# *Herbaspirillum lusitanum* sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of *Phaseolus vulgaris*

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Several bacterial strains were isolated from root nodules of *Phaseolus vulgaris* plants grown in a soil from Portugal. The strains were Gram-negative, aerobic, curved rod-shaped and motile. The isolates were catalase- and oxidase-positive. The TP-RAPD (two-primer randomly amplified polymorphic DNA) patterns of all strains were identical, suggesting that they belong to the same species. The complete 16S rDNA sequence of a representative strain was obtained and phylogenetic analysis based on the neighbour-joining method indicated that this bacterium belongs to the *β-Proteobacteria* and that the closest related genus is *Herbaspirillum*. The DNA G+C content ranged from 57·9 to 61·9 mol%. Growth was observed with many different carbohydrates and organic acids including caprate, malate, citrate and phenylacetate. No growth was observed with maltose, *meso*-inositol, *meso*-erythritol or adipate as sole carbon source. According to the phenotypic and genotypic data obtained in this work, the bacterium represents a novel species of the genus *Herbaspirillum*, and the name *Herbaspirillum lusitanum* sp. nov. is proposed. The type strain is P6-12$^T$ (=LMG 21710$^T$=CECT 5661$^T$).

The genus *Herbaspirillum* was first described with a single species, *Herbaspirillum seropedicae*, that included bacterial strains associated with roots of several cereals (Baldani *et al.*, 1986). Three other species have since been included: *Herbaspirillum rubrisubalbicans* (Baldani *et al.*, 1996), a mild pathogen of sugarcane formerly named *Pseudomonas rubrisubalbicans*, *Herbaspirillum* species 3 (Baldani *et al.*, 1996; Gillis *et al.*, 1990), which contains mainly strains of clinical origin, and, more recently, *Herbaspirillum frisingense* (Kirchhof *et al.*, 2001), which occurs in C4-fibre plants. With the exception of *Herbaspirillum* species 3, all species of *Herbaspirillum* are nitrogen-fixing bacteria able to establish close associations with plants (Reinhold-Hurek & Hurek, 1998), even as endophytes in apoplastic (Elbeltagy *et al.*, 2001; Olivares *et al.*, 1997) or intracellular (James *et al.*, 1997; Olivares *et al.*, 1997) locations. When associated with plants, either as a causal agent of mild disease or as an asymptomatic bacterium, *Herbaspirillum* species have been found predominantly in species of the family Gramineae (Baldani *et al.*, 1996; Kirchhof *et al.*, 2001), and only exceptionally in other plants (Baldani *et al.*, 1996).

Although it has been suggested for several years that the species of *Herbaspirillum* form a type of endophytic association in gramineous plants, in which they liberate fixed nitrogen and supply it to the plant (Döbereiner *et al.*, 1993), direct experimental evidence for this observation has been recently obtained in rice (*Oryza officinalis*) inoculated with *Herbaspirillum* sp. strain B501 by using acetylene reduction and $^{15}N_2$ gas incorporation assays (Elbeltagy *et al.*, 2001).

Olivares *et al.* (1996) described the isolation of *H. seropedicae* not only from gramineae but also from roots of a legume species (*Cajanus cajan*); however, as these authors pointed

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Abbreviation: TP-RAPD, two-primer randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Herbaspirillum lusitanum* P6-12$^T$ is AF543312.

Details of DNA G+C contents, levels of DNA–DNA relatedness and pellicle formation by the novel isolates, transmission electron micrographs of cells, a 16S rDNA-based phylogenetic tree and RFLP and TP-RAPD patterns of the novel isolates are available as supplementary material in IJSEM Online.

out, the exact origin of this isolate is uncertain because small pieces of maize root may have been included in the sample. In the course of isolating bacteria from nodules of *Phaseolus vulgaris* plants growing in a soil from Portugal, we have isolated several bacteria that, based on their genotypic and phenotypic characterization, should be classified as a novel species of the genus *Herbaspirillum*, for which we propose the name *Herbaspirillum lusitanum* sp. nov. To our knowledge, this is the first report of the undoubted association of a bacterium of the genus *Herbaspirillum* with a leguminous plant.

The reference strains and novel isolates used in this study are listed in Table 1. A total of six novel herbaspirillum isolates was obtained from young nodules of five *Phaseolus vulgaris* plants growing in a soil from Sierra da Peneda in the north-east of Portugal. Isolations were made according to Vincent (1970) using YMA (Bergersen, 1961). The cultures used in further studies were purified from single colonies after 10 days of incubation at 28 °C. On YMA, colonies were mucoid, circular convex, white, slightly translucent and usually 1–2 mm in diameter within 2 days at 28 °C.

Gram-staining was performed as described by Doetsch (1981). Cell morphology was observed by scanning electron microscopy under a Zeiss EM900 electron microscope, with cells grown for 3 days at 28 °C in liquid YED medium. Cells were Gram-negative and showed a short, curved rod morphology ($1{\cdot}6 \times 0{\cdot}5$ μm). Cells were also grown in nutrient agar for 48 h to check for motility by phase-contrast microscopy. To observe flagella, cells were treated with 2 % uranyl acetate and were observed under a Zeiss EM209 transmission electron microscope. Cells were motile and showed one or two polar flagella (see Supplementary Fig. A in IJSEM Online).

The 16S rRNA gene of a representative strain, P6-12$^T$, was sequenced as described by Rivas *et al.* (2002). The sequence obtained was compared with those from GenBank using the FASTA program (Pearson & Lipman, 1988). Sequences were aligned using CLUSTAL X software (Thompson *et al.*, 1997) and distances were calculated according to Kimura's two-parameter method (Kimura, 1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was based on 1000 resamplings. The MEGA 2.1 package (Kumar *et al.*, 2001) was used for all analyses. The trees were rooted using *Comamonas testosteroni* ATCC 11996$^T$ as the outgroup (Supplementary Fig. B). Sequence similarity calculations after neighbour-joining analysis indicated that the organism was phylogenetically related to members of the family '*Oxalobacteraceae*'. The 16S rDNA sequence of strain P6-12$^T$ showed 97·9 % similarity to that of *H. frisingense*, its closest relative, indicating that strain P6-12$^T$ could constitute a novel species of the genus *Herbaspirillum*.

Determinations of DNA base composition and DNA–DNA hybridization analysis (Supplementary Table A) were performed as described by Arahal *et al.* (2001). The G+C contents of the six strains isolated in this study ranged from 57·9 to 61·9 mol%. These values are similar to those obtained for *Herbaspirillum* species. The results of DNA–DNA hybridization showed 92–98 % relatedness between strain P6-12$^T$ and the other five strains isolated. The relatedness of strain P6-12$^T$ to *H. frisingense* DSM 13128$^T$, *H. seropedicae* DSM 6445$^T$ and *H. rubrisubalbicans* DSM 9440$^T$ was respectively 28, 10 and 29 %. These results indicate that the strains isolated in this study do not belong to any of the known species of *Herbaspirillum* when the recommendation of a threshold value of 70 % DNA–DNA relatedness for definition of species is considered (Wayne *et al.*, 1987).

PCR products of 16S rDNA amplification were digested with the restriction endonucleases *Dde*I and *Cfo*I (Amersham-Pharmacia Biotech) as recommended by the manufacturer and electrophoresed in 2 % agarose gels. Each endonuclease produced the same RFLP pattern in all strains isolated in this study, which was different from those obtained for *Herbaspirillum* species already described (Supplementary Fig. C).

TP-RAPD (two-primer randomly amplified polymorphic DNA) patterns were obtained according to Rivas *et al.*

**Table 1.** Strains used in this study

| Strain | Source | Geographical origin |
|---|---|---|
| P6-12$^T$ | *Phaseolus vulgaris* nodules | Portugal |
| P6-13 | *Phaseolus vulgaris* nodules | Portugal |
| P6-14 | *Phaseolus vulgaris* nodules | Portugal |
| P6-15 | *Phaseolus vulgaris* nodules | Portugal |
| P6-16 | *Phaseolus vulgaris* nodules | Portugal |
| P6-17 | *Phaseolus vulgaris* nodules | Portugal |
| *H. seropedicae* DSM 6445$^T$ | *Oryza sativa* roots | Brazil |
| *H. seropedicae* DSM 6447 | *Zea mays* roots | Brazil |
| *H. frisingense* DSM 13128$^T$ | *Miscanthus sacchariflorus* washed stems | Germany |
| *H. rubrisubalbicans* DSM 9440$^T$ | *Saccharum officinarum* roots | USA |

(2001) by using the primer 849F (5′-GCCTGGGGAGT-ACGGCCGCA-3′; *Escherichia coli* positions 829–849) and the reverse primer 1522R (5′-AAGGAGGTGATCCANCC-RCA-3′; *E. coli* positions 1502–1522), both originally designed for amplification of 16S rDNA. Each species of the genus *Herbaspirillum* showed a different pattern and all strains from this study showed the same pattern that was different from those of the species of the genus *Herbaspirillum* (Supplementary Fig. D). According to our previous results, strains showing different TP-RAPD patterns belong to different species (Rivas *et al.*, 2001, 2002). Therefore, the TP-RAPD and RFLP patterns confirm the results obtained by 16S rDNA sequence analyses, DNA base composition and DNA–DNA hybridization analyses and indicate that the novel isolates belong to a novel species of the genus *Herbaspirillum*.

The six strains isolated in this study, together with the type strain of the previously described species of *Herbaspirillum*, were subjected to several phenotypic tests. The ability to grow at temperatures between 20 and 40 °C and at pH values between 5 and 8 was determined on YMA medium. Catalase production was assayed by using 0·3 % hydrogen peroxide with one colony taken from YMA plates. Oxidase activity was detected by using *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride. For testing antibiotic resistance, API ATB G− strips (bioMérieux) were used following the manufacturer's instructions. Other physiological and biochemical tests were carried out using API 20NE and API ZYM strips (bioMérieux) following the manufacturer's instructions.

The six strains isolated in this work showed the same physiological and biochemical characteristics. Their range of temperature for growth was 20–35 °C. However, the known species of *Herbaspirillum* were able to grow at up to 40 °C, which is in agreement with previous reports (Baldani *et al.*, 1996; Kirchhof *et al.*, 2001). All isolates were able to grow at pH 5–8. The strains isolated in this study can be distinguished from previously described *Herbaspirillum* species on the basis of phenotypic properties such as nitrate reduction, *β*-galactosidase production, assimilation of *N*-acetyl D-glucosamine, *meso*-inositol, *meso*-erythritol, L-rhamnose and arabinose and resistance to gentamicin, cefotaxime, ceftazidime, tobramicin, netilmicin and amikacin (Table 2).

Microaerophilic, dinitrogen-fixation-dependent growth was assessed by monitoring pellicle-forming ability on a nitrogen-free, semi-solid medium (Döbereiner, 1995), as described for *H. frisingense* (Kirchhof *et al.*, 2001). All strains isolated in this study were able to grow, forming a pellicle, in such medium supplemented with malate, D-glucose, *N*-acetyl D-glucosamine, L-arabinose, mannitol, D-fructose, L-tartrate and L-rhamnose, but not when supplemented with *meso*-inositol or *meso*-erythritol (Supplementary Table B). Furthermore, the presence of the *nifD* gene was confirmed by using a PCR approach with universal *nifD* primers (Stoltzfus *et al.*, 1997). After electrophoresis in a 1·5 % agarose gel, a band of 390 bp was observed (data not shown), coinciding with the results obtained in *H. frisingense* (Kirchhof *et al.*, 2001) and in other endophytic bacteria (Stoltzfus *et al.*, 1997).

**Table 2.** Phenotypic characteristics of the type strains of *Herbaspirillum* species

Strains: 1, *H. seropedicae* DSM 6445$^T$; 2, *H. rubrisubalbicans* DSM 9440$^T$; 3, *H. frisingense* DSM 13128$^T$; 4, P6-12$^T$. All strains were positive for assimilation of glucose*, mannose*, mannitol*, gluconate*, caprate*, malate*, citrate* and phenylacetate* and production of alkaline phosphatase†, esterase (C4)†, esterase lipase (C8)†, leucine arylamidase†, valine arylamidase†, cystine arylamidase†, acid phosphatase†, naphthol-AS-BI-phosphohydrolase† and urease*. All strains were negative for assimilation of maltose* and adipate*, production of lipase (C14)†, trypsin†, chymotrypsin†, α-galactosidase†, *β*-glucuronidase†, α-glucosidase†, *β*-glucosidase†, *N*-acetyl-*β*-glucosaminidase†, α-mannosidase†, α-fucosidase†, arginine dihydrolase* and protease*, indole production* and glucose acidification*.

| Test | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Reduction of nitrates to nitrites* | + | + | + | − |
| *β*-Galactosidase*† | + | + | + | − |
| Assimilation of: | | | | |
| *N*-Acetyl D-glucosamine* | + | − | + | + |
| *meso*-Inositol‡ | + | − | − | − |
| L-Rhamnose‡ | + | − | − | + |
| *meso*-Erythritol‡ | − | + | − | − |
| Arabinose* | + | + | − | + |
| Resistance to (μg ml$^{-1}$): | | | | |
| Gentamicin (8) | + | − | − | − |
| Cefotaxime (32) | − | + | + | + |
| Ceftazidime (32) | + | − | − | + |
| Tobramicin (8) | + | + | − | − |
| Netilmicin (8) | + | − | − | − |
| Amikacin (16) | + | − | − | − |

*Included in API 20NE strip.
†Included in API ZYM strip.
‡The mannitol component in YMA medium was replaced by the corresponding substrate.

The infectivity of strain P6-12$^T$ was assayed in *Phaseolus vulgaris* plants. Seeds of *Phaseolus vulgaris* were surface-sterilized for 10 min using 5 % sodium hypochlorite and then washed repeatedly with sterile, distilled water. After sterilization, the seeds were sown in pots containing autoclaved vermiculite. One week after germination, roots were inoculated with 1 ml ($10^5$ cells) of a 48 h culture in YMB medium or with sterile water as a negative control. Two weeks after inoculation, plants were withdrawn from the pots and bacteria were reisolated from the roots as described by Elbeltagy *et al.* (2001). Finally, decimal dilutions were inoculated on YMA plates and incubated for 48 h at 28 °C. From these plates, several colonies were chosen at

random and the identity of these isolates was checked by TP-RAPD fingerprinting (Rivas *et al.*, 2001). Strain P6-12$^T$ was recovered from root tissues at concentrations of 2·3–3·7 × $10^3$ c.f.u. (g fresh weight)$^{-1}$, which is in accordance with results reported for the species of *Herbaspirillum* known to be endophytes (Elbeltagy *et al.*, 2000).

Therefore, on the basis of phylogenetic, genotypic and phenotypic data, we propose that the isolates from this study should be classified as the novel species *Herbaspirillum lusitanum* sp. nov.

### Description of *Herbaspirillum lusitanum* sp. nov.

*Herbaspirillum lusitanum* (lu.si.ta′num. L. neut. adj. *lusitanum* of *Lusitania*, the Roman name of Portugal, where the strains reported in this study were isolated).

Gram-negative, aerobic, non-spore-forming curved cells, 1·6 μm long and 0·5 μm in diameter. Motile by polar flagella. Colonies on YMA are circular convex, white, slightly translucent and usually 1–2 mm in diameter within 2 days at 28 °C. Carbon source utilization, hydrolytic enzyme production and resistance to antibiotics (including differentiating characters for all *Herbaspirillum* species) are indicated in Table 2. The G+C content of the DNA is 59·9±2 mol%.

The type strain is P6-12$^T$ (=LMG 21710$^T$=CECT 5661$^T$), isolated from root nodules of *Phaseolus vulgaris* plants grown in a soil from Sierra da Peneda (Portugal). Its DNA G+C content is 57·9 mol%.

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