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 rrr 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 IS123T (=USDA 4950T =DSM 14623T) 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 IS123T (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 vicifolia*). 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 notcouple nitrate or nitrite reduction with energy conservation (Casella et al., 1994; Toffilanin 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 nodABC, nodD, syrM, nodFE, nodL and nodMNO. 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 fragment, comparing different rhizobia, was done (Selenksa-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 (Selenksa-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 rrr 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 BHI (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 hydroxypatite by the procedure of Cashon et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970), with the modifications described by Hübl et al. (1983) and Escara & Hutton (1980). The reaction was performed at 69 °C in 2 x 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 rRNA region and the whole rrr 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 (Selenksa-Pobell et al., 1998). The set of primers used for PCR is listed in Selenksa-Pobell et al. (1996). Restriction endonucleases used included EcoRI, HaeIII, AvaI, DdeI, HincII, MspI, NcoI, SalI and TaqI (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 vicifolia* 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Plant host</th>
<th>Geographical origin</th>
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<tr>
<td><em>Rhizobium sullae</em> sp. nov. RHA6</td>
<td>A. Benguedouar</td>
<td><em>H. coronarium</em></td>
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<td><em>H. coronarium</em></td>
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<td><em>R. sullae</em> RHIS123* ( = IS123*)</td>
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<td><em>H. coronarium</em></td>
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</tr>
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<td><em>H. coronarium</em></td>
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<td><em>H. coronarium</em></td>
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<td><em>O. vicieaefolia</em></td>
<td>Souhodol, Bulgaria</td>
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<td><em>O. vicieaefolia</em></td>
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<td>Cicer arietinum</td>
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<td>Astragalus sinicus</td>
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<td>Phaseolus vulgaris</td>
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<td>N. Amarger</td>
<td>Phaseolus vulgaris</td>
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<td>Medicago ruthenica</td>
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<td>Sinorhizobium fredii USDA 205*</td>
<td>P. van Berkum</td>
<td>Glycine max</td>
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<td>( = ATCC 35423*)</td>
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<td>Acacia laeua</td>
<td>Senegal</td>
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<td>Sinorhizobium saheli LMG 7837*    ( = USDA 4102*)</td>
<td>P. van Berkum</td>
<td>Sesbania cannabina</td>
<td>Senegal</td>
</tr>
</tbody>
</table>

* 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 BHI 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
The ultrastructure of vegetative cells of strain IS123<sup>T</sup> 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.

**Sequence alignment of nodA**

The nodA sequence from strain IS123<sup>T</sup> is most similar to that of *Rhizobium* sp. (*Oxytropsis 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. (*Oxytropsis arctobia*) and strain IS123<sup>T</sup> 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<sup>T</sup> (Alberghini *et al.*, 1998),
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 vicieae folia*. 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 other *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 vicieae folia* 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\(^3\) 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 RNA 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\(^3\) (accession no. Y10169) from *Onobrychis vicieae folia* 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 rDNA 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 *z*-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 rDNA sequences (Huber & Selenska-Pobell, 1994). However, significant differences occur in the structure of the 23S rDNA 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
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 *Hedy-sarum* tribe not far from sulla, shows that they are very closely affiliated with *Agrobacterium tumefaciens* spp. *sarta* (Israel), *Hedy-sarum* (Alaska). The strains nodulating *Onobrychis viciaefolia* also is in agreement with the separation of the group *R. sullae* strains HCNT1, ATCC 10004T, 12, *S. saheli* USDA 1844T, 13, *S. fredii* ATCC 35423T.

### 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
study have the same LMM RNA profile (Fig. 4, lanes 1–6); this supports the homogeneous nature of the group. All strains of Rhizobium 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 Rhizobium and Sinorhizobium are shown in lanes 7–14 of Fig. 4. The SS rRNA profile is different between the two genera. The strains nodulating H. coronarium show a profile in the SS 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 Rhizobium sullae falling in the former. According to the LMM RNA data, the species of Rhizobium most closely related to Rhizobium sullae are Rhizobium mongolense (nodulating Medicago ruthenica), Rhizobium 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 (IS123T, 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 Rhizobium sullae to be screened against all others, as well as the target Rhizobium 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 Rhizobium 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 Rhizobium sullae and any of the other validly described rhizobium species included in this study were between 32.4% (Rhizobium sullae to Rhizobium hainanense) and 53.2% (Rhizobium sullae to Rhizobium 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,
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 sula, 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, mannotol, raffinose, rhamnose and lactose as carbon sources and glutamate, valine, proline, isoleucine, arginine and asparagine as nitrogen sources. The strains 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 IS123ᵀ, which has been deposited in the USDA National Rhizobium Germplasm Culture Collection (Beltsville, MD, USA) as USDA 4950ᵀ and in the DSMZ as DSM 14623ᵀ.

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**REFERENCES**


FEMS Microbiol Lett 110, 217–222.


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A. Squartini and others


