Description of new *Ensifer* strains from nodules and proposal to transfer *Ensifer adhaerens* Casida 1982 to *Sinorhizobium* as *Sinorhizobium adhaerens* comb. nov. Request for an Opinion

Anne Willems,[1] Manuel Fernández-López,[2] Estefania Muñoz-Adelantado,[2] Johan Goris,[1] Paul De Vos,[1] Esperanza Martínez-Romero,[3] Nicolas Toro[2] and Monique Gillis[1]

Correspondence
Anne Willems
anne.willems@ugent.be

[1]Laboratorium voor Microbiologie, Vakgroep Biochemie, Fysiologie, Microbiologie, Faculteit Wetenschappen, Universiteit Gent, Ledeganckstraat 35, B-9000 Belgium

[2]Grupo de Ecología Genética, Estación Experimental del Zaidin, CSIC, E-18008, Granada, Spain

[3]Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Ap. P. 565-A, Cuernavaca, Mexico

A group of four diverse rhizobial isolates and two soil isolates that are highly related to *Ensifer adhaerens* were characterized by a polyphasic approach. On the basis of DNA–DNA hybridizations and phenotypic features, these strains cannot be distinguished clearly form *Ensifer adhaerens*, a soil bacterium that was described in 1982, mainly on the basis of phenotypic characteristics. Phylogenetically, *Ensifer* and *Sinorhizobium* form a single group in the 16S rDNA dendrogram of the *α-Proteobacteria*, as well as in an analysis of partial *recA* gene sequences. They may therefore be regarded as a single genus. Because *Sinorhizobium* was proposed in 1988, according to the *Bacteriological Code* (1990 Revision) the older name, *Ensifer*, has priority. However, there are several reasons why a change from *Sinorhizobium* to *Ensifer* may not be the best solution and making an exception to Rule 38 may be more appropriate. We therefore propose the species *Sinorhizobium adhaerens* comb. nov. and put forward a Request for an Opinion to the Judicial Commission regarding the conservation of *Sinorhizobium adhaerens* over *Ensifer adhaerens*.

INTRODUCTION

In studies of populations of rhizobia, a large number of non-symbiotic rhizobial strains is often recovered (Segovia *et al.*, 1991; Sivakumaran *et al.*, 1997; Laguerre *et al.*, 1993). These strains can often become effective symbionts again when provided with a suitable symbiotic plasmid, as reported for strains from the rhizosphere of bean plants (Segovia *et al.*, 1991) and white clover (Sivakumaran *et al.*, 1997). The symbiotic phenotype can also be conferred by transfer of chromosomal symbiotic genes as demonstrated for *Mesorhizobium loti* ICMP 3153 and *Lotus corniculatus* rhizobia (Sullivan *et al.*, 1995). It was proposed that non-symbiotic rhizobia persist in soils in the absence of legume plants, and upon introduction of legumes may acquire symbiotic genes from inoculant strains (Sullivan *et al.*, 1996).

Ensifer adhaerens is a soil bacterium that is capable of adhering to and lysing other soil bacteria, although it is not an obligate predator and is not nutritionally fastidious (Casida, 1982). Because of this unusual predatory activity and other mostly morphological characteristics, the organism was considered to be most similar to the budding and appendaged bacteria *sensu Bergey's Manual of Determinative Bacteriology*, 8th edition (Buchanan & Gibbons, 1974). Because it differed significantly from all known genera in that group, it was placed in a new genus and species (Casida, 1982).

Recently the 16S rDNA sequence of *Ensifer adhaerens* has become available (Balkwill, 2003) and comparative analysis places this organism inside the genus *Sinorhizobium*. Together with the observation that non-symbiotic *Ensifer adhaerens* could effectively nodulate *Phaseolus vulgaris* and

Published online ahead of print on 19 September 2002 as DOI 10.1099/ijs.0.02264-0.

The GenBank/EMBL/DDBJ accession numbers for the new 16S rDNA sequences are AJ420773 (strain 5D19), AJ420774 (HAMBI 1631), AJ420775 (LMG 20582) and AJ420776 (LMG 20571) and for new *recA* gene sequences: AJ505595 (ATCC 33212^T), AJ505596(LMG 20582), AJ505597 (5D19), AJ505598 (HAMBI 1631), AJ505599 (BR819), AJ505600 (BR8606) and AJ505601 (LMG 21331^T).

Leucaena leucocephala when provided with symbiotic plasmids of *Rhizobium tropici* CFN 299 (Rogel *et al.*, 2001), this evidence suggests that *Ensifer adhaerens* may represent a non-symbiotic rhizobial group. Its remarkable predatory activity and the fact that phylogenetic methods were not widely used at the time have contributed to the fact that it was not readily recognized as a rhizobium when first isolated (Casida, 1982).

We report the characterization of a group of diverse rhizobial and soil isolates that are highly related to *Ensifer adhaerens*. In view of the phylogenetic position of the genus *Ensifer*, it should be merged with the genus *Sinorhizobium* which, according to the *Bacteriological Code* (1990 Revision) (Lapage *et al.*, 1992), should then be called *Ensifer*. However, we believe that in this case there are several arguments against such a change and we therefore request the opinion of the Judicial Commission on a proposal to transfer *Ensifer adhaerens* to *Sinorhizobium* as *Sinorhizobium adhaerens*.

METHODS

Bacterial strains. The strains used are listed in Table 1. They were grown on yeast extract mannitol agar (YMA) or, for the preparation of protein extracts, on tryptone yeast extract (TY) agar (de Lajudie *et al.*, 1998).

SDS-PAGE of total cellular proteins. Protein extracts were prepared as described previously (Pot *et al.*, 1994). SDS-PAGE, digitization, normalization and numerical analyses of the protein patterns were performed with the GelCompar 4.0 software (Applied Maths) as described by Pot *et al.* (1994).

Nodulation assays. Seeds of *Sesbania rostrata*, *Vigna unguiculata* UC46 and *Leucaena gigante* were surface-sterilized and germinated for 2 days on paper plates according to Fernández-López *et al.* (1998). Thereafter, the seedlings were transferred to sterile Leonard jars filled with vermiculite, inoculated with 1 ml of a 24-h culture of the appropriate strain and covered with sterile perlite. Two replicas with four plants each, per strain, were performed. Plants were grown on nitrogen-free medium (Rigaud & Puppo, 1975). Growth conditions were 16 h day and 8 h night with a temperature of 25 and 20 °C, respectively. The plants were harvested 25 days after inoculation and checked for nodulation.

DNA hybridization with *nod* gene probes. Total DNA was extracted as previously described (Laguerre *et al.*, 1992), digested with *Hin*dIII and separated on 0·8 % agarose gel. The DNA was transferred to nylon filters (Roche) using a VacuGene XL blotting apparatus according to the manufacturer's instructions (Pharmacia Biotech). Probes were obtained by PCR amplification of internal fragments of *nodC* and *nodD* genes of *Sinorhizobium meliloti* GR4 and *Rhizobium* sp. LPU83 (Del Papa *et al.*, 1999). Oligonucleotides for the *nodD* gene were: RD1 (5′-TCATCTGCGATATGGATGC-3′) and RD2 (5′-GTCTCGAACGAGATGTTCC-3′) (Van Dillewijn, 2000); and for *nodC*: C1 (5′ ACATGGAGTATTGGCTTGCC-3′) and C2 (5′-AGTTGTTGGCGCAGATATGG-3′). Amplified fragments were isolated from agarose gels and labelled with dioxigenin-11-dUTP (Roche Molecular Biochemicals) by PCR. PCR was performed using 1 cycle at 95 °C for 5 min; 30 cycles at 95 °C for 1 min, at 60 °C for 1 min, at 72 °C for 1 min; followed by a final extension at 72 °C for 5 min. Hybridizations were carried out under high-stringency conditions [formamide 50 %, 5× SSC (1× SSC is 0·15 M NaCl plus 0·015 M trisodium citrate, pH 7·0 ± 0·2), 42 °C]. Washing was carried out twice, 5 min each, in 2× SSC, 0·1 % SDS at room temperature and twice, 15 min each, in 0·1× SSC, 0·1 % SDS at 68 °C. Alternatively, the last step of the washing was carried out at 50 °C and 1× SSC. Detection of the hybridization signals was performed by using as substrate CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenylphosphate) from Roche Molecular Biochemicals with an exposure of 5 h and 18 h to an X-ray film.

DNA–DNA hybridization. DNA was prepared according to a slightly modified procedure of Marmur (1961) as described previously (Willems *et al.*, 2001). Hybridizations were carried out using a microplate method in which unlabelled DNA, non-covalently bound to a microplate, is hybridized with biotinylated probe DNA (Ezaki *et al.*, 1989; Willems *et al.*, 2001). Hybridizations were performed at 45 °C in 2× SSC in the presence of 50 % formamide.

Determination of the DNA base composition. DNA was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The resulting nucleoside mixtures were separated by HPLC using a Waters SymmetryShield C8 column at 37 °C. The solvent was 0·02 M $NH_4H_2PO_4$ (pH 4·0) with 1·5 % acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

Sequencing and phylogenetic analysis of the 16S rDNA and part of the *recA* gene. A large part of the 16S rRNA gene (corresponding to positions 39–1521 of the *Escherichia coli* rDNA) was amplified by PCR using conserved primers at the 5′ and 3′ ends of the gene. Approximately 550 bases of the *recA* gene (corresponding to positions 6–555 of the *recA* gene of *Rhizobium leguminosarum*, GenBank no. X59956) were amplified as described by Gaunt *et al.* (2001). PCR products were purified using a Qiaquick PCR purification kit (Qiagen). The 16S rDNA PCR products were sequenced using conserved primers, a BigDye DideoxyTerminator Cycle Sequencing kit (Perkin Elmer) and an ABI 310 Genetic Analyzer (Perkin Elmer) according to the manufacturer's instructions. The *recA* PCR products were sequenced using primers described by Gaunt *et al.* (2001) on an ABI 3100 Genetic Analyzer. Consensus sequences were constructed using the AutoAssembler software (Perkin Elmer). The new 16S rDNA sequence data were aligned with those of related strains from the EMBL database with the program PILEUP of the GCG software (Devereux *et al.*, 1984) and phylogenetic analyses were performed using the TREECON package (Van de Peer & De Wachter, 1994). The phylogenetic analysis of *recA* sequences was performed using the program Bionumerics (Applied Maths). Distances were calculated using the Kimura correction and clustering was performed with the neighbour-joining algorithm. Bootstrap analysis was performed using 500 (16S rDNA) or 1000 (*recA*) replications. For comparison maximum-likelihood and maximum-parsimony trees were also calculated using the program Bionumerics (Applied Maths).

Phenotypic characterization. Strains were studied using API 20NE systems (bioMérieux) according to the manufacturer's instructions. We also used API 50CH systems (bioMérieux) to study growth on different carbon sources following a previously described protocol (Kersters *et al.*, 1984). Growth was scored after 1, 2, 4 and 7 days incubation at 28 °C. Each strain, except for LMG 20582, was tested in API 50CH on two separate occasions and agreement was very good. The only differences observed occasionally were quantitative (e.g. a later onset of a positive reaction) and not qualitative.

Antibiotic sensitivities. We used the disk-diffusion method to study antibiotic sensitivities. Because the presence or concentrations of certain ions (Mg^{2+}, Ca^{2+}), sulphates, pyruvate and some trace elements can influence resistance patterns (Phillips, 1991), we modified the composition of YMA by using, per litre, 5 instead of 10 g

Table 1. Strains used

Name as received	No.*	Other no.*	Proposed classification	Source	Reference
Ensifer adhaerens	R-14065^T	ATCC 33212^T	*Sinorhizobium adhaerens* genomovar C	Soil, Central Pennsylvania, USA	Casida, (1982)
Ensifer adhaerens	R-14067	ATCC 33499	*Sinorhizobium adhaerens* genomovar C	Soil, Central Pennsylvania, USA	Casida (1982)
Ensifer sp.	R-14063	4FB6	*Sinorhizobium adhaerens* genomovar C	Agricultural soil, Wyoming, USA	Song *et al.* (2000)
Sinorhizobium sp.	LMG 20582		*Sinorhizobium adhaerens* genomovar C	Agricultural soil, Pittem, Belgium	Dejonghe *et al.* (2000)
Sinorhizobium sp.	R-9451	HAMBI 1631, MPI 3051	*Sinorhizobium adhaerens* genomovar A	*Sesbania grandiflora*, Sri Lanka	Lindström & Lethomäki (1988)
Sinorhizobium sp.	LMG 9954	BR819	*Sinorhizobium adhaerens* genomovar A	*Leucaena leucocephala*, Brazil	Moreira *et al.* (1993)
Sinorhizobium sp.	LMG 10007	BR8606	*Sinorhizobium adhaerens* genomovar A	*Pithecellobium dulce*, Brazil	Moreira *et al.* (1993)
Sinorhizobium sp.	R-7457	5D19	*Sinorhizobium adhaerens* genomovar B	*Medicago sativa*, Spain	Muñoz *et al.* (2001)
Sinorhizobium morelense	LMG 21331^T	Lc-04^T		*Leucaena leucocephala* cv. Cunnigham in cultivated soils	Wang *et al.* (2002)
Sinorhizobium sp.	LMG 20571		*Sinorhizobium morelense*	Agricultural soil, Pittem, Belgium	Dejonghe *et al.* (2000)
Sinorhizobium fredii	LMG 6217^T	USDA 205^T		*Glycine max*, China	Scholla & Elkan (1984)
Sinorhizobium xinjiangense	LMG 17930^T	CCBAU 110^T		*Glycine max*, China	Chen *et al.* (1988)
Sinorhizobium meliloti	GR4			*Medicago sativa*, Granada, Spain	Villadas *et al.* (1995)
Rhizobium sp.	LPU83			*Medicago sativa*, INTA Castelar, Argentina	Segundo *et al.* (1999)
Rhizobium tropici	CFNER90			*Gliricidia sepium*, Mexico	Acosta-Durán & Martínez-Romero (2002)

*ATCC, American Type Culture Collection, Manassas, VA, USA; CCBAU, Culture Collection of Beijing Agricultural University, Beijing, People's Republic of China; CFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de Mexico, Cuernavaca, Mexico; HAMBI, Culture Collection of the Department for Microbiology, University of Helsinki, Finland; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie Gent, Universiteit Gent, Belgium; MPI, Max-Planck-Institute, Cologne, Germany; R, Research Collection, Laboratorium voor Microbiologie Gent, Universiteit Gent, Belgium; USDA, US Department of Agriculture, Beltsville, MD, USA. T, Type strain.

mannitol and 0·0747 g $MgCl_2.6H_2O$ instead of 0·1 g $MgSO_4.7H_2O$. Care was taken to produce agar plates of uniform thickness. For each strain, a cell suspension in physiological water (0·86 % NaCl in distilled water, sterilized) was prepared with a density of 0·5 on the McFarland scale. Using sterile swabs, plates were then uniformly inoculated. Antibiotic disks were applied with a dispenser and plates were incubated for 2 days at 28 °C. Inhibition zones were determined using a digital caliper.

RESULTS AND DISCUSSION

This study started as an investigation into the identity of strain 5D19 that was isolated in the course of a large screening for *Sinorhizobium meliloti* GR4-type field isolates at the Estacíon Experimental del Zaidin (EEZ), Granada, Spain (Muñoz *et al.*, 2001), in order to analyse and characterize the diversity within a natural bacterial population. *Sinorhizobium meliloti* GR4 exhibits a highly competitive phenotype linked to the *nfe* (nodule formation efficiency) locus (Soto *et al.*, 1993) and belongs to the major infective groups of the indigenous *Sinorhizobium meliloti* population at EEZ (Villadas *et al.*, 1995). Strain 5D19 was isolated from an alfalfa nodule, which was also occupied by a *Sinorhizobium meliloti* GR4-type isolate. Strain 5D19 is unable to elicit nodules on alfalfa roots by itself (Nod^-), but it can occupy nodules when co-inoculated with *Sinorhizobium meliloti* strain GR4. It is of particular interest because it contains a group II intron closely related to *Sinorhizobium meliloti* intron RmInt1, which will be reported on separately. In a first step towards its identification, we determined the 16S rDNA sequence of strain 5D19 and found that the isolate belongs to *Sinorhizobium*, where it formed a separate line.

To search among our collection of unnamed rhizobial isolates for similar strains, we prepared a protein extract from strain 5D19 and compared the SDS-PAGE protein profile with our database. This way we found three additional strains with a similar protein profile: BR819, BR8606 and HAMBI 1631. The first two of these strains formed SDS-PAGE cluster 3 in the study of Moreira *et al.* (1993). The third strain is an unclassified fast-growing rhizobial isolate from *Sesbania grandiflora* in Sri Lanka (Lindström & Lehtomäki, 1988). The 16S rDNA of a representative of these three, HAMBI 1631, was sequenced. A sequence analysis (Fig. 1) was performed with more organisms that had meanwhile become available from the EMBL database and from a study of soil isolates in our own laboratory (Goris *et al.*, 2002). This revealed that strains 5D19 and HAMBI 1631 are closely related to the new species *Sinorhizobium morelense* (Wang *et al.*, 2002), to *Ensifer adhaerens*, a soil bacterium capable of lysing other bacteria (Casida, 1982), to two other soil isolates, LMG 20571 and LMG 20582 (Dejonghe *et al.*, 2000), and to an isolate from the gut of the termite *Nasutitermes nigriceps* (EMBL/GenBank accession no. AJ298869). All these strains form a relatively tight cluster among *Sinorhizobium* species. Although this cluster was strongly supported in the bootstrap analysis, the internal branching is poorly resolved because of the high internal sequence similarity (Fig. 1, 99–100 %). *Sinorhizobium morelense* and strain LMG 20571 form a small subgroup inside this cluster, with sequence similarities of 99·0–99·6 % with the other members of the cluster. The maximum-likelihood and parsimony trees (data not shown) gave essentially the same groupings as the neighbour-joining tree (Fig. 1).

Previously, Gaunt *et al.* (2001) demonstrated the phylogeny of *recA* supports that based on 16S rRNA genes among the rhizobia. We took this study as a starting point and extended

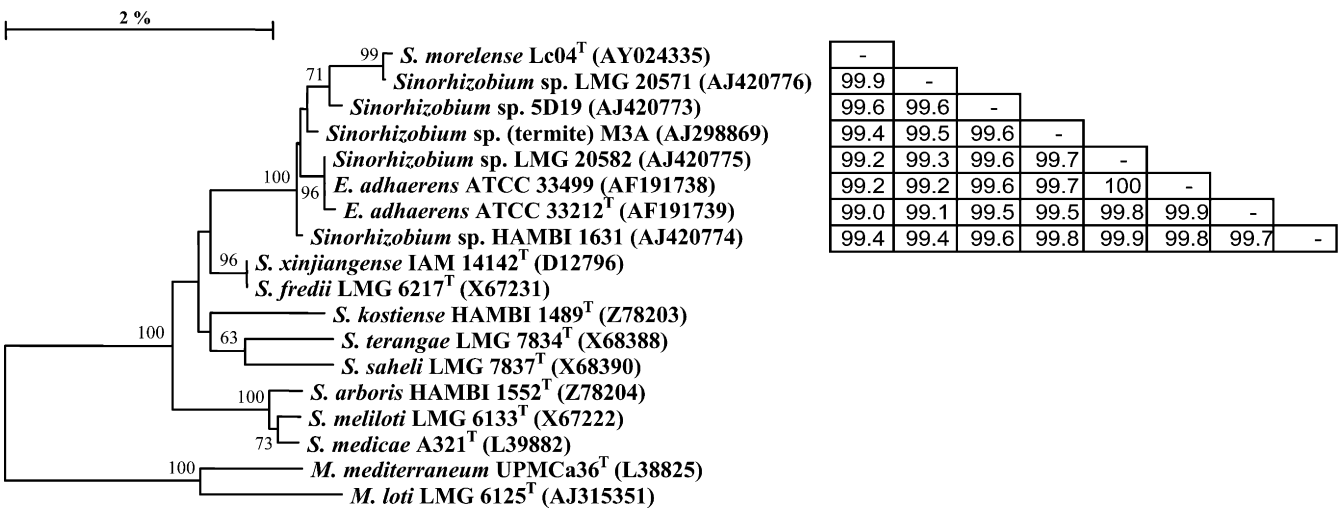

Fig. 1. Unrooted neighbour-joining tree of 16S rDNA sequences including representatives of all *Sinorhizobium* species. *Mesorhizobium loti* and *Mesorhizobium mediterraneum* were used as outgroups. Bootstrap values, calculated as percentages of 500 replications, that are higher than 50 % are given at the branching points. The small matrix gives pairwise sequence similarities within the *Sinorhizobium adhaerens–Sinorhizobium morelense* cluster.

it by determining the partial *recA* gene sequences for our unnamed strains and for *Ensifer* and *Sinorhizobium morelense* strains. The resulting neighbour-joining dendrogram (Fig. 2) confirms that *Ensifer* and *Sinorhizobium* form a single well-supported cluster (bootstrap value 93). The partial *recA* sequences within the *Sinorhizobium–Ensifer* cluster had similarity levels ranging from 85·4 to 100 %. Within this cluster a small well-supported subcluster (bootstrap value 98) was formed by the newly sequenced strains. Furthermore, partial *recA* sequences for strains HAMBI 1631, BR819 and BR8606 were identical, as were the sequences for strains LMG 20582 and ATCC 33212^T. Parsimony and maximum-likelihood analyses showed essentially the same grouping (data not shown).

We compared total cellular protein extracts of the strains by SDS-PAGE (Fig. 3). This shows that strains BR 819, BR 8606 and HAMBI 1631 had a highly similar protein profile. The protein profile of strain 5D19 is similar to that of these strains, but overall shows more similarity to the pattern of strains ATCC 33212^T, ATCC 33499, 4FB6 and LMG 20582. The protein pattern of strain LMG 20571 is distinct from all these strains and most similar to that of *Sinorhizobium morelense* Lc04^T.

We performed DNA–DNA hybridizations with the same set of strains and included also the type strains of the two *Sinorhizobium* species that are phylogenetically the closest, *Sinorhizobium fredii* (LMG 6217^T) and *Sinorhizobium xinjiangense* (LMG 17930^T) (Table 2). These data demonstrate the presence of five groups among the strains, all different from *Sinorhizobium fredii* and *Sinorhizobium xinjiangense*. The first group (group A) consists of strains BR 819, BR 8606 and HAMBI 1631. Strain 5D19 shows hybridization values of 62–67 % with group A and is therefore highly related. In view of its distinct SDS-PAGE protein profile (Fig. 3), it can be regarded as representing a separate group (group B). The third DNA group (group C) contains two *Ensifer adhaerens* strains, *Ensifer* sp. strain 4FB6 and soil isolate LMG 20582. With hybridization values above 92 % with *Ensifer adhaerens* strains, these latter two strains can be identified as *Ensifer adhaerens*. Hybridization values of DNA groups A and B with C were rather high: 43–62 % and 46–57 %, respectively. The type strain of

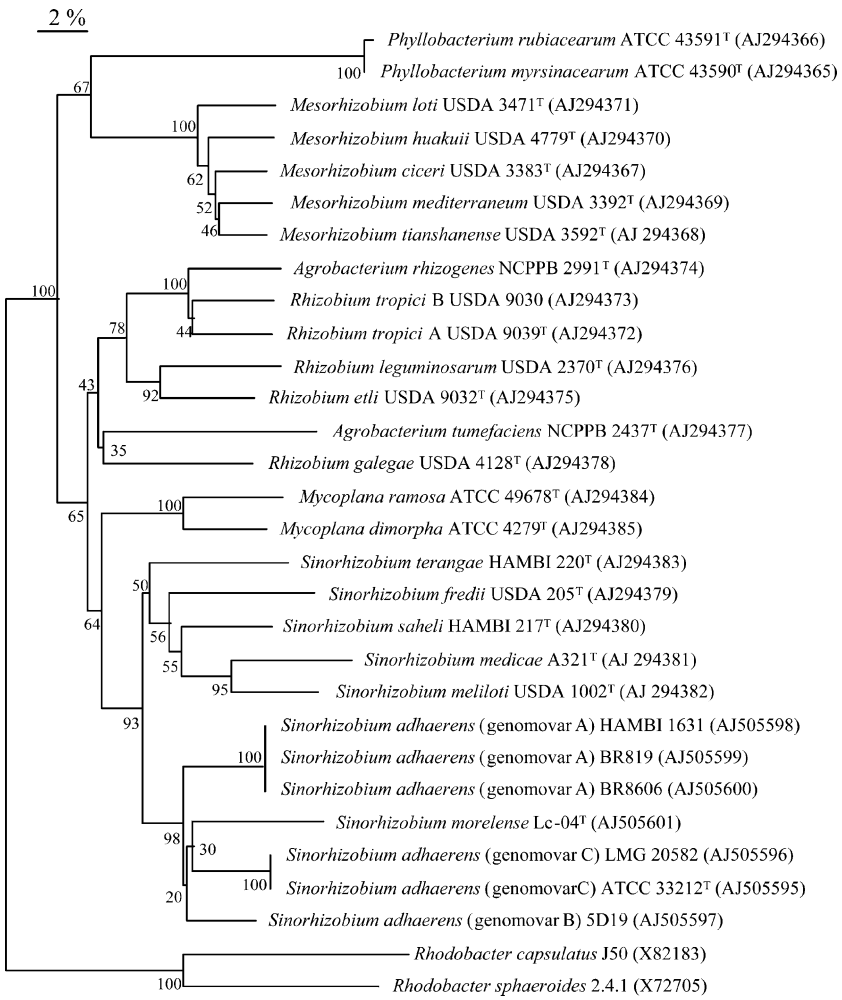

Fig. 2. Unrooted neighbour-joining tree of partial *recA* gene sequences of rhizobia and close relatives. *Rhodobacter* was used as an outgroup. Bootstrap values were calculated as a percentage of 1000 replications and are shown at branching points.

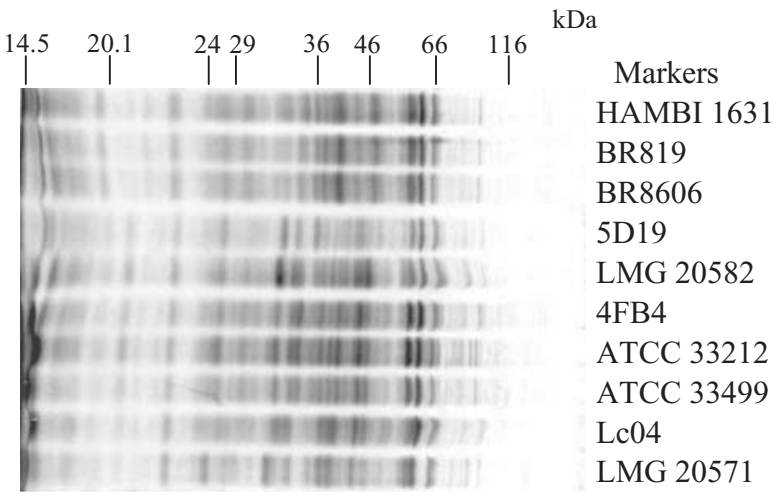

Fig. 3. SDS-PAGE protein profiles of *Sinorhizobium adhaerens* and *Sinorhizobium morelense* strains. Markers, molecular mass markers (kDa): lysozyme, 14·5; trypsin inhibitor, 20·1; trypsinogen, 24; carbonic anhydrase, 29; glyceraldehyde-3-phosphate dehydrogenase, 36; egg albumin, 45; bovine albumin, 66; and β-galactosidase, 116.

Sinorhizobium morelense (Lco4^T) shows a mean hybridization value of 59 % with *Sinorhizobium* strain LMG 20571. If we use 70 % as a cut-off value, both can be regarded as representing two further DNA groups D and E. It is striking that the grouping found by DNA–DNA hybridizations is neatly corroborated by the *recA* grouping (Fig. 2).

To clarify whether DNA groups A and B form separate species distinct from *Ensifer adhaerens* (DNA group C), and whether strain LMG 20571 was phenotypically similar to *Sinorhizobium morelense*, we characterized the strains phenotypically using API 20NE and API 50CH tests. We also tested the sensitivity of the strains to 12 antibiotics. Our data (Table 3) show that DNA groups A and B cannot be differentiated from *Ensifer adhaerens* (DNA group C) on the basis of these tests. In view of the relatively high DNA–DNA hybridization between the groups (43–62 %, Table 2), we conclude that, for now, they have to be regarded as a single species, *Ensifer adhaerens*, in line with international recommendations (Wayne *et al.*, 1987). However, in view of the genomic diversity that clearly exists in this species and that is also reflected in the protein profiles (Fig. 3) and partial *recA* sequences, we propose to create three highly related genomovars (A, B and C) inside *Ensifer adhaerens*. This terminology was proposed for phenotypically similar genomic groups (Ursing *et al.*, 1995) and a comparable genomovar system exists in *Burkholderia cepacia* (Vandamme *et al.*, 1997), where genomovars are gradually being named as differentiating data become available (Vandamme *et al.*, 2000; Coenye *et al.*, 2001).

Strain LMG 20571 is phenotypically very similar to *Sinorhizobium morelense* Lc04^T and also has a very similar protein profile (Fig. 3). It can therefore be designated a member of this species. *Sinorhizobium morelense* is distinct from *Ensifer adhaerens* in that it is urease-negative and is sensitive to nalidixic acid (Table 3).

To explore the host range of *Ensifer adhaerens* and *Sinorhizobium morelense*, nodulation tests with different

Table 2. DNA–DNA hybridization results

Strain used for labelled probe	DNA group	G+C (mol%)	Strain providing fixed DNA											
			1	2	3	4	5	6	7	8	9	10	11	12
1. *Sinorhizobium xinjiangense* LMG 17930^T		62·3	**100**				22	23			22	19	19	25
2. *Sinorhizobium fredii* LMG 6217^T		62·2		**100**			25	29		25	26	23	22	29
3. *Sinorhizobium* sp. HAMBI 1631	A	61·7			**100**	**70**	**84**	64		54	51			28
4. *Sinorhizobium* sp. BR 819	A	61·2			**100**	**100**	**100**			61	62			32
5. *Sinorhizobium* sp. BR 8606	A	61·7	23	24	**96**	**92**	**100**	67		57	51	43	31	35
6. *Sinorhizobium* sp. 5D19	B	61·4	27	28	62		68	**100**		55	54	46	34	37
7. *Sinorhizobium* sp. LMG 20582	C	62·3							**100**		**93**	**93**	46	
8. *Ensifer* sp. 4FB6	C	62·3		23			46	54		**100**	**99**			
9. *Ensifer adhaerens* ATCC 33212^T	C	62·3	25	25			45	53	**97**	**92**	**100**	**92**	41	46
10. *Ensifer adhaerens* ATCC 33499	C	62·1	23	25			49	57	**100**		**96**	**100**	40	41
11. *Sinorhizobium morelense* Lc04^T	D	61·7	22	26			32	39	41	40	41	39	**100**	66
12. *Sinorhizobium* sp. LMG 20571	E	60·8	23	23	25	19	28	29			37	36	52	**100**

Table 3. Phenotypic characters

Strains: 1, *Sinorhizobium adhaerens* HAMBI 1631; 2, *Sinorhizobium adhaerens* BR 819; 3, *Sinorhizobium adhaerens* BR 8606; 4, *Sinorhizobium adhaerens* 5D19; 5, *Sinorhizobium adhaerens* LMG 20582; 6, *Sinorhizobium adhaerens* 4FB6; 7, *Sinorhizobium adhaerens* ATCC 33212^T; 8, *Sinorhizobium adhaerens* ATCC 33499; 9, *Sinorhizobium morelense* Lc04^T; 10, *Sinorhizobium morelense* LMG 20571.

Character	Strain (genomic group)									
	1 (A)	2 (A)	3 (A)	4 (B)	5 (C)	6 (C)	7 (C)	8 (C)	9 (D)	10 (E)
API 20NE* Urease	+	+	+	+	+	+	+	+	−	−
API 50CH† L-Lyxose, xylitol	+	+	+	+	+	+	+	+	+	−
L-Sorbose	W	W	W	−	W	W	W	−	W	W
Dulcitol	W	W	W	−	−	W	W	W	W	+
Amygdalin	+	+	+	+	+	+	W	W	W	−
Melezitose	+	+	+	+	+	+	+	+	−	+
Starch	W	−	W	W	W	W	W	W	−	−
D-Tagatose	+	+	+	W	+	+	+	+	+	+
D-Fucose	−	−	−	−	−	W	−	−	W	−
Gluconate	+	−	−	−	−	W	−	W	−	−
5-Ketogluconate	+	−	−	−	−	−	−	−	−	−
Sensitivity to:‡										
Netilmycin (30)	I	I	I	I	R	I	I	I	I	R
Novobiocin (30)	R	R	R	S	S	S	S	S	S	S
Chloramphenicol (30)	R	R	R	S	R	R	R	R	I	S
Amikacin (30)	S	I	I	S	I	I	I	S	I	I
Nalidixic acid (30)	R	R	R	R	R	R	R	R	S	S
Gentamicin (10)	R	R	R	R	R	R	R	R	I	I
Kanamycin (30)	R	R	R	I	R	R	R	R	R	R

*In the API 20NE tests, all strains reduced nitrates to nitrites, hydrolysed aesculin, possessed β-galactosidase, were oxidase-positive, used glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose and malate; none of the strains produced indole, fermented glucose, possessed arginine dihydrolase or gelatinase, used gluconate, caprate, adipate, citrate or phenyl-acetate.

†In API 50CH tests, all strains used glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, adonitol, galactose, D-glucose, D-fructose, D-mannose, rhamnose, inositol, mannitol, sorbitol, methyl α-D-glucoside, *N*-acetylglucosamine, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, D-raffinose, β-gentiobiose, D-turanose, D-lyxose, L-fucose, D-arabitol and L-arabitol; none of the strains used: methyl β-xyloside, methyl α-mannoside, inulin, glycogen or 2-ketogluconate.

‡The disk-diffusion method was used; the concentration in μg per disk of the antibiotic is given in parentheses. R, Resistant (diameter inhibition zone 6–8 mm); I, intermediate (diameter 9–12 mm); S, sensitive (diameter >12 mm). All strains were sensitive to tetracycline (30) and resistant to cefuroxime (30), carbenicillin (100), streptomycin (10) and erythromycin (10).

host plants were performed with strains 5D19, BR 819, BR 8606, HAMBI 1631, Lc04^T, LMG 20571, LMG 20582, 4FB6, ATCC 33212^T, ATCC 33499, *Sinorhizobium meliloti* GR4 and *Rhizobium tropici* CNFER90. On *Leucaena gigante* plants, all the strains tested were Nod$^-$, including *Sinorhizobium morelense* Lc04^T and *Sinorhizobium* sp. strain LMG 9954 that were isolated from nodules of *Leucaena leucocephala*. A similar Nod$^-$ phenotype was obtained with *Vigna unguiculata* UC46, a tropical crop, for all strains except *Sinorhizobium* sp. HAMBI 1631 and *Rhizobium tropici* CFNER90 that exhibited a Nod$^+$ Fix$^-$ phenotype. On *Sesbania rostrata*, strains of DNA groups C, D and E (*Sinorhizobium morelense*) were Nod$^-$, while the strains that belong to DNA groups A and B and *Sinorhizobium meliloti* strain GR4 elicited empty pseudo-nodules. Controls with *Rhizobium tropici* were Nod$^+$, Fix$^+$. To determine the presence of *nod* genes, DNA hybridizations were performed with PCR probes derived from *nodC* and *nodD* genes of *Sinorhizobium meliloti* strain GR4 and *Rhizobium* strain LPU83. The following strains were tested: 5D19, HAMBI 1631, BR819, BR8606, ATCC 33212^T, ATCC 33499, LMG 20582, 4FB6, Lc04^T and LMG 20571; *Sinorhizobium meliloti* GR4 was used as a positive control. The washing steps were performed at 68 and 50 °C. At 68 °C, only the positive control gave a hybridization signal. At 50 °C, a positive signal was observed for strain BR819, but its size (20 kb) was different from that of the positive control (500 bp; data not shown). No hybridization signals were observed for the other strains tested. Whether these strains carry *nod* genes will require further investigation, although strains HAMBI 1631, BR819 and BR8606 were isolated from nodules and must have a set of nodulation genes. Our results are in line with those of Rogel *et al.* (2001), who reported that strain ATCC 33499

did not form nodules on bean, *Leucaena leucocephala*, *Vigna mungo*, *Macroptilium atropurpureum* and alfalfa and that it did not carry *nodC* or *nifH* genes.

Casida (1980) described the ability of a streak of the type strain of *Ensifer adhaerens* to track an intersecting perpendicular streak of *Micrococcus luteus*. When we performed the same experiments with all *Ensifer adhaerens* and *Sinorhizobium morelense* strains in this study, we did observe clear tracking for strains ATCC 33212^T, ATCC 33499 and HAMBI 1631 (2–5 mm after 7 days; in one instance >10 mm) and to a lesser extent for strains 5D19, Lc04^T and LMG 20571 (1–2 mm). *Escherichia coli* LMG 2092^T did not show tracking. However, tracking of 1–14 mm was observed for 8 of 11 strains tested when a perpendicular line was made with a sterile loop. Thus, the tracking experiment may not provide a reliable indication of predatory behaviour. To assess this direct microscopic observation may be needed.

Request for an Opinion

Comparative 16S rDNA sequence analysis (Fig. 1, and Balkwill, 2003) shows that *Ensifer adhaerens* is phylogenetically clearly a member of the *Sinorhizobium* subgroup of the α-*Proteobacteria*, with sequence similarities of approximately 97·9–99·9 % with *Sinorhizobium* species. This observation is corroborated by *recA* gene sequence analysis (Fig. 2) and also by probe hybridization experiments with *nolR*, a regulatory gene involved in nodulation. A probe consisting of the 5′ end of the *Sinorhizobium meliloti nolR* gene and its upstream region gave hybridization bands with different *Sinorhizobium* species as well as with *Ensifer adhaerens*, but not with eight *Rhizobium* species and *Allorhizobium undicola*. The same *Rhizobium* species did yield hybridization bands with a *Rhizobium etli nolR* probe (Toledo *et al.*, 2003). Comparison of phenotypic data (our own API 20NE, API 50CH and antibiotic-sensitivity data, as well as the literature) indicates that *Sinorhizobium* and *Ensifer* are highly similar genera. *Ensifer adhaerens* strain ATCC 33499 did not nodulate *Phaseolus vulgaris* or *L. leucocephala*, but with symbiotic plasmids of *Rhizobium tropici* it did form nitrogen-fixing nodules on these hosts (Rogel *et al.*, 2001). Our own finding of symbiotic strains very close to *Ensifer adhaerens* is further evidence of the similarities between *Ensifer* and *Sinorhizobium*. In view of all these data, it would seem logical to propose the merger of both genera.

According to Rule 38 of the *Bacteriological Code* (1990 Revision), when two genera, including their respective type strains, are merged, the oldest genus name should be retained (Lapage *et al.*, 1992). The genus *Ensifer* was proposed in 1982 (Casida, 1982) with a single species, *Ensifer adhaerens*, and only two strains. *Sinorhizobium* was proposed in 1988 (Chen *et al.*, 1988) with *Sinorhizobium fredii* (the former *Rhizobium fredii*) as type species and *Sinorhizobium xinjiangense* as a second species. Therefore, *Ensifer* is the oldest valid name and should have priority over *Sinorhizobium*. However, several arguments can be raised against this change and do require consideration.

1. *Sinorhizobium* is a well-known genus of rhizobia. Its genetics are extensively studied and the genome sequence of *Sinorhizobium meliloti* has been determined (Capela *et al.*, 2001; Finan *et al.*, 2001; Barnett *et al.*, 2001). Its is also widely studied for agronomic applications. *Ensifer*, on the other hand, is a rather less well-known organism.

2. Since its proposal, the genus *Sinorhizobium* was enlarged several times and it now contains nine species of symbiotic nitrogen-fixers, while *Ensifer* has only one species that until now contained just two strains (Nick *et al.*, 1999; Casida, 1982).

3. Replacing the name *Sinorhizobium* with *Ensifer* would seem regrettable because the word 'rhizobium' – immediately clarifying the nature of the organisms – would be lost. The name *Ensifer*, meaning 'sword-bearer', does not have the same relevance for symbiotic, nitrogen-fixing bacteria. Replacing the name *Sinorhizobium* by the name *Ensifer* is likely to meet with resistance and misunderstanding or even derision by non-taxonomists.

4. *Ensifer adhaerens* was proposed in 1982, after an extensive morphological and phenotypic characterization of one strain. On this basis, the author placed this organism in part 4 (budding and appendaged bacteria) of division II (the bacteria) in *Bergey's Manual of Determinative Bacteriology*. However, because it differed significantly from all the genera in part 4, he proposed a new genus and species for this strain (Casida, 1982). More recent studies (Balkwill, 2003), determining the 16S rDNA sequence of *Ensifer* and *Sinorhizobium* strains, demonstrate clearly that *Ensifer* is phylogenetically a member of the *Sinorhizobium* cluster and we have now confirmed this finding using *recA* sequences. When *Ensifer adhaerens* was proposed, 16S rDNA sequence analysis was not yet widely used. Had it been used, the species would have been placed close to *Rhizobium meliloti* and therefore would probably have been described as a *Rhizobium* (and later *Sinorhizobium*) species since the diversity of rhizobia and their relatedness with other, non-rhizobial, genera was not well-known at the time. Therefore, renaming *Ensifer adhaerens* to *Sinorhizobium adhaerens* can be regarded as being in accordance with Principle 9 of the *Bacteriological Code* (1990 Revision), which states that 'the name of a taxon should not be changed without sufficient reason based either on further taxonomic studies or on the necessity of giving up a nomenclature that is contrary to the Rules of this Code'. In this case, we propose that the above arguments represent 'sufficient reason based on further taxonomic studies'.

In view of these arguments, we would prefer to preserve the genus name *Sinorhizobium* over the older name *Ensifer* for *Ensifer adhaerens*. This view was also expressed at a recent meeting of the Subcommittee on *Rhizobium* and *Agrobacterium* (Lindström & Martínez-Romero, 2002).

Rule 38 also states 'if however, this choice would lead to confusion in bacteriology, the author should refer this matter to the Judicial Commission'. We believe that replacing *Sinorhizobium* by *Ensifer* would cause considerable misunderstanding and confusion among rhizo-microbiologists and we therefore ask for the opinion of the Judicial Commission on this matter. We propose to transfer *Ensifer adhaerens* to the genus *Sinorhizobium* as *Sinorhizobium adhaerens* comb. nov. The species now includes symbiotic nitrogen-fixing strains and an emended description is provided below.

Description of *Sinorhizobium adhaerens* (Casida 1982) comb. nov.

Sinorhizobium adhaerens (ad′hae.rens. L. neut. adj. *adhaerens* adherent).

The original description of *Ensifer adhaerens* was based solely on the type strain (Casida, 1982). The data presented here include the type strain and seven other strains. Some of the characteristics we studied were also reported by Casida, (1982) and are in good agreement with our findings.

Colonies on TY medium (3 days, 30 °C) are smooth, shiny, creamy-white, convex, round with a diameter of 0·5–2 mm. In some strains, neighbouring colonies merge to form a single uniform area of growth because of exopolysaccharide production. Cells are motile rods, 0·3–0·6 × 1–3·5 μm, occurring singly or in pairs. It is not known if the predatory activity towards other bacteria reported for the type strain is also present in other strains of the species. Detailed phenotypic features for all strains are given in Table 3. The G+C content of the DNA, as determined by HPLC, ranges from 61·2 to 62·3 mol%. Using DNA–DNA hybridization, three highly related genomovar groups (43–62 % hybridization) were found. Strains of groups A and B were isolated from root-nodules on various host plants; strains of group C were isolated from soil. Group A and group B strains form empty pseudonodules on *Sesbania rostrata*; they do not nodulate *Leucaena gigante* or *Vigna unguiculata*, except for strain HAMBI 1631, which is Nod^+ Fix^- on the latter plant species. Group C strains do not nodulate *Sesbania rostrata*, *Vigna unguiculata* or *Leucaena gigante*. *nodC* or *nodD* genes are not detected by using hybridization probes derived by PCR from *Sinorhizobium meliloti* GR4 and *Rhizobium* sp. LPU83. No phenotypic differentiation of the three genomovars is currently possible, but they can be distinguished by their distinct protein profiles and *recA* gene sequences. The species has a very wide geographic distribution with strains so far recognized originating from the USA, Spain, Belgium, Sri Lanka and Brazil. The possible signature sequence for *Ensifer adhaerens* proposed by Balkwill (2003) is present in strains ATCC 33499, ATCC 33212^T, LMG 20582 and 5D19, but has one mismatch in strain HAMBI 1631, two in the termite strain M3A and one in *Sinorhizobium morelense*. Of the signature positions reported for *Ensifer adhaerens* (Balkwill, 2003), only those at bases 658 (A), 659 (T), 746 (A), 747 (T) apply to *Sinorhizobium adhaerens*; they can differentiate this species from all other *Sinorhizobium* species except *Sinorhizobium morelense*.

The type strain is ATCC 33212^T (=LMG 20216^T). It was isolated from soil and is able to attach to *Micrococcus luteus* cells, causing their lysis. It is not, however, an obligate predator and is not nutritionally fastidious in the absence of prey cells. The DNA G+C content is 62·3 mol% (HPLC). The EMBL/GenBank accession number for the 16S rDNA sequence is AF191739. The type strain belongs to genomovar C.

ACKNOWLEDGEMENTS

We are grateful to David L. Balkwill for providing us with *Ensifer* strains and a copy of the unpublished chapter on *Ensifer* for *Bergey's Manual of Systematic Bacteriology*. We also thank Max M. Häggblom and Kristina Lindström for providing us with strains of *Ensifer* and *Sinorhizobium*. The work by the Grupo de Ecología Genética was supported by grant BIO99-0905 from the Comisión Asesora de Investigación Científica y Técnica; M. F.-L. is grateful to the Spanish Ministerio de Ciencia y Tecnología for a Postdoctoral contract. A. W. and M. G. are grateful to the Fund for Scientific Research – Flanders for a position as Postdoctoral Research fellow and for research and personnel grants, respectively. J. G. was supported by grant Concerted Research Action 12050797 from the Ministerie van de Vlaamse Gemeenschap, Bestuur Wetenschappelijk Onderzoek, Belgium.

REFERENCES

Acosta-Durán, C. & Martínez-Romero, E. (2002). Diversity of rhizobia from nodules of the leguminous tree *Gliricidia sepium*, a natural host of *Rhizobium tropici*. *Arch Microbiol* **178**, 161–164.

Balkwill, D. L. (2003). The genus *Ensifer*. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2 (in press). Edited by G. M. Garrity. New York: Springer Verlag.

Barnett, M. J., Fisher, R. F., Jones, T. & 23 other authors (2001). Nucleotide sequence and predicted functions of the entire *Sinorhizobium meliloti* pSymA megaplasmid. *Proc Natl Acad Sci U S A* **98**, 9883–9888.

Buchanan, R. E. & Gibbons, N. E. (editors) (1974). *Bergey's Manual of Determinative Bacteriology*, 8th edn. Baltimore: Williams & Wilkins.

Capela, D., Barloy-Hubler, F., Gouzy, J. & 25 other authors (2001). Analysis of the chromosome sequence of the legume symbiont *Sinorhizobium meliloti* strain 1021. *Proc Natl Acad Sci U S A* **98**, 9877–9882.

Casida, L. E., Jr (1980). Bacterial predators of *Micrococcus luteus* in soil. *Appl Environ Microbiol* **39**, 1035–1041.

Casida, L. E., Jr (1982). *Ensifer adhaerens*, gen. nov., sp. nov. a bacterial predator of bacteria in soil. *Int J Syst Bacteriol* **32**, 339–345.

Chen, W. X., Yan, G. H. & Li, J. L. (1988). Numerical taxonomic study of fast-growing soybean rhizobia and proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *Int J Syst Bacteriol* **38**, 392–397.

Coenye, T., Mahenthiralingam, E., Henry, D., LiPuma, J. J., Laevens, S., Gillis, M., Speert, D. P. & Vandamme, P. (2001). *Burkholderia ambifaria* sp nov., a novel member of the *Burkholderia cepacia*

complex including biocontrol and cystic fibrosis-related isolates. *Int J Syst Evol Microbiol* **51**, 1481–1490.

Dejonghe, W., Goris, J., El Fantroussi, S., Höfte, M., De Vos, P., Verstraete, W. & Top, E. (2000). Effect of dissemination of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation plasmids on 2,4-D degradation and on bacterial community structure in two different soil horizons. *Appl Environ Microbiol* **66**, 3297–3304.

de Lajudie, P., Willems, A., Nick, G. & 9 other authors (1998). Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. *Int J Syst Bacteriol* **48**, 369–382.

Del Papa, M. F., Balagué, L. J., Castro-Sowinski, S. & 9 other authors (1999). Isolation and characterization of alfalfa-nodulating rhizobia present in acidic soils of central Argentina and Uruguay. *Appl Environ Microbiol* **65**, 1420–1427.

Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Fernández-López, M., D'Haeze, W., Van Montagu, M. & Holsters, M. (1998). Changes in the glycosylation pattern at the reducing end of azorhizobial Nod factors affect nodulation efficiency. *FEMS Microbiol Lett* **158**, 237–242.

Finan, T. M., Weidner, S., Wong, K. & 9 other authors (2001). The complete sequence of the 1,683-kb pSymB megaplasmid from the N_2-fixing endosymbiont *Sinorhizobium meliloti*. *Proc Natl Acad Sci U S A* **98**, 9889–9894.

Gaunt, M. W., Turner, S. L., Rigottier-Gois, L., Lloyd-Macgilp, S. A. & Young, J. P. W. (2001). Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. *Int J Syst Evol Microbiol* **51**, 2037–2048.

Goris, J., Dejonghe, W., Falsen, E., De Clerck, E., Geeraerts, B., Willems, A., Top, E., Vandamme, P. & De Vos, P. (2002). Diversity transconjugants that acquired plasmid pJP4 or pEMT1 after inoculation of a donor strain in the A- & B-horizon of an agricultural soil and description of *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. *Syst Appl Microbiol* **25**, 340–352.

Kersters, K., Hinz, K.-H., Hertle, A., Segers, P., Lievens, A., Siegmann, O. & De Ley, J. (1984). *Bordetella avium* sp. nov., isolated from the respiratory tracts of turkeys and other birds. *Int J Syst Bacteriol* **34**, 56–70.

Laguerre, G., Mauzier, S. I. & Amarger, N. (1992). Plasmid profiles and restriction fragment lenght polymorphism of *Rhizobium leguminosarum* bv. viciae in field populations. *FEMS Microbiol Ecol* **101**, 17–26.

Laguerre, G., Bardin, M. & Amarger, N. (1993). Isolation from soil of symbiotic and nonsymbiotic *Rhizobium leguminosarum* by DNA hybridization. *Can J Microbiol* **39**, 1142–1149.

Lapage, S. P., Sneath, P. H. A., Lessel, E. F., Skerman, V. B. D., Seeliger, H. P. R. & Clark, W. A. (editors) (1992). *International Code of Nomenclature of Bacteria (1990 Revision). Bacteriological Code.* Washington, DC: American Society for Microbiology.

Lindström, K. & Lehtomäki, S. (1988). Metabolic properties, maximum growth temperature and phage sensitivity of *Rhizobium* sp. (*Galega*) compared with other fast-growing rhizobia. *FEMS Microbiol Lett* **50**, 277–287.

Lindström, K. & Martínez-Romero, M. E. (2002). International Committee on the Systematics of Prokaryotes Subcommittee on the taxonomy of Agrobacterium and Rhizobium. Minutes of the meeting, 4 July 2001, Hamilton, Canada. *Int J Syst Evol Microbiol* **52**, 2337.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* **3**, 208–218.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Moreira, F. M. S., Gillis, M., Pot, B., Kersters, K. & Franco, A. F. (1993). Characterization of rhizobia isolated from different divergence groups of tropical Leguminosae by comparative polyacrylamide gel electrophoresis of their total proteins. *Syst Appl Microbiol* **16**, 135–146.

Muñoz, E., Villadas, P. J. & Toro, N. (2001). Ectopic transposition of a group II intron in natural bacterial populations. *Mol Microbiol* **41**, 645–652.

Nick, G., de Lajudie, P., Eardley, B. D., Suomalainen, S., Paulin, L., Zhang, X., Gillis, M. & Lindström, K. (1999). *Sinorhizobium arboris* sp. nov. and *Sinorhizobium kostiense* sp. nov., isolated from leguminous trees in Sudan and Kenya. *Int J Syst Bacteriol* **49**, 1359–1368.

Phillips, I. (1991). A guide to sensitivity testing. *J Antimicrob Chemother* **27** (Suppl. D), 1–50.

Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Modern Microbial Methods. Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: Wiley.

Rigaud, J. & Puppo, A. (1975). Indole-3 acetic catabolism by soybean bacteroids. *J Gen Microbiol* **88**, 223–228.

Rogel, M. A., Hernández-Lucas, I., Kuykendall, L. D., Balkwill, D. L. & Martínez-Romero, E. (2001). Nitrogen fixing nodules with *Ensifer adhaerens* harboring *Rhizobium tropici* symbiotic plasmids. *Appl Environ Microbiol* **67**, 3264–3268.

Scholla, M. H. & Elkan, G. H. (1984). *Rhizobium fredii* sp. nov., a fast-growing species that effectively nodulates soybeans. *Int J Syst Bacteriol* **34**, 484–486.

Segovia, I., Pinero, D., Palacios, R. & Martínez-Romero, E. (1991). Genetic structure of a soil population of nonsymbiotic *Rhizobium leguminosarum*. *Appl Environ Microbiol* **57**, 426–433.

Segundo, E., Martínez-Abarca, F., van Dillewijn, P., Fernández-López, M., Lagares, A., Martínez-Drets, G., Niehaus, K., Pühler, A. & Toro, N. (1999). Characterization of symbiotically efficient alfalfa-nodulating rhizobia isolated from acid soils of Argentina and Uruguay. *FEMS Microbiol Ecol* **28**, 169–176.

Sivakumaran, S., Lockhart, P. J. & Jarvis, B. D. W. (1997). Identification of soil bacteria expressing a symbiotic plasmid from *Rhizobium leguminosarum* bv. *trifolii*. *Can J Microbiol* **43**, 164–177.

Song, B. K., Palleroni, N. J. & Häggblom, M. M. (2000). Isolation and characterization of diverse halobenzoate-degrading denitrifying bacteria from soils and sediments. *Appl Environ Microbiol* **66**, 3446–3453.

Soto, M. J., Zorzano, A., Mercado-Blanco, J., Lepek, V., Olivares, J. & Toro, N. (1993). Nucleotide sequence and characterization of *Rhizobium meliloti* competitiveness genes *nfe*. *J Mol Biol* **229**, 570–576.

Sullivan, J. T., Patrick, H. N., Lowther, W. L., Scott, D. B. & Ronson, C. W. (1995). Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. *Proc Natl Acad Sci U S A* **92**, 8985–8989.

Sullivan, J. T., Eardly, B. D., Van Berkum, P. & Ronson, C. W. (1996). Four unnamed species of nonsymbiotic rhizobia isolated from the

rhizosphere of *Lotus corniculatus*. *Appl Environ Microbiol* **62**, 2818–2825.

Toledo, I., Lloret, L. & Martínez-Romero, E. (2003). *Sinorhizobium americanus*, *sp. nov.*, a new *Sinorhizobium* species nodulating native *Acacia* spp. in Mexico. *Syst Appl Microbiol* **26**, 54–64.

Ursing, J. B., Rosselló-Mora, R. A., García-Valdés & Lalucat, J. (1995). Taxonomic note: a pragmatic approach to the nomenclature of phenotypically similar genomic groups. *Int J Syst Bacteriol* **45**, 604.

Vandamme, P., Holmes, B., Vancanneyt, M., Coenye, T., Hoste, B., Coopman, R., Revets, H., Lauwers, S., Gillis, M., Kersters, K. & Govan, J. R. W. (1997). Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. *Int J Syst Bacteriol* **47**, 1188–1200.

Vandamme, P., Mahenthiralingam, E., Holmes, B., Coenye, T., Hoste, B., De Vos, P., Henry, D. & Speert, D. P. (2000). Identification and population structure of *Burkholderia stabilis* sp. nov. (formerly *Burkholderia cepacia* genomovar IV). *J Clin Microbiol* **38**, 1042–1047.

Van de Peer, Y. & De Wachter, R. (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosc* **10**, 569–570.

Van Dillewijn, P. (2000). *Constucción de cepas de Sinorhizobium meliloti altamente competitivas para la nodulación de alfalfa (Medicago sativa L.) y su liberación en campo*. PhD thesis, University of Granada.

Villadas, P. E., Velázquez, E., Martínez-Molina, E. & Toro, N. (1995). Identification of nodule-dominant *Rhizobium meliloti* strains carrying pRmeGR4b type plasmid within indigenous soil populations by PCR using primers derived from specific DNA sequences. *FEMS Microbiol Ecol* **17**, 161–168.

Wang, E. T., Tan, Z. Y., Willems, A., Fernández-López, M., Reinhold-Hurek, B. & Martínez-Romero, E. (2002). *Sinorhizobium morelense* sp. nov., a *Leucaena leucocephala*-associated bacterium that is highly resistant to multiple antibiotics. *Int J Syst Evol Microbiol* **52**, 1687–1693.

Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Willems, A., Doignon-Bourcier, F., Goris, J., Coopman, R., de Lajudie, P. & Gillis, M. (2001). DNA–DNA hybridization study of *Bradyrhizobium* strains. *Int J Syst Evol Microbiol* **51**, 1315–1322.