International Journal of Systematic and Evolutionary Microbiology (2002), **52**, 1267–1276 DOI: 10.1099/ijs.0.01821-0

Rhizobium sullae sp. nov. (formerly '*Rhizobium hedysari*'), the root-nodule microsymbiont of *Hedysarum coronarium* L.

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This work is the completion of a series of reports describing the nitrogen-fixing bacterial symbionts of sulla (*Hedysarum coronarium* L., Leguminosae) and providing the grounds for their proposal as a new taxon. The introduction summarizes a large amount of previous evidence gathered on the physiology, genetics and ecology of such organisms, which have in the past been referred to provisionally as '*Rhizobium hedysari*'. Upon adding 16S RNA sequencing, amplified rDNA restriction analysis of the *rrn* operon, DNA–DNA hybridization homology and analysis of low-molecular-mass RNA species, it is concluded that the group of strains that specifically nodulate sulla consists of a coherent set of isolates that differ from previously described rhizobia to an extent that warrants the constitution of the species boundary. The name *Rhizobium sullae* sp. nov. is proposed, with isolate IS123^T (=USDA 4950^T =DSM 14623^T) as the type strain.

Keywords: taxonomy, *Rhizobiaceae*, sulla, Mediterranean legume, alkaline soil

INTRODUCTION

The genus *Hedysarum*, including over 100 species, is distributed throughout Europe, Africa, Asia and North America. The species *Hedysarum coronarium* L. (tribe Hedysareae, family Leguminosae), known by the Italian name of sulla, ranges within the Mediterranean basin from northern Africa to southern Spain and centrally to southern Italy. Tolerance to the stress factors of drought, salinity and alkaline soil (pH up to 9·6) renders sulla well-adapted to marginal areas, deserts and basic clays. The plant also appears well-suited to grow in soils containing low amounts of phosphorus and not to rely on mycorrhizal infection (Lioi & Giovannetti, 1987). The quality of its forage and a rapid productivity have made sulla a popular agronomic crop in Spain and Italy. Bacteria isolated from the nitrogen-fixing root nodules of sulla have been described and studied by different authors. In several previous reports, we have referred to them using the provisional name of '*Rhizobium hedysari*'. The first report of bacteria isolated from sulla nodules dates back to the late 1800s (Mottareale, 1898). Indeed, the first description of the symbionts of sulla was contemporary with the discoveries of biological nitrogen fixation and the first bacillary forms associated with other legume root nodules. Other comprehensive physiological studies were due to the efforts of Nicolai (1900) and Severini (1908). In more recent studies, the high degree of host specificity of rhizobia from sulla was reported by Cabrera & Ruiz-Argüeso (1979), who tested a number of isolates on different legumes and, in parallel, different rhizobia on sulla, recording no cross-nodulation. Glatzle *et al.* (1986) tested bacteria isolated in Morocco from nodules of *H. coronarium* and from the very closely related *Hedysarum flexuosum* and found that nodules were formed on both hosts, but were ineffective in nitrogen fixation in the heterologous plant. The extremely broad-host-range *Rhizobium*

Abbreviations: ARDRA, amplified rDNA restriction analysis; TEM, transmission electron microscopy; UPGMA, unweighted pair group matrix analysis.

The EMBL accession numbers for the 16S rDNA sequences of strains IS123^T (1370 nt), RH44 (first 651 nt), RH44 (last 508 nt), HCNT1 (1307 nt) and CC1335 (1306 nt) are respectively Y10170–Y10174.

strain NGR234 is also unable to nodulate sulla (W. J. Broughton, personal communication). The introduction of sulla to countries such as Australia, outside the natural distribution range of the genus *Hedysarum*, showed the strict requirement for the inoculation of specific strains in order to achieve nodulation (Casella *et al*., 1984a). In the same study, a strain from *H. coronarium* was able sporadically to nodulate sainfoin (*Onobrychis viciaefolia*). A certain degree of ineffective nodulation has also been observed on clover (Espuny *et al*., 1987). Sulla rhizobia can be used as commercial inoculants (Lupi *et al*., 1988; Rodriguez-Navarro *et al*., 1991). A combined light and transmission electron microscopy analysis of the infection process in sulla was published previously (Squartini *et al*., 1993). All rhizobia isolated from sulla are of the fast-growing type (Cabrera & Ruiz-Argüeso, 1979). Studies on their G+C content (61·6 mol%) and on general metabolic properties were presented previously (Struffi *et al*., 1998). We also investigated specific metabolic pathways of these rhizobia, such as their metabolism of nitrogen oxides and denitrification activities *in planta* (Casella *et al*., 1984b), in free-living bacteria (Casella *et al*., 1986) and in bacteroids *ex planta* (Casella *et al*., 1988). A unique behaviour was shown in this respect by strain HCNT1, the nitrite reductase of which is induced when grown in low oxygen conditions and, unlike other rhizobia, this strain does not couple nitrate or nitrite reduction with energy conservation (Casella *et al*., 1994; Toffanin *et al*., 1996). We also examined storage polymers in rhizobia from sulla and showed that they accumulate poly-β-hydroxybutyrate (Tombolini & Nuti, 1989). Subsequently, we tested the biotechnological suitability for poly-β-hydroxybutyrate production by wild-type (Chiellini *et al*., 1989) and genetically engineered sulla rhizobia (Casini *et al*., 1993).

Various components of the cell envelope and extracellular glycocalyx of sulla rhizobia have been examined, including lipopolysaccharide (Casella *et al*., 1992), the glycoconjugate and membrane lipid components (Orgambide *et al*., 1996; Navarini *et al*., 1997).

The genetics of rhizobia from sulla has also received thorough attention (Espuny *et al*., 1987; Mozo *et al*., 1988, 1990; Ollero *et al*., 1989, 1991, 1993). Our own data (Meneghetti *et al*., 1996; A. Squartini, unpublished) indicate the presence of *nodABCH*, *nodD*, *syrM*, *nodFE*, *nodL* and *nolMNO*. Two new insertion elements are present in all tested wild-type strains from sulla rhizobia and absent from the symbionts of other legumes (Meneghetti *et al*., 1996, 1997; Alberghini *et al*., 1998).

In terms of systematic evaluation, several strains from nodules of *H. coronarium* were included in tests aimed at both individual fingerprinting and polyphasic taxonomy. A MIDI cellular fatty acid analysis was performed on different strains (Tighe *et al*., 1994) and the results indicate that rhizobia from sulla are, in that respect, related to *Rhizobium etli*, *Rhizobium leguminosarum* and *Sinorhizobium meliloti*. An analysis of the 23S rRNA variable 5′-end fragmentation, comparing different rhizobia, was done (Selenska-Pobell & Evguenieva-Hackenberg, 1995). In a different study, we characterized different strains of rhizobia from sulla by RFLP analysis of amplified rDNA and by genomic fingerprinting (Selenska-Pobell *et al*., 1996). Specific phage typing, macromolecular cell profiles as plasmids, proteins and large genomic fragments were analysed (Struffi *et al*., 1998). Immunological studies allowed us to distinguish sulla strains (Casella *et al*., 1992). Multilocus enzyme electrophoresis was also applied to a group of strains (Benguedouar *et al*., 1997).

In the present paper, we have sought to complete the requirements for the description of a novel rhizobium species of the root-nodule symbiont of sulla by comparing the 16S rRNA sequences of four strains with the Ribosomal Database Project databank, by analysing the polymorphism of the *rrn* operon via amplified rDNA restriction analysis (ARDRA), by performing DNA–DNA homology studies and by staircase electrophoresis of low-molecular-mass (LMM) RNA molecules (Cruz-Sánchez *et al*., 1997; Velázquez *et al*., 1998).

METHODS

Bacterial strains and culture conditions. Strains are listed in Table 1. Rhizobia were routinely grown on either YMB, defined BIII (Dazzo, 1982) or TY (Beringer, 1974) media at 28 °C.

DNA–DNA hybridization. The analysis was custom-performed by the DSMZ service using the spectroscopic method. DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al*. (1977). DNA–DNA hybridization was carried out as described by De Ley *et al*. (1970), with the modifications described by Huß *et al*. (1983) and Escara & Hutton (1980). The reaction was performed at 69 °C in 2 × SSC. A Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter was used. Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992).

ARDRA. Both the 16S rDNA region and the whole *rrn* operon (from position 8 of the 16S rDNA to position 2759 of the 23S rDNA; *Escherichia coli* numbering) were analysed, as described previously for other species (Selenska-Pobell *et al*., 1998). The set of primers used for PCR is listed in Selenska-Pobell *et al*. (1996). Restriction endonucleases used included *Alu*I, *Ava*II, *Cfo*I, *Dde*II, *Hae*III, *Msp*I, *Nde*II, *Rsa*I and *Taq*I (Gibco-BRL). Results were processed by the unweighted pair group matrix analysis (UPGMA).

Sequence analysis of the 16S rDNA. 16S rRNA genes of several rhizobial strains recovered from nodules of the host plants *H. coronarium*, *Hedysarum alpinum* and *Onobrychis viciaefolia* were sequenced by using standard sequencing primers (Huber & Selenska-Pobell, 1994). Sequences covering almost the whole 16S rRNA genes of the strains studied were obtained using an Automatic ALFexpress Sequencer (Pharmacia). The sequences were aligned using CLUSTAL W and analysed with the software package PHYLIP version 3.5c (Felsenstein, 1993). Jukes–Cantor distances were derived

Table 1. Bacterial strains included in this study

Strain	Source	Plant host	Geographical origin
Rhizobium sullae sp. nov. RHA6	A. Benguedouar	*H. coronarium*	Northern Algeria
R. sullae RHA10	A. Benguedouar	*H. coronarium*	Northern Algeria
R. sullae RHIS123^T* (= IS123^T)	F. J. Ollero	*H. coronarium*	Southern Spain
R. sullae RH100	A. Toffanin	*H. coronarium*	Balearic Islands
R. sullae RH44*	A. Toffanin	*H. coronarium*	Southern Spain
R. sullae RHF	S. Casella	*H. coronarium*	Pisa, Italy
R. sullae HCNT1*	S. Casella	*H. coronarium*	Volterra, Italy
R. sullae RH19	S. Casella	*H. coronarium*	Sicily, Italy
R. sullae CC1335*	J. Brockwell	*H. coronarium*	Southern Spain
Mesorhizobium sp. CIAM 1414*	N. Novikova	*H. alpinum*	Moscow, Russia
Mesorhizobium sp. CIAM 1415	N. Novikova	*H. alpinum*	Moscow, Russia
Isolate Esp3*	L. Raitcheva	*Onobrychys viciaefolia*	Souhodol, Bulgaria
Isolate EspTr3	L. Raitcheva	*Onobrychys viciaefolia*	Trestenic, Bulgaria
Isolate EspTr4	L. Raitcheva	*Onobrychys viciaefolia*	Trestenic, Bulgaria
Isolate EspP16	L. Raitcheva	*Onobrychys viciaefolia*	Pleven, Bulgaria
Isolate Esp820	L. Raitcheva	*Onobrychys viciaefolia*	Russia
Mesorhizobium ciceri UPM-Ca7^T	S. Nour	*Cicer arietinum*	Spain
Mesorhizobium huakuii CCBAU 2609^T (= USDA 4779^T)	P. van Berkum	*Astragalus sinicus*	Nanjing, China
Mesorhizobium loti ATCC 33669^T (= USDA 3471^T)	P. van Berkum	*Lotus corniculatus*	New Zealand
Rhizobium etli CFN 42^T	DSMZ	*Phaseolus vulgaris*	Mexico
Rhizobium tropici IIa CFN 299	P. van Berkum	*Phaseolus vulgaris*	Central America
R. tropici IIb ATCC 49672^T (= CIAT 899^T)	DSMZ	*Phaseolus vulgaris*	Central America
Rhizobium galegae ATCC 43676^T (= HAMBI 540^T)	K. Lindström	*Galega orientalis*	Finland
Rhizobium leguminosarum bv. phaseoli ATCC 14482$^{(T)}$	DSMZ	*Phaseolus vulgaris*	USA
R. leguminosarum bv. trifolii ATCC 14480$^{(T)}$	DSMZ	*Trifolium pratense*	USA
R. leguminosarum bv. viciae USDA 2370^T (= ATCC 10004^T)	DSMZ	*Pisum sativum*	USA
Rhizobium gallicum bv. gallicum MSDJ 1109^T (= R602sp^T)	N. Amarger	*Phaseolus vulgaris*	France
Rhizobium giardinii bv. giardinii MSDJ 0144^T (= H152^T)	N. Amarger	*Phaseolus vulgaris*	France
Rhizobium hainanense CCBAU 57015^T (= I66^T)	DSMZ	*Desmodium sinuatum*	China
Rhizobium mongolense USDA 1844^T	P. van Berkum	*Medicago ruthenica*	Mongolia
Sinorhizobium meliloti ATCC 9930^T	P. van Berkum	*Medicago sativa*	USA
Sinorhizobium fredii USDA 205^T (= ATCC 35423^T)	P. van Berkum	*Glycine max*	China
Sinorhizobium terangae LMG 7834^T (= ORS 1009^T)	P. de Lajudie	*Acacia laeta*	Senegal
Sinorhizobium saheli LMG 7837^T (= USDA 4102^T)	P. van Berkum	*Sesbania cannabina*	Senegal

* 16S rDNA sequenced.

The designation $^{(T)}$ indicates biovar reference strains (former species type strain).

from the aligned sequences to construct a tree using the UPGMA method.

Transmission electron microscopy (TEM). Cells were grown to mid-exponential phase in shaken flask cultures containing 25 ml BIII broth and harvested by centrifugation at 4000 ***g***. The cell pellet was resuspended to about 1 ml from the residual broth and processed using the agarose block fixation procedure of Beaman *et al.* (1972), except that the primary fixation was in 2·5 (v/v) glutaraldehyde in 0·1 M sodium/potassium phosphate buffer, pH 7·2. Fixed samples were embedded in WPE 144 poly/bed resin, ultrathin-sectioned, post-stained with aqueous lead citrate and uranyl acetate and examined with a Philips CM-10 TEM.

To examine encapsulation, carbon-coated Formvar copper

grids were floated directly on 1 ml broth cultures in wells of a glass Petri dish and then blotted and stained with the glutaraldehyde/ruthenium red/uranyl acetate method as described by Mutaftschiev *et al.* (1982). Individual whole-cell mounts were examined by TEM. Photographic negatives were digitally scanned at 200 d.p.i. and examined by computer-assisted image analysis (Dazzo & Petersen, 1989) using UTHSCSA image tool/CMEIAS software.

Sequencing of the nodulation genes and phylogenetic alignment of *nodA*. From the previously identified (Meneghetti *et al.*, 1996) and partially sequenced genetic region essential for nodulation, the complete *nodA* gene sequence was aligned with all the corresponding rhizobial *nodA* gene sequences present in GenBank, using the CLUSTAL method with weighted residue weight table.

RNA extraction and LMM RNA profile analysis. The following commercial molecules from Boehringer Mannheim and Sigma were used as references: 5S rRNA from *E. coli* MRE 600 (120 and 115 nt) (Bidle & Fletcher, 1995), tRNA specific for tyrosine from *E. coli* (85 nt) and tRNA specific for valine from *E. coli* (77 nt) (Sprinzl *et al.*, 1985). Samples were prepared as reported elsewhere (Höfle, 1988). The RNA of the strains studied was extracted according to the method described by Höfle (1998). LMM RNA profiles were obtained using staircase electrophoresis in a 14% polyacrylamide gel under denaturing conditions in steps of 10 min, rising through a constant ramp with 50 V increases from 100 to 2300 V, as reported previously (Cruz-Sánchez *et al.*, 1997). After electrophoresis, the gels were silver-stained according to the method described by Haas *et al.* (1994). The bands in each profile were coded for input into a database including all the strains studied and Jaccard's similarity coefficient was calculated to construct the distance matrix. A dendrogram was constructed from the distance matrix using UPGMA.

RESULTS AND DISCUSSION

TEM

The ultrastructure of vegetative cells of strain IS123^T grown in BIII broth culture was examined by two different methods of specimen preparation for TEM. The first method was designed to illustrate the ultrastructure of the cells in thin section. The rod-shaped bacteria had a typical Gram-negative cell envelope and a cytoplasm that accumulated granules having the typical ultrastructure of polyhydroxyalkanoate and polyphosphate granules (Fig. 1a). The second technique was performed to reveal the delicate surface capsule and filamentous appendages. This revealed a fibrillar capsule of ruthenium red-staining acidic polymer and several negatively stained flagella (Fig. 1b). Digital image analysis indicated a mean cell width of 0·44 μm, a cell length of 1–2 μm and a flagellar wavelength periodicity of 0·9 μm.

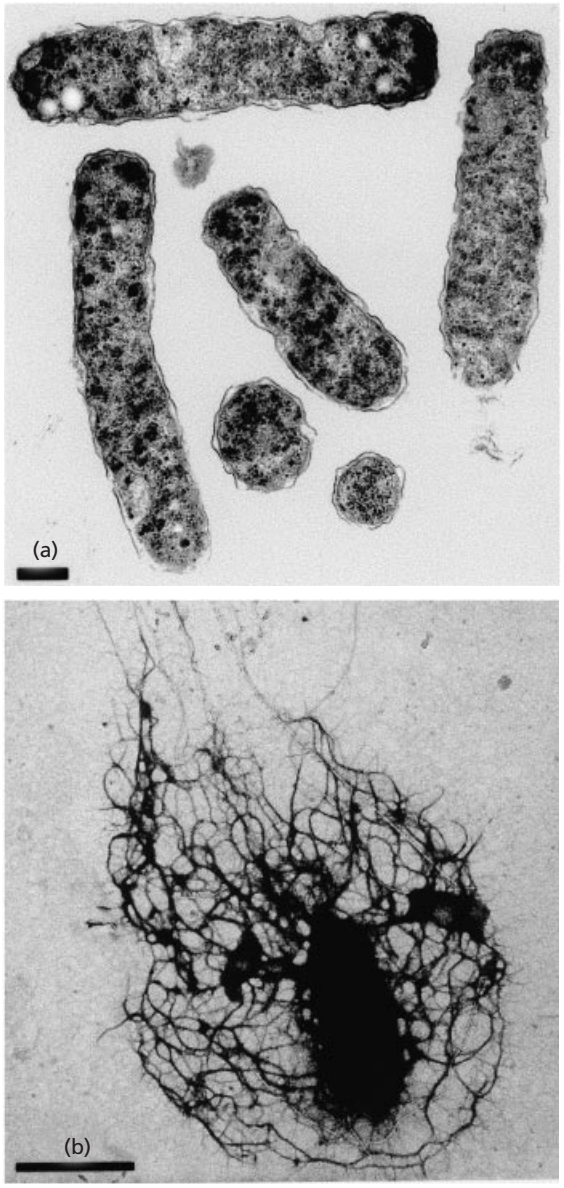

Fig. 1. TEM of *R. sullae* grown in defined BIII medium. (a) Ultrathin section of rods in longitudinal and cross-section. Note the electron-transparent and electron-opaque granules indicative of polyhydroxybutyrate and polyphosphate, respectively. Bar, 0·25 μm. (b) Whole-cell mount processed by the glutaraldehyde/ruthenium red/uranyl acetate method to reveal the various exostructures. Bar, 1 μm.

Sequence alignment of *nodA*

The *nodA* sequence from strain IS123^T is most similar to that of *Rhizobium* sp. (*Oxytropis arctobia*) strain N33 (Cloutier *et al.*, 1996), a symbiont of both arctic and temperate legumes (data not shown). Interestingly, the latter group of hosts includes *Onobrychis viciaefolia*, a species very close to *H. coronarium*, which was itself formerly included in the genus *Onobrychis*. Another similarity between *Rhizobium* sp. (*Oxytropis arctobia*) and strain IS123^T is in the organization of the *nod* genes. Cloutier *et al.* (1996) reported the presence of a vestigial truncated portion of *nodA* in front of the *nodBC* genes in strain N33 and a full *nodA* copy in a distant locus. A similar situation is observed in our proposed type strain, IS123^T (Alberghini *et al.*, 1998),

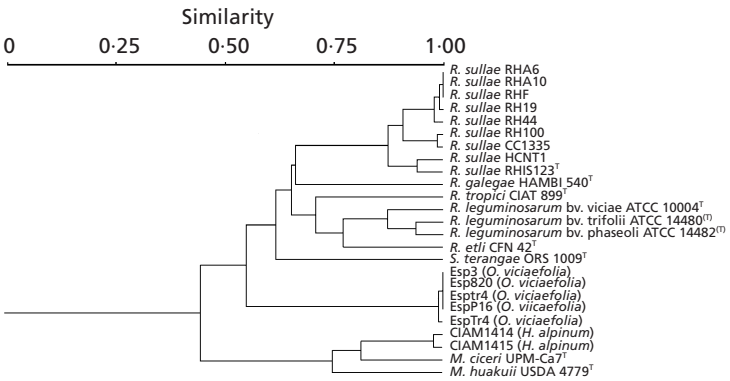

Fig. 2. UPGMA dendrogram derived from ARDRA of the *rrn* operon.

where the *nodA* copy preceding *nodBC* carries an internal deletion.

ARDRA

In addition to the comparison among rhizobia isolated from sulla, also included in the analysis were two other groups of newly isolated strains that nodulate the related legume species *H. alpinum* and *Onobrychis viciaefolia*. As seen from the UPGMA dendrogram of the whole *rrn* operon (Fig. 2), all strains recovered from *H. coronarium* (*Rhizobium sullae* sp. nov.) cluster in a group that is separated from the other species tested. These strains are more related to the genera *Rhizobium* and *Sinorhizobium* than to *Mesorhizobium*. In contrast, strains nodulating *H. alpinum* have similar *rrn* ARDRA patterns that are closely related to those of the genus *Mesorhizobium*. The five isolates from *Onobrychis viciaefolia* form a separate cluster that is not very closely related to other rhizobia, with a similarity of about 66%. ARDRA of the entire *rrn* operon, as opposed to that based on the 16S RNA region alone (data not shown), yielded considerably finer resolution. This is mostly due to the fact that the whole *rrn* operon includes two highly variable regions. One of them is the intergenic spacer between the 16S and 23S rRNA-encoding genes. As we have shown previously (Selenska-Pobell *et al*., 1996), strain IS123^T possesses the most variable intergenic spacer in the group of rhizobia from *H. coronarium*. The second highly variable region is the intervening sequence, situated in helix 9 of the 23S rRNA gene, present in all members of the family *Rhizobiaceae* (Selenska-Pobell & Evguenieva-Hackenberg, 1995; Selenska-Pobell & Döring, 1998).

16S rRNA sequence and database alignment

The 16S rDNA genes of four strains of rhizobia from *H. coronarium* were sequenced completely. The four sequences, which are identical, were subsequently subjected to comparison with the RDP database. In addition, two strains from other host plants related to *H. coronarium* were sequenced, namely strain Esp3 (accession no. Y10169) from *Onobrychis viciaefolia* and strain CIAM 1414 (accession no. Y10175) from *H. alpinum*.

The tree (Fig. 3), an UPGMA output, constructed by aligning database sequences of the 16S rRNA molecules between positions 106 and 1469 (*E. coli* numbering), shows the phylogenetic relatedness between the proposed novel species, *R. sullae* sp. nov., and its closest matches, as well as its position relative to different α-proteobacteria. Our isolates group in one of the *Rhizobium* branches. This is in agreement with the earlier report by Terefework *et al*. (1998), who aligned the partial 16S sequence of strain IMAP 835, isolated from *H. coronarium* in Italian soils, among several isolates from Asian and African legumes. The highest similarity (98·8% identity) was found to *Rhizobium* sp. strain USDA 1920, from *Medicago ruthenica*. This isolate has been indicated as a potential genomic species distinct from *Rhizobium mongolense*, which more typically nodulates the same host (van Berkum *et al*., 1998). Next in similarity to *R. sullae* is *Rhizobium gallicum* (98·4%), followed by the type strain of *R. mongolense* and by *R. leguminosarum* strain IAM 12609 (both at 97·7%). Progressively lower values are found between *R. sullae* and the other *R. mongolense* strain sequences available in the database.

Strain CIAM 1414 from *H. alpinum* belongs to the *Mesorhizobium* branch. Thus, comparing the position of strains from hosts related to sulla highlights that these two members of the genus *Hedysarum* have symbionts that are phylogenetically very unrelated. The situation is the opposite in other legume genera, e.g. *Galega*, in which the rhizobial symbionts of the species *Galega orientalis* and *Galega officinalis* are also very host-specific but have almost identical 16S rRNA sequences (Huber & Selenska-Pobell, 1994). However, significant differences occur in the structure of the 23S rRNA genes of these two rhizobia (Selenska-Pobell & Döring, 1998). Additional 16S sequence alignment analyses against sequences published from bacteria isolated from other *Hedysarum* species (Wernegreen & Riley, 1999) revealed that, within the symbionts of this genus, our isolates from *H. coronarium* share the highest similarity to *Rhizobium* sp. from *Hedysarum mongolicum* growing in China and much lower values

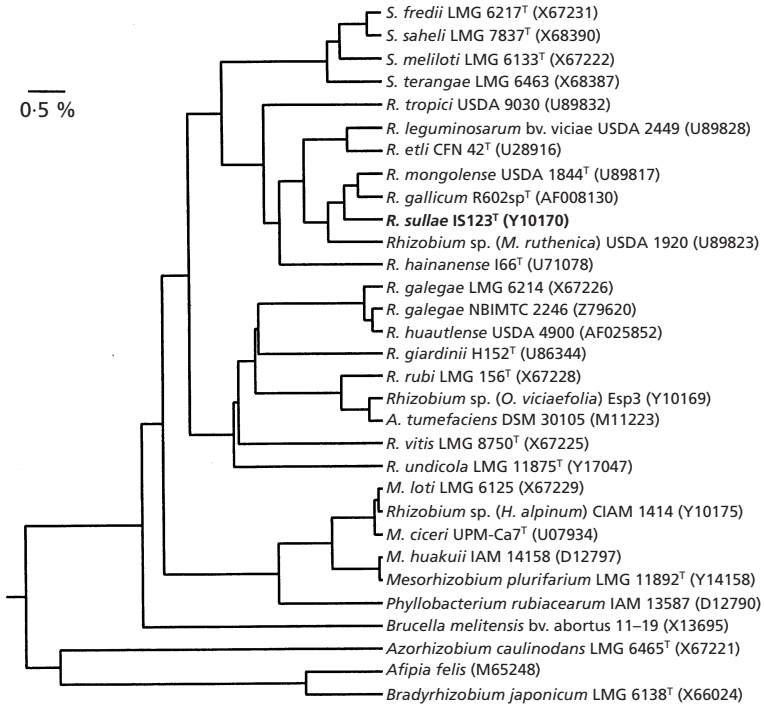

Fig. 3. UPGMA phylogenetic tree, derived from a Jukes–Cantor pairwise distance matrix, based on the comparative analysis of 16S rRNA gene sequences of bacteria related to *R. sullae*. GenBank accession numbers are indicated. Bar, 0·5 % nucleotide difference.

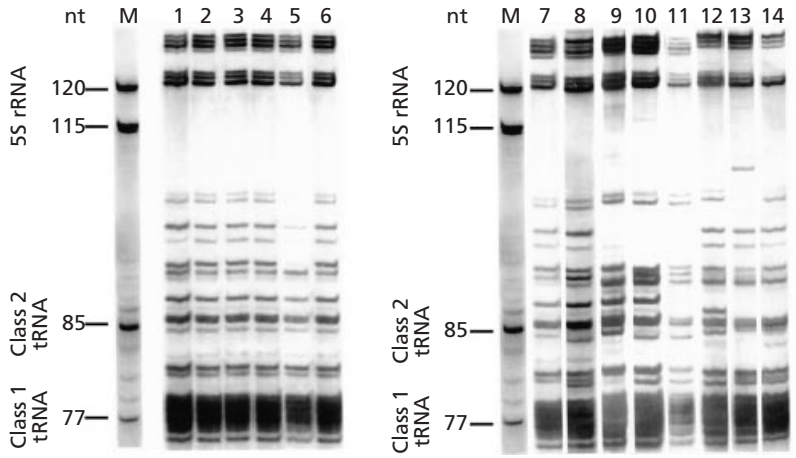

Fig. 4. LMM RNA profiles of the strains used in this study. Lanes: M, markers (see Methods for details); 1 and 7, *R. sullae* IS123^T; 2, RHA6; 3, RH19; 4, HCNT1; 5, RH44; 6, RHF; 8, *R. mongolense* USDA 1844^T; 9, *R. tropici* IIb CIAT 899^T; 10, *R. leguminosarum* ATCC 10004^T; 11, *R. etli* ATCC 51251^T (identical to CFN 42^T); 12, *S. meliloti* ATCC 9930^T; 13, *S. saheli* USDA 4102^T; 14, *S. fredii* ATCC 35423^T.

with rhizobia from *Hedysarum pallens* (Israel), *H. alpinum* (Russia) or *Hedysarum boreale* (Alaska). The 16S rRNA sequence of strain Esp3, isolated from *Onobrychis viciaefolia*, another member of the *Hedysareae* tribe not far from sulla, shows that they are very clearly affiliated with *Agrobacterium tumefaciens* but not with *R. sullae*. This is in agreement with data on *rrn* fragmentation (Selenska-Pobell & Döring, 1998). In contrast with the strains of the two other groups studied, a central break occurs in the 23S rRNA of the strains belonging to this group of isolates, which is typical for all agrobacteria except *Rhizobium vitis*. This is also in agreement with the separation of the group from other *Rhizobia* obtained by ARDRA.

Considering the resolution power of the techniques used, neither 16S rDNA sequence analyses nor ARDRA of the 16S rRNA (not shown) demonstrated differences among the *R. sullae* strains HCNT1, CC1335 and IS123^T. ARDRA of the whole *rrn* operon, however, could discriminate these strains. This shows that RFLP analysis of the intergenic spacer and 23S rDNA may be used, in addition to 16S rDNA RFLP, for rapid grouping and characterization of novel rhizobia. On the basis of this analysis, the most appropriate representatives for sequencing can be chosen. In addition, by sequencing the variable regions evaluated by ARDRA, strain-specific probes may be designed for monitoring of individual strains.

LMM RNA profile analysis

Previous studies indicate that staircase electrophoresis reveals patterns of stable LMM RNA that can be of reliable value for the taxonomy of *Rhizobium* species (Cruz-Sánchez *et al.*; 1997; Velázquez *et al.*, 1998). All the strains nodulating *H. coronarium* analysed in this

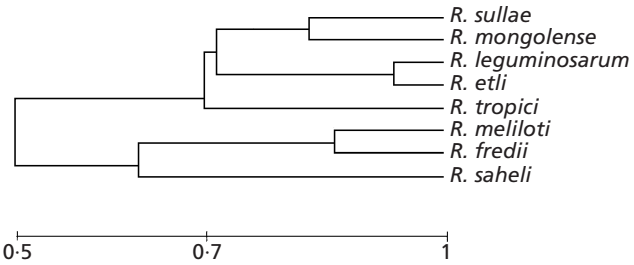

Fig. 5. UPGMA dendrogram based on Jaccard's coefficient derived from LMM RNA profiles of the type strains of species tested.

study have the same LMM RNA profile (Fig. 4, lanes 1–6); this supports the homogeneous nature of the group. All strains of *R. sullae* tested are included in the fast-growing rhizobia and, according to the 16S rRNA sequences, their most closely related genera are *Rhizobium* and, secondly, *Sinorhizobium*. The LMM RNA profiles of type strains of species of *Rhizobium* and *Sinorhizobium* are shown in lanes 7–14 of Fig. 4. The 5S rRNA profile is different between the two genera. The strains nodulating *H. coronarium* show a profile in the 5S rRNA zone that is typical of the genus *Rhizobium*, further supporting the inclusion of these strains in this genus.

The above profiles were used to construct a dendrogram by using Jaccard's coefficient and the UPGMA clustering method (Fig. 5). The taxa tested in this study grouped into two clusters; one encompassed the species of the genus *Rhizobium* and the other those of *Sinorhizobium*, the proposed novel species *R. sullae* falling in the former. According to the LMM RNA data, the species of *Rhizobium* most closely related to *R. sullae* are *R. mongolense* (nodulating *Medicago ruthenica*), *R. leguminosarum* and *R. etli*. This result is in agreement with that obtained by 16S rRNA sequence analysis.

DNA–DNA hybridization

Three strains among the *H. coronarium* symbionts (IS123^T, A6 and RHF, respectively isolated in Spain, Africa and Italy) were chosen for a series of pairwise hybridizations with different rhizobia. These included other representatives of the group of sulla isolates and other species of rhizobia, selected on the basis of relatedness to our proposed species, indicated by other taxonomic approaches such as small-subunit RNA sequencing, ARDRA and staircase electrophoresis. The results are shown in Table 2. The three strains of *R. sullae* to be screened against all others, as well as the target *R. sullae* strains, were selected as examples of cases showing variability in the ARDRA clusters. The information arising from the DNA–DNA similarity test can be outlined as follows: (i) the tested *R. sullae* strains, within their group, displayed homology values ranging from 77·3 to 100 %, with a mean of 92·1 %. This evidence, while confirming the differences observed under ARDRA, argues in favour of a fair amount of homogeneity within the group of symbionts of *H. coronarium*; (ii) the mean values recorded between *R. sullae* and any of the other validly described rhizobium species included in this study were between 32·4 % (*R. sullae* to *Rhizobium hainanense*) and 53·2 % (*R. sullae* to *R. gallicum*); within the latter group, the highest value observed was 62·7 %, which is below the threshold value of 70 % that is considered discriminatory for the species rank.

Adding the present information to the body of literature that has been accumulated on these bacteria,

Table 2. DNA relatedness based on percentage spectroscopic DNA–DNA hybridization

Source of unlabelled DNA	Source of labelled DNA			Mean ± SD
	IS123^T	RHA6	RHF	
R. sullae IS123^T	108·0	83·5	87·8	93·1 ± 13·1
R. sullae HCNT1	98·1	86·3	95·5	93·3 ± 6·2
R. sullae RH44	77·3	96·1	94·3	89·2 ± 10·4
R. sullae RHF	87·8	110·0	105·0	100·9 ± 11·6
R. sullae RHA6	83·5	103·3	110·0	98·9 ± 13·8
R. gallicum bv. gallicum R602^T	43·3	62·7	53·7	53·2 ± 9·7
R. mongolense USDA 1844^T	45·0	50·0	53·9	49·6 ± 4·5
R. giardinii bv. giardinii H152^T	19·8	34·9	42·3	32·3 ± 11·5
R. leguminosarum bv. trifolii ATCC 14480$^{(T)}$	41·4	54·3	49·6	48·4 ± 6·5
R. leguminosarum bv. viciae ATCC 10004^T	26·3	41·2	31·3	32·9 ± 7·6
R. leguminosarum bv. phaseoli ATCC 14482$^{(T)}$	24·0	36·9	41·2	34·0 ± 9·0
R. etli CFN 42^T	39·6	44·8	49·9	44·8 ± 5·2
R. tropici CIAT 899^T	34·1	40·7	39·4	38·1 ± 3·5
R. hainanense I66^T	14·4	37·0	45·8	32·4 ± 16·2
M. huakuii USDA 4799^T	14·7	43·2	44·5	34·1 ± 16·8

we consider the data sufficient to propose *Rhizobium sullae* as a novel species. As Elkan (1992) pointed out in his review on *Rhizobium* taxonomy, 'it is perceived that newly described species will result from a series of publications in which initial phenotypic characteristics useful in identification, are followed by a balance of chromosomally determined and plasmid mediated traits, and later by phylogenetic studies'. In proposing yet another rhizobium, we are conscious at the same time that the concept of the prokaryotic species itself is drifting more and more towards the need for a redefinition of its boundaries, especially in the case of rhizobia. Surely, the polyphyletic origin of legume-nodulating bacteria, sharing branches of their tree with non-nitrogen-fixing species, must warn against a phylogeny based on a simple time-related divergence. The processes of horizontal gene transfer, in many instances enhanced by the mobility of replicons carrying the symbiotic genes, are constantly contributing to the reshaping of the relations among different plant symbionts. In this sense, we feel the need to recommend the inclusion of analyses of *nod* gene homology or plasmid-borne specific insertion sequences as indicative signs to evaluate functional evolution of plant–microbe interacting traits besides the slower evolution of the cellular background that hosts the genetic changes.

After this manuscript was submitted for publication, the novel species *Rhizobium yanglingense* was described (Tan *et al*., 2001). The 16S rRNA genes of the type strains of *R. yanglingense* and *R. sullae* share 97·9% similarity.

Description of *Rhizobium sullae* sp. nov.

Rhizobium sullae (sul'la.e. N.L. gen. n. *sullae* of sulla, the common Italian name of *Hedysarum coronarium*, the host plant).

Gram-negative, aerobic, non-spore-forming rods, motile by multiple polar flagella. Colonies on YMA are circular and opaque, reaching a diameter of 3–5 mm within 3 days at 28 °C. Growth rate (10 strains measured) is between 0·28 and 0·32 h^{-1} (Cabrera & Ruiz-Argüeso, 1979). The G+C content is 61·6 mol% (Struffi *et al*., 1998). Growth on YEM is inhibited between 0·5 and 1% (w/v) NaCl and below pH 5·5. Most strains are resistant to carbenicillin and nalidixic acid, all strains tested utilize D-(+)-galactose, mannitol, raffinose, rhamnose and lactose as carbon sources and glutamate, valine, proline, isoleucine, arginine and asparagine as nitrogen sources. The strains considered for the inclusion in this taxon are listed in Table 1. A complete table of the metabolic reactions of each strain, including those of the type strain, has been presented previously (Struffi *et al*., 1998). The characteristics considered essential for membership in the taxon, and that distinguish *R. sullae* from related species, are (i) specific nitrogen-fixing endosymbiosis with *H. coronarium* and (ii) DNA–DNA hybridization values among taxon members above 70%. Other characteristics considered non-essential but indicative of possible membership in the taxon are: (i) presence of insertion element ISRH1 (Meneghetti *et al*., 1996), (ii) double-layered infection thread (Squartini *et al*., 1993), (iii) Latin cross-shaped bacteroids (Squartini *et al*., 1993) and (iv) sensitivity to one or more of the following bacteriophages: f835a, f123c, fHA5, fHC, f100c, f19a, f19c, f44a and f44c1 (Struffi *et al*., 1998). Characteristics that qualify the species for inclusion in the genus *Rhizobium* are the results of (i) 16S sequencing, (ii) *rrn* ARDRA, (iii) LMM RNA electrophoresis and (iv) DNA–DNA hybridization, which consistently cluster all isolates within the genus *Rhizobium*.

The type strain is IS123^T, which has been deposited in the USDA National Rhizobium Germplasm Culture Collection (Beltsville, MD, USA) as USDA 4950^T and in the DSMZ as DSM 14623^T.

ACKNOWLEDGEMENTS

Peter van Berkum and Nöelle Amarger are kindly acknowledged for counselling and several of the reference strains. Gisèle Laguerre and Anne Willems are thanked for method communications and discussion. Kristina Lindström is remembered for her precious advice in initiation of the taxonomical study. Special thanks are due to Francisco J. Ollero for his long-standing collaboration and fruitful discussions.

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