likelihood (ML) approach by using PAUP version 4.0b10 (17). Two types of substitution (the substitution matrix being estimated by ML), three classes of site rate variation based on the codon structure of the DNA sequence, and base frequencies were estimated from the data by the ML approach. The same model was applied for both *nifH* and *nodA* phylogenies. Node robustness was esimated by bootstrap analysis by combining the Seqboot and DnaML programs from the PHYLIP package (5).

Plant tests. Seeds were surface sterilized with concentrated sulfuric acid for 10 min and then with 3% sodium hyperchlorite for 10 min. Plant cultivation and nodulation tests were carried out as described previously (8). Nitrogen fixation was estimated by visual observation of the vigor and foliage color of 60-day-old plants. Fresh nodules were observed under an Olympus SHZ 10 stereomicroscope. Sections 80 μm thick were prepared by using a Leica VT1000S Vibratome. Microscopic preparations were cleared with sodium hypochlorite and stained with methylene blue as described by Truchet et al. (21).

Nucleotide sequence accession numbers. EMBL accession numbers for the 16S rRNA genes are as follows: AJ505296 (TJ167), AJ505297 (TJ170), AJ505298 (TJ171), AJ505299 (TJ172), AJ505300 (TJ173), and AJ505301(TJ182). Accession numbers for the *nifH* genes are as follows: AJ505312 (TJ173), AJ505313 (TJ172), AJ505314 (TJ171), AJ505315 (TJ170), AJ505316 (TJ167), AJ505317 (TJ182), AJ505319 (STM815), AJ505320 (LMG 19424), and AJ505321 (LMG 19425). Accession numbers for the *nodA* genes are as follows: AJ505304 (LMG 19425), AJ505305 (TJ167), AJ505306 (TJ171), AJ505307 (TJ172), AJ505308 (TJ173), AJ505309 (TJ182), AJ55310 (STM1441), AJ505311 (LMG 19424), and AJ505318 (STM815). The accession number for *nodBCI* of LMG 19424 is AJ505303.

RESULTS

Most rhizobia isolated from *M. pudica* and *M. diplotricha* in Taiwan belong to the genus *Ralstonia*. To further examine the taxonomic diversity of *Mimosa* nodule isolates, 190 new iso-

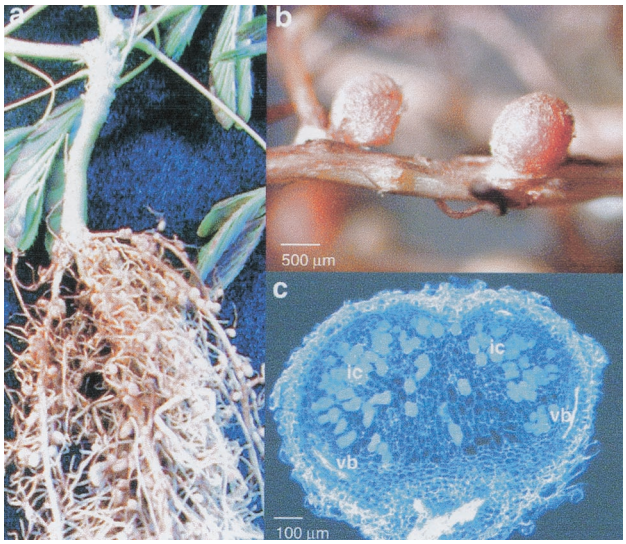

FIG. 3. Nodules of *M. pudica* 4 weeks after inoculation with *R. taiwanensis* LMG 19424. (a) Nodulated roots. (b) Root segment with pink nodules. (c) Longitudinal section showing the structure of a nodule. Plant tissue was cleared with sodium hypochlorite and stained with methylene blue as described by Truchet et al. (21). ic, infected cells; vb, vascular bundles.

lates were recovered from root nodules of *M. pudica* and *M. diplotricha* plants growing in 14 different areas in Taiwan (Table 1).

16S rDNA PCR-RFLP analysis grouped 177 isolates together with *R. taiwanensis* reference strains (Table 1). Additional PFGE and *nodA* PCR-RFLP (see below) analyses showed that these *R. taiwanensis* isolates represented at least 44 different strains (Table 1). The remaining 13 isolates fell into six PFGE pattern groups. 16S rDNA sequencing of representative strains of these groups showed that they belonged to the genus *Burkholderia* (strains TJ182 and TJ183) (Fig. 2), the genus *Rhizobium* (TJ167 to TJ169, TJ171 to TJ176, and TJ189) (data not shown), or the genus *Sinorhizobium* (TJ170) (data not shown) (Table 1). Further taxonomical analysis identified TJ182 and TJ183 as *B. caribensis* (24) (Fig. 2).

Representatives of the *Mimosa* isolate collection were double-checked for their ability to nodulate *M. pudica* under axenic laboratory conditions. The four *R. taiwanensis* strains tested, LMG 19424, LMG 19425, LMG 19426, and LMG 19430, formed nitrogen-fixing nodules (Fig. 3a and b) from which the original inoculated bacteria could be reisolated. The nodules displayed a genuine nodule structure, with central infected tissue containing cells with intracellular bacteria and peripheral tissue with vascular bundles (Fig. 3c). *B. caribensis* TJ182 and TJ183, as well as *Rhizobium* sp. strains TJ167 and TJ173, were able to effectively nodulate *M. pudica* (data not shown). On the other hand, *Sinorhizobium* sp. strain TJ170 was unable to nodulate either *M. pudica* or *M. diplotricha* (data not shown).

***Ralstonia* and *Burkholderia nod* and *nif* genes are very similar to those of α-rhizobia.** In α-rhizobia, the ability to nodulate requires the *nodABC* genes, responsible for the synthesis of the Nod factor core structure (13, 14). These three genes are all present in the first β-rhizobium identified, *B. tuberum* STM678.

TABLE 2. Sequence identities among NodA amino acid sequences[a]

Sequence	% Identity to the following sequence:						
	Bca	Bp	Ac	Sme	WM9	TJ167	TJ172
Rt	99.4	79.8	60.1	63.0	66.5	69.4	68.2
Bca		80.2	60.0	63.2	66.8	70.0	67.9
Bp			64.3	68.7	69.2	69.8	72.0
Ac				55.6	56.1	57.4	58.4
Sme					61.7	64.5	61.6
WM9						69.5	70.0
TJ167							81.6

[a] Rt, *R. taiwanensis* LMG 19425; Bca, *B. caribensis* TJ182; Bp, *B. phymatum* STM815; Ac, *A. caulinodans* ORS571; Sme, *Sinorhizobium meliloti* 2011; WM9, *Bradyrhizobium* sp. strain WM9; TJ167, *Rhizobium* sp. strain TJ167; TJ172, *Rhizobium* sp. strain TJ172. NodA sequence lengths ranged from 170 amino acids (partial sequences) to 197 amino acids (complete sequences). Percent identities were calculated from 170-amino-acid sequences.

To investigate whether the other β-rhizobial species identified so far also possess essential *nod* genes, we searched for the presence of *nodA* in a collection of β-rhizobial strains. So far, sequences homologous to *nodA* have not been identified in nonrhizobial bacteria, and this gene therefore constitutes—together with the *nodBC* genes—a molecular signature for rhizobia. We found that an internal *nodA* sequence (from bp 44 to 574) could be amplified from 181 *Ralstonia* isolates (177 new isolates and 4 *R. taiwanensis* reference strains) (data not shown). The *nodA* genes of *R. taiwanensis* LMG 19424 and LMG 19425 were sequenced, as were those of *B. caribensis* TJ182 and *B. phymatum* STM815. Partial *nodA* sequences of four α-rhizobial isolates were also determined. Sequence similarities between α- and β-rhizobial NodA proteins ranged from 60% (*B. caribensis* and *Azorhizobium caulinodans*) to 72% (*B. phymatum* and *Rhizobium* sp. strain TJ172) (Table 2), confirming that the *nodA* genes of β-proteobacteria are very similar to those of α-rhizobia. Interestingly, α- and β-rhizobial symbionts of *M. diplotricha* possess unrelated NodA sequences (Table 2).

We also searched for the presence of other nodulation genes, besides *nodA*, in *R. taiwanensis* LMG 19424 by PCR amplification (see Materials and Methods). Analysis of the amplified DNA sequences revealed the presence of the common *nodBC* genes preceded by a NodD-dependent regulatory sequence (*nod* box), as well as part of the *nodH* gene, involved in Nod factor sulfation, and the *nodI* gene, presumably involved in Nod factor transport. Sequencing indicated that *nodA* is separated from *nodBC*. Such genetic nonlinkage of *nodABC* was found previously for *B. tuberum* (10) as well as for several rhizobia (25). Sequence similarity with rhizobial Nod proteins available in databases ranged from 32% (*A. caulinodans*) to 58% (*Mesorhizobium* sp. strain N33) for NodB and from 49% (*Rhizobium gallicum*) to 74% (*Rhizobium etli*) for NodC.

Part of the *nifH* gene, encoding dinitrogenase reductase, a key enzyme in nitrogen fixation, was also amplified and sequenced in representative strains of *R. taiwanensis*, *B. caribensis*, *B. phymatum*, and *Rhizobium* spp. (data not shown).

***nod* and *nif* genes are located on a plasmid in *R. taiwanensis* and *B. phymatum*.** Genes required for nodulation and symbiotic nitrogen fixation are often clustered and located on large plasmids (9) or mobile symbiotic islands (15). To determine

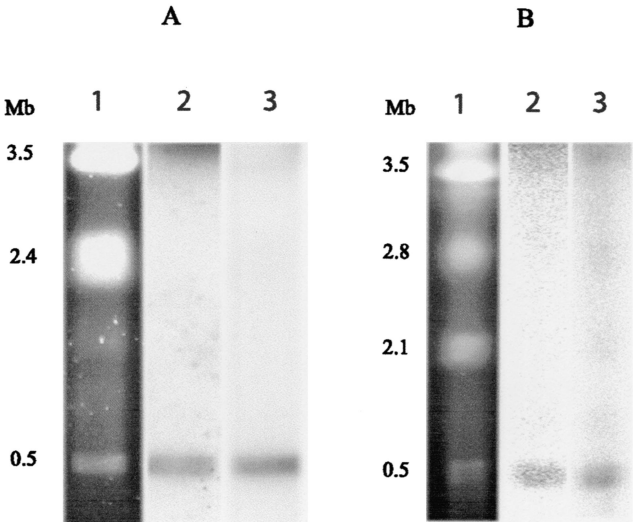

FIG. 4. Locations of *nodA* and *nifH* genes on replicons of *R. taiwanensis* LMG 19424 (A) and *B. phymatum* STM815 (B). Lane 1, PFGE of undigested genomic DNA stained with ethidium bromide; lanes 2 and 3, autoradiographs of blotted PFGE gels hybridized with *nodA* and *nifH* probes, respectively. Sizes of replicons are indicated on the left.

the locations of symbiotic genes in the genomes of the new β-rhizobia, we first examined the genome organization of *Ralstonia* and *Burkholderia* representatives by using PFGE (Fig. 4). Two high-molecular-weight replicons with apparent sizes of 3.5 and 2.4 Mb and a smaller replicon of about 0.5 Mb were identified for *R. taiwanensis* LMG 19424, while *B. phymatum* STM815 possesses replicons of approximately 3.5, 2.8, 2.1, and 0.5 Mb. No readable PFGE profile could be obtained with *B. tuberum*. To determine which replicons carry the symbiotic genes, Southern blots of PFGE agarose gels were hybridized with *nifH* and either *nodA* or *nodC* probes. Both *nod* and *nif* probes hybridized with the smallest 0.5-Mb replicons of *R. taiwanensis* and *B. phymatum* (Fig. 4). These symbiotic replicons did not hybridize with parental strain 16S rRNA, suggesting that they are genuine plasmids (data not shown).

Hence, the clustering of nodulation and nitrogen fixation genes is a common feature of α- and β-rhizobia.

Phylogenetic analysis of *nodA* and *nifH* genes of α- and β-rhizobia. Phylogenetic analysis of 42 *nodA* sequences—including most available α-rhizobial and four β-rhizobial sequences—resulted in the ML tree shown in Fig. 5. The four β-proteobacteria fell into two strongly supported clades. *B. phymatum*, *B. caribensis*, and *R. taiwanensis* strains clustered in the same clade. The *nodA* sequence closest to this clade comes from the highly divergent and atypical *A. caulinodans*, although this finding may have resulted from a long branch attraction artifact (4). *B. tuberum* and *Methylobacterium nodulans* fell into a separate and strongly supported cluster. Interestingly, the β-rhizobia *R. taiwanensis* and *B. caribensis* and the α-rhizobia *Rhizobium* sp. strains TJ167 and TJ172 isolated from *M. diplotricha* clustered separately in the *nodA* tree, suggesting that their nodulation genes have different origins.

The clustering of the β-rhizobial sequences in different *nodA* lineages intertwined with α-rhizobial sequences suggested that multiple *nod* gene transfers have occurred between the two subclasses of proteobacteria. Indeed, a single transfer of nodulation genes between α- and β-proteobacteria would have led to a single branch of β-proteobacteria within the rest of the tree, which is composed of α-proteobacteria. Constraining the four β-rhizobial symbionts to the same clade led to a tree that was only marginally less likely than the ML tree (*P* value, 0.083, as determined by the Shimodeira-Hasegawa test implemented in PAUP) and thus did not clearly support or infer the hypothesis of multiple *nod* gene transfers. On the other hand, the different lengths of the NodA proteins from *B. tuberum* and *R. taiwanensis* that clustered in two clades support the hypothesis of different origins for the corresponding genes. The NodA sequence from *B. tuberum* possesses at the N terminus an additional 13-amino-acid segment that is characteristic of bradyrhizobial NodA sequences (9a), while *R. taiwanensis* and the genera *Azorhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Rhizobium* all lack this NodA N-terminal extension. Moreover, the similarity between *B. caribensis* TJ182 and *R. taiwanensis* LMG 19424 *nodA* sequences (97.4% identity) indicates that *nod* gene transfer may have occurred between β-proteobacteria, as already suggested for α-proteobacteria (16).

Interestingly, the phylogeny of the nitrogen fixation gene *nifH* provides a representation of the rhizobia different from that of the phylogeny of *nodA* (Fig. 6). Indeed, some of the groupings within the *nifH* tree corresponded to the phylogeny of the organisms as deduced from comparative 16S rDNA analysis, although α- and β-proteobacteria did not form distinct and monophyletic clades. Moreover, the *nifH* tree grouped together free-living and symbiotic nitrogen-fixing *Burkholderia* and *Ralstonia* strains. An example of a representative organism is *B. tuberum*, which grouped with *M. nodulans* in the *nodA* phylogeny but grouped with other β-proteobacteria in the *nifH* phylogeny. Constraining either α-and β-rhizobia or *M. nodulans* and *B. tuberum* to the same clade led to a tree that was statistically less probable than the ML tree (both with *P* values of $<10^{-4}$). These results suggest that *nod* and *nif* genes of β-rhizobia have different origins.

DISCUSSION

In this study, we have confirmed and extended the phylogenetic diversity of rhizobia initially presented in articles by Moulin et al. (10) and Chen et al. (1). We have identified as rhizobia two additional *Burkholderia* strains as well as at least 48 different *R. taiwanensis* strains isolated from *M. pudica* and *M. diplotricha*. Representative *Burkholderia* and *Ralstonia* strains fix nitrogen in symbiosis with their respective host plants, demonstrating that the root nodule β-proteobacteria are indeed true rhizobia. Detailed studies have shown that *R. taiwanensis*-induced nodule ontogeny and development are similar to those described for other, mimosa-like legumes (1a). Moreover, *R. taiwanensis* is the favored partner of *M. pudica* and *M. diplotricha* in Taiwan, indicating that nodulation by β-proteobacteria is not a rare phenomenon exhibited by certain opportunistic strains. In this respect, it should be noted that *R. taiwanensis* strains also have been isolated from *M. pudica* in India (20).

The widespread character of nodulation by β-proteobacteria

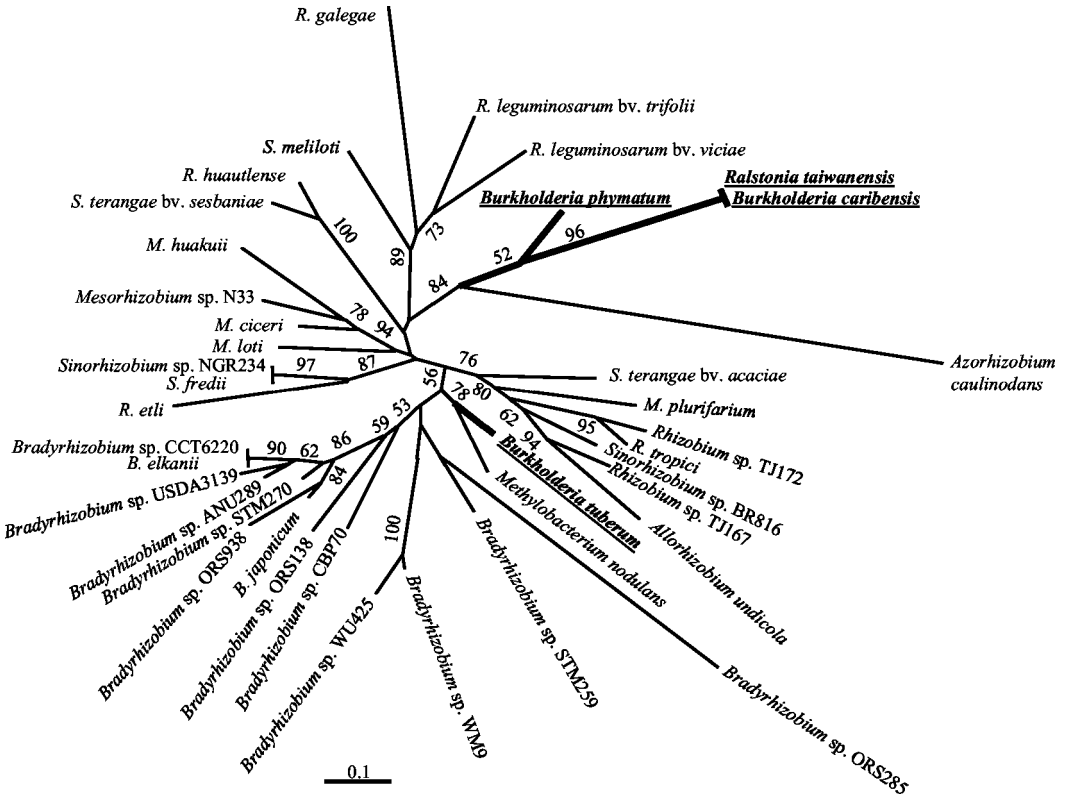

FIG. 5. Unrooted *nodA* phylogenetic tree of rhizobia. β-Proteobacterial strains are in shown in bold type and underlined. The tree was reconstructed by using an ML approach based on a 597-bp alignment (excluding the additional segment at the N-terminal part). Sequence lengths included ranged from 558 bp (*B. phymatum* STM815; partial sequence) to 597 bp (most strains). Values along branches indicate bootstrap percentages higher than 50%, based on 100 replicates. *nodA* sequences for published bacteria are available from GenBank. EMBL accession numbers and *nodA* sequences for unpublished bacteria were AJ505318 (*B. phymatum*), AJ505311 (*R. taiwanensis*), AJ505309 (*B. caribensis*), AJ300229 (*S. terangae* bv. acaciae), AJ300249 (*M. plurifarium*), AJ505307 (TJ172), AJ300234 (BR816), AJ505305 (TJ167), AJ300242 (*A. undicola*), AJ302321 (*B. tuberum*), AJ303088 (STM259), AJ430707 (WU425), AJ430730 (CBP70), AJ430715 (ORS938), AJ430712 (USDA3139), AJ430728 (CCT6220), AJ300260 (STM270), AJ300247 (*M. ciceri*), AJ300228 (*S. terangae* bv. sesbaniae), and J300235 (*R. huautlense*).

is also attested to by the phylogenetic diversity of the β-rhizobia identified so far, including one *Ralstonia* species (1) and three *Burkholderia* species (*B. caribensis*, *B. tuberum*, and *B. phymatum*) (24), as well as the fact that they have been isolated from Asia, Africa, and South America. Since many legumes and environments remain to be explored, it is highly likely that further characterization of rhizobia will reveal an even greater diversity. For many decades, standard isolation procedures have been used for rhizobia, and identification as legume symbionts through nodulation tests has required the availability of host plant seeds. These traditional approaches, coupled with the difficulty of obtaining seeds for many tropical legumes, have probably contributed to masking of the natural diversity of rhizobia.

Nitrogen fixation, which is widespread in eubacteria and archaea, is thought to be an ancestral function now lost by many bacteria (3). Conversely, nodulation is thought to have appeared recently in evolution, at the same time as the appearance of legumes on Earth, about 70 to 130 millions years ago. At that period of history, the α- and β-proteobacteria and the different rhizobial lineages already had diverged (22). The genes required for legume nodulation are thought to have been acquired subsequently by lateral transfer from undefined sources, thus converting soil saprophytes into symbionts (7). This hypothesis has been confirmed by recent data (6, 15). The presence in α- and β-rhizobia of very similar and phylogenetically related *nodABC* genes strongly supports the hypothesis of a unique origin for the common *nod* genes. However, it is not clear whether a single transfer event was responsible for the spread of nodulation genes from one subclass to the other or whether recurrent transfers occurred between the two subclasses. Our phylogenetic and NodA length analyses together suggest the occurrence of at least two lateral transfers between these two unrelated subclasses of proteobacteria, although statistical analysis did not allow this hypothesis to be ascertained. Further identification of other β-rhizobia may be useful for confirming such a hypothesis. Moreover, a comparative analysis of the *nodA*, *nifH*, and 16S rDNA trees suggests that β-rhizobia emerged through the transfer of *nod* genes to diazotrophic β-proteobacteria. Since the *nod* and *nif* genes are located on the same plasmid in the β-rhizobia investigated, it is possible that exogenous *nod* and *nif* genes were cotransferred prior to the replacement of the exogenous *nifH* gene by the indigenous gene. This level of complexity is in line with the

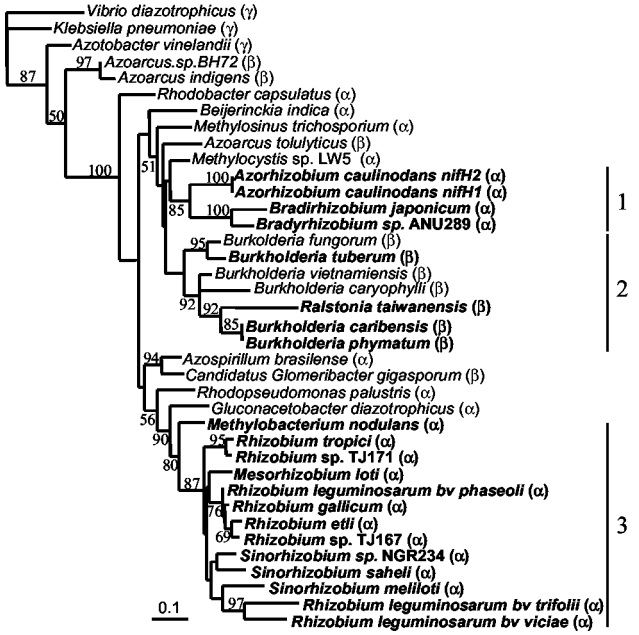

FIG. 6. *nifH* phylogenetic tree. The tree was reconstructed by using an ML approach based on an 800-bp alignment matrix (partial and full sequence lengths ranged from 336 to 797 bp). Values along branches indicate bootstrap percentages higher than 50%. The tree was rooted by using sequences from *V. diazotrophicus*, *K. pneumoniae*, and *A. vinelandii*. Rhizobia are shown in bold type, and the α-, β-, or γ-proteobacterial classification is indicated in parentheses. Clusters 1 and 3 contain α-rhizobia only, while cluster 2 includes both symbiotic and nonsymbiotic diazotrophic β-proteobacteria. *nifH* sequences for published bacteria are available from GenBank EMBL. EMBL accession numbers and *nifH* sequences for unpublished bacteria were AJ302315 (*B. tuberum*), AJ505320 (*R. taiwanensis*), AJ512206 (*B. vietnamensis*), AJ512207 (*B. caryophylli*), AJ505317 (*B. caribensis*), AJ505319 (*B. phymatum*), and AJ512205 (*M. nodulans*). *nifH* sequences from *B. fungorum*, *R. palustris*, and *R. leguminosarum* were from partially completed genome Web sites.

highly complex evolutionary history of the legume-rhizobium symbiosis.

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REFERENCES

1. **Chen, W. M., S. Laevens, T. M. Lee, T. Coenye, P. de Vos, M. Mergeay, and P. Vandamme.** 2001. *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. Int. J. Syst. Evol. Microbiol. **51:**1729–1735.

1a. **W.-M. Chen, E. K. James, A. R. Prescott, M. Kierans, and J. I. Sprent.** Nodulation of *Mimosa* spp. by the β-proteobacterium *Ralstonia taiwanensis*. Mol. Plant-Microbe Interact., in press.

2. **Deschodt, C. C., and B. W. Strijdom.** 1976. Effective nodulation of *Aspalathus linearis* by rhizobia from other *Aspalathus* species. Phytophylactica **8:**103–104.

3. **Fani, R., R. Gallo, and P. Lio.** 2000. Molecular evolution of nitrogen fixation: the evolutionary history of the nifD, nifK, nifE, and nifN genes. J. Mol. Evol. **51:**1–11.

4. **Felsenstein, J.** 1978. Cases in which parsimony or compatibility methods will be positively misleading. Syst. Zool. **27:**401–410.

5. **Felsenstein, J.** 1993. PHYLIP (phylogeny inference package), version 3.5c. University of Washington, Seattle.

6. **Galibert, F., T. M. Finan, S. R. Long, A. Puhler, P. Abola, F. Ampe, F. Barloy-Hubler, M. J. Barnett, A. Becker, P. Boistard, G. Bothe, M. Boutry, L. Bowser, J. Buhrmester, E. Cadieu, D. Capela, P. Chain, A. Cowie, R. W. Davis, S. Dreano, N. A. Federspiel, R. F. Fisher, S. Gloux, T. Godrie, A. Goffeau, B. Golding, J. Gouzy, M. Gurjal, I. Hernandez-Lucas, A. Hong, L. Huizar, R. W. Hyman, T. Jones, D. Kahn, M. L. Kahn, S. Kalman, D. H. Keating, E. Kiss, C. Komp, V. Lalaure, D. Masuy, C. Palm, M. C. Peck, T. M. Pohl, D. Portetelle, B. Purnelle, U. Ramsperger, R. Surzycki, P. Thebault, M. Vandenbol, F. J. Vorholter, S. Weidner, D. H. Wells, K. Wong, K. C. Yeh, and J. Batut.** 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science **293:**668–672.

7. **Hirsch, A. M., M. R. Lum, and J. A. Downie.** 2001. What makes the rhizobia-legume symbiosis so special? Plant Physiol. **127:**1484–1492.

8. **Lortet, G., N. Méar, J. Lorquin, B. Dreyfus, P. de Lajudie, C. Rosenberg, and C. Boivin.** 1996. Nod factor thin-layer chromatography profiling as a tool to characterize symbiotic specificity of rhizobial strains: application to *Sinorhizobium saheli*, *S. teranga* and *Rhizobium* sp. strains isolated from *Acacia* and *Sesbania*. Mol. Plant-Microbe Interact. **9:**736–747.

9. **Martinez-Romero, E., and J. Caballero-Mellado.** 1996. *Rhizobium* phylogenies and bacterial genetic diversity. Crit. Rev. Plant Sci. **15:**113–140.

9a. **L. Moulin, G. Béna, C. Boivin-Masson, and T. Stpkowski.** Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene co-transfer within the Bradyrhizobium genus. Mol. Phylogenet. Evol., in press.

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

11. **Nicholas, K. B., H. B. Nicholas, Jr., and D. W. Deerfield.** 1997. GeneDoc: analysis and visualization of genetic variation. EMBNEW News **4:**14.

12. **Parker, M. A., and A. Lunk.** 2000. Relationships of bradyrhizobia from *Platypodium* and *Machaerium* (Papilionoideae: tribe Dalbergieae) on Barro Colorado Island, Panama. Int. J. Syst. Evol. Microbiol. **50:**1179–1186.

13. **Perret, X., C. Staehelin, and W. J. Broughton.** 2000. Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. **64:**180–201.

14. **Schultze, M., and A. Kondorosi.** 1998. Regulation of symbiotic root nodule development. Annu. Rev. Genet. **32:**33–57.

15. **Sullivan, J. T., and C. W. Ronson.** 1998. Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc. Natl. Acad. Sci. USA **95:**5145–5149.

16. **Suominen, L., C. Roos, G. Lortet, L. Paulin, and K. Lindstrom.** 2001. Identification and structure of the *Rhizobium galegae* common nodulation genes: evidence for horizontal gene transfer. Mol. Biol. Evol. **18:**907–916.

17. **Swofford, D.** 1998. PAUP*: Phylogenetic Analysis Using Parsimony (and other methods), version 4. Sinauer Associates, Sunderland, Mass.

18. **Sy, A., E. Giraud, P. Jourand, N. Garcia, A. Willems, P. de Lajudie, Y. Prin, M. Neyra, M. Gillis, C. Boivin-Masson, and B. Dreyfus.** 2001. Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J. Bacteriol. **183:**214–220.

19. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.

20. **Tripathi, A. K.** 2002. Rhizobia of the β-subclass of Proteobacteria: a tale of losing the race. Curr. Sci. **82:**8–9.

21. **Truchet, G., S. Camut, F. de Billy, R. Odorico, and J. Vasse.** 1989. The *Rhizobium*-legume symbiosis. Protoplasma **149:**82–88.

22. **Turner, S. L., and J. P. W. Young.** 2000. The glutamine synthetases of rhizobia: phylogenetics and evolutionary implications. Mol. Biol. Evol. **17:** 309–319.

23. **van Berkum, P., and B. D. Eardly.** 1998. Molecular evolutionary systematics of the *Rhizobiaceae*, p. 1–24. *In* H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (ed.), The *Rhizobiaceae*. Molecular biology of plant-associated bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.

24. **Vandamme, P., J. Goris, W. M. Chen, P. de Vos, and A. Willems.** 2002. *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. Syst. Appl. Microbiol. **25:**507–512.

25. **Zhang, X. X., S. L. Turner, X. W. Guo, H. J. Yang, F. Debellé, G. P. Yang, J. Dénarié, J. P. W. Young, and F. D. Li.** 2000. The common nodulation genes of *Astragalus sinicus* rhizobia are conserved despite chromosomal diversity. Appl. Environ. Microbiol. **66:**2988–2995.