Mesorhizobium metallidurans sp. nov., a metal-resistant symbiont of *Anthyllis vulneraria* growing on metalicolous soil in Languedoc, France

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A polyphasic taxonomic approach was used to characterize 31 rhizobial isolates obtained from *Anthyllis vulneraria*, a metalicolous legume species, growing close to a zinc mine in the south of France (Saint Laurent le Minier). Comparative analysis of nearly full-length 16S rRNA gene sequences showed that these Gram-negative bacteria belonged to the genus *Mesorhizobium* and that they were related most closely to *Mesorhizobium tianshanense* ORS 2640^T. The phylogenetic relationships of these isolates with other *Mesorhizobium* species were confirmed by sequencing and analysis of the *recA* and *atpD* genes, which were used as alternative chromosomal markers. These novel mesorhizobial strains tolerated high concentrations of heavy metals: 16–32 mM Zn and 0.3–0.5 mM Cd. DNA–DNA hybridizations revealed >73% relatedness between the strains isolated from *A. vulneraria*, but only 19–33% relatedness between these and the type strains of *M. tianshanense* and *Mesorhizobium mediterraneum*. These results, together with other phenotypic characteristics, support the conclusion that these isolates represent a single, novel species of the genus *Mesorhizobium*, for which the name *Mesorhizobium metallidurans* sp. nov. is proposed. The type strain is STM 2683^T (=CFBP 7147^T=LMG 24485^T).

In the Languedoc region of the south of France, the zinc-mining area at Saint Laurent le Minier had been exploited for 120 years, and closed in 1991. One of the few plant species growing on the heavy metal-enriched spoil heaps of the area (5–15% Zn) is *Anthyllis vulneraria*, a plant associated with a nitrogen-fixing *Rhizobium*. Together, they are an interesting tool for post-mining restoration (Frérot *et al.*, 2006). Indeed, plants of the family Leguminosae are of general environmental interest; bacteria, in association with legumes, can reduce atmospheric dinitrogen to organic nitrogen and thus

can greatly increase plant growth and stimulate soil fertility. The bacteria that form nitrogen-fixing nodules on leguminous plants belong to both the *Alphaproteobacteria* and *Betaproteobacteria*. Some of these species belong to the genus *Mesorhizobium*, which was first described by Jarvis *et al.* (1997). At the time of writing, this genus comprises 12 recognized species, namely *Mesorhizobium loti* (Jarvis *et al.*, 1982), *M. huakuii* (Chen *et al.*, 1991), *M. ciceri* (Nour *et al.*, 1994), *M. mediterraneum* (Nour *et al.*, 1995), *M. tianshanense* (Chen *et al.*, 1995), *M. plurifarium* (de Lajudie *et al.*, 1998), *M. amorphae* (Wang *et al.*, 1999), *M. chacoense* (Velazquez *et al.*, 2001), *M. septentrionale* (Gao *et al.*, 2004), *M. temperatum* (Gao *et al.*, 2004), *M. thiogangeticum* (Ghosh & Roy, 2006) and *M. albiziae* (Wang *et al.*, 2007).

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are AM930381–AM930393.

A table showing GenBank accession numbers for individual sequences is available with the online version of this paper.

The presence of nodules on *A. vulneraria* root systems was first observed from plants excavated directly from the mine site of Saint Laurent le Minier. Bacterial isolates nodulating this plant species were subsequently obtained from soil samples by trapping with *A. vulneraria*. Seeds of *A. vulneraria* used for both trapping and nodulation investigation were collected in bulk from the same site of Saint Laurent le Minier. Thirty-one rhizobia were isolated from nodules harvested from 30 plants. Nodules were surface-sterilized with 300 µl H₂O₂ (70 %) for 2 min, rinsed with distilled water (five times) and crushed in 50 µl sterile distilled water. The suspension was streaked on yeast extract mannitol (YEM) medium (Vincent, 1970). Colonies appeared after 2–3 weeks incubation at 28 °C under aerobic conditions and were checked for purity by repeated streaking on YEM agar. All strains were kept in 20 % glycerol (v/v) at –80 °C.

The nodulating ability of the 31 mesorhizobial isolates was verified. Seeds were scarified and surface-sterilized with sulfuric acid for 2 min and then rinsed with distilled water (five times). Seeds were incubated to allow germination in sterile Petri dishes with 1 % water agar for 48 h at 4 °C and then for 48 h at 28 °C. Plants were transferred to tubes containing clay granules as rooting substrate (Damolin) and 40 ml mineral nutritive solution lacking nitrogen (Bertrand *et al.*, 2000). Each tube was inoculated with one rhizobial suspension from an early-stationary-phase culture. Ten replicates were prepared for each treatment. After 7 weeks, plants were harvested and analysed for nodule occurrence. Observations confirmed that all bacterial isolates had the ability to nodulate the original host plant, *A. vulneraria*.

In order to assess the taxonomic position of the 31 rhizobia, a phylogenetic analysis was performed. The nearly full-length 16S rRNA genes (1399 bp) were amplified and their sequences were determined from the PCR products by using universal primers (Normand *et al.*, 1992). The genus *Mesorhizobium* contains several species that share high 16S rRNA gene sequence similarity (Gao *et al.*, 2004; Kwon *et al.*, 2005). Therefore, data from additional gene sequences are usually needed for species delineation within this genus. Phylogenies of the *recA* gene (encoding part of the DNA recombination and repair system) and *atpD* gene (encoding the β chain of ATP synthase) in rhizobial taxa (Gaunt *et al.*, 2001; Vinuesa *et al.*, 2005; Weir *et al.*, 2004) have been shown to be congruent with those of the 16S rRNA gene, with well-supported clades grouping *Mesorhizobium* species with high levels of bootstrap support (Gaunt *et al.*, 2001). The two housekeeping genes *recA* and *atpD* were amplified by using published primers (Gaunt *et al.*, 2001) and resulted in two DNA fragments of 469 and 433 bp, respectively. Sequences obtained were compared with sequences retrieved from GenBank by using the BLASTN program (Altschul *et al.*, 1990). Multiple nucleotide sequence alignments were generated by using CLUSTAL_X version 1.83 (Thompson *et al.*, 1997) and were optimized manually by using GeneDoc 2.6.002 (Nicholas & Nicholas, 1997). Phylogenetic trees were inferred with the

neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Yang, 1997) methods by using PAUP version 4.0b10 (Swofford, 1998). For *recA* and *atpD* gene sequence analyses, we estimated the best likelihood model for each dataset by using MODELTEST (Posada & Crandall, 1998), and we consequently applied a GTR+G model (general time-reversible with rates across sites assumed to follow a continuous gamma distribution). Trees were obtained by using a heuristic search implemented in PAUP. Bootstrap analysis was based on 1000 resamplings to validate the reliability of the branching pattern of the tree. All trees were visualized by using TreeView version 1.6.6 (Page, 1996). Trees were rooted by using the type strain of *Bradyrhizobium japonicum* as an outgroup. The neighbour-joining and maximum-likelihood methods produced similar results for the 16S rRNA gene sequence analyses (data not shown).

The 16S rRNA gene sequences of the 31 new isolates analysed showed 100 % similarity. Fig. 1 shows the neighbour-joining phylogenetic tree based on full-length 16S rRNA gene sequences. The closest relative to the novel strains was the type strain of *M. tianshanense* (99.7 % similarity), and in the neighbour-joining tree these clustered together with >90 % bootstrap support.

These phylogenetic relationships among the new isolates and other *Mesorhizobium* species were confirmed by *recA*

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain STM 2683^T and the type strains of reference species. The analysis was based on 1399 nt. GenBank accession numbers are given in parentheses, and the significance of each branch is indicated by a bootstrap value (when ≥ 700) calculated for 1000 subsets at major branch points. Bar, 1 substitution per 100 nt.

and *atpD* gene sequence analyses (Fig. 2). The 31 rhizobia isolated from *A. vulneraria* clustered together and diverged from *M. tianshanense* ORS 2640^T and *M. mediterraneum* ORS 2739^T as sister clades. Compared with the 16S rRNA gene sequences of the novel strains, which were identical, the *recA* and *atpD* gene sequences displayed some variability (95.9–99.8 and 96.5–99.7 % similarity, respectively), and their analyses allowed the distinction of six closely related clusters among the 31 strains (Fig. 2). GenBank accession numbers for individual sequences are given in Supplementary Table S1 (available in IJSEM Online).

DNA–DNA hybridization was carried out with a fluorometric method by using photobiotin-labelled DNA probes (Ezaki *et al.*, 1989), following the recommendations of

Willems *et al.* (2001). The five strains showing highest *recA* and *atpD* gene sequence dissimilarity were analysed. The hybridization temperature was 42 °C. At least four repetitions were performed, and self-hybridization of the probe with homologous target DNA was set at 100 %. The novel mesorhizobial strains isolated from *A. vulneraria* showed >73 % DNA–DNA relatedness, compared with 19–33 % DNA–DNA relatedness between these strains and *M. tianshanense* ORS 2640^T and *M. mediterraneum* ORS 2739^T (Table 1). Given the recommended threshold value of 70 % DNA–DNA relatedness for the definition of a species (Wayne *et al.*, 1987), these results indicate that the strains isolated from *A. vulneraria* should be considered as representing a novel species.

Among the 31 isolates from *A. vulneraria*, five strains (STM 2683^T, STM 3291, STM 3292, STM 3293 and STM 3294), chosen as described above, and reference strains of *Mesorhizobium* were characterized phenotypically. Classical phenotypic features and the ability to tolerate zinc and cadmium were examined. In total, 138 phenotypic characteristics were analysed. Assimilation of substrates was determined by using the API 100, API 50CH and API 20NE systems (bioMérieux). Tests for antibiotic resistance, tolerance to different concentrations of NaCl, and temperature and pH ranges for growth were performed in YEM. The optimal growth temperature was 28 °C. None of the strains was able to grow at 4 or 41 °C. Table 2 shows the key phenotypic characteristics that can be used to distinguish the rhizobial strains isolated from metalicolous *A. vulneraria* from the other *Mesorhizobium* species and *Rhizobium galegae*. As shown in Table 2, the novel strains differed from recognized *Mesorhizobium* species in the assimilation of 21 carbon sources. No difference in antibiotic resistance was found.

To test for metal resistance, the strains were grown on YEM liquid medium with increasing concentrations of zinc (in the form of ZnSO₄) of 0.02, 0.05, 0.1, 0.3, 0.5, 1, 2, 4, 6, 8, 10, 12, 16 and 32 mM, and cadmium (in the form of CdCl₂) of 0.02, 0.05, 0.1, 0.3, 0.5, 0.8 and 1 mM. Each metal concentration was tested in triplicate and each bacterial culture was incubated at 28 °C. OD₆₂₀ was measured after 2 weeks growth. The lowest concentration that prevented growth was considered the minimum inhibitory concentration (Andrews, 2001). The strains isolated from *A. vulneraria* tolerated high concentrations of heavy metals, 16–32 mM Zn and 0.3–0.5 mM Cd, whereas *M. tianshanense* ORS 2640^T and *M. mediterraneum* ORS 2739^T were able to tolerate only low concentrations, 0.05–0.5 mM Zn and 0.05 mM Cd.

Microbes apply various types of resistance mechanisms in response to heavy metals (Nies, 2003). These mechanisms, often based on efflux systems, may be encoded by chromosomal genes, but loci conferring resistance are more usually located on plasmids (Cervantes & Gutierrez-Corona, 1994; Wuertz & Mergeay, 1997). The resistance systems of the strains isolated from *A. vulneraria*, which

Fig. 2. Phylogenetic trees constructed by using (a) *recA* and (b) *atpD* gene sequences. The analyses were based on 469 and 433 nt, respectively. Maximum-likelihood trees were constructed by using a GTR+G model via the program PAUP. GenBank accession numbers are given in parentheses, and the significance of each branch is indicated by a bootstrap value (when ≥ 700) calculated for 1000 subsets at major branch points. Bars, 10 substitutions per 100 nt.

Table 1. Levels of DNA–DNA relatedness among five *Mesorhizobium* strains isolated from *Anthyllis vulneraria* and the type strains of *M. tianshanense* and *M. mediterraneum*

ND, Not determined.

Strain	DNA–DNA relatedness (%) with DNA of strain:			
	1	2	3	4
1 <i>M. tianshanense</i> ORS 2640 ^T	100			
2 STM 2683 ^T	29	100		
3 STM 3293	28	82	100	
4 STM 3297	ND	78	88	100
5 STM 3294	33	77	77	89
6 STM 3292	18	73	91	ND
7 <i>M. mediterraneum</i> ORS 2739 ^T	19	27	24	ND

tolerated high concentrations of Zn and Cd, remain unknown.

From the above data, the 31 heavy metal-tolerating isolates from root nodules of *A. vulneraria* are considered to

represent a single, novel species of the genus *Mesorhizobium*, for which the name *Mesorhizobium metallidurans* sp. nov. is proposed.

Description of *Mesorhizobium metallidurans* sp. nov.

Mesorhizobium metallidurans (me.tal.li.du'rans. L. n. *metallum* metal; L. pres. part. *durans* enduring; N.L. part. adj. *metallidurans* enduring metal, referring to the metal resistance of strains of the species).

Gram-negative, aerobic, non-spore-forming rods. Colonies appearing on YEM agar within 3–5 days incubation at 28 °C are circular, opaque, convex and cream-coloured. The maximum temperature for aerobic growth is 37 °C; some strains can tolerate 1 % NaCl and grow over a pH range of 5–11. Nodulates the legume *Anthyllis vulneraria*. Tolerates high concentrations of heavy metals: 16–32 mM zinc and 0.3–0.5 mM cadmium. Resistant to penicillin G (10 µg ml⁻¹), erythromycin (15 µg ml⁻¹) and cefuroxime (30 µg ml⁻¹); susceptible to ampicillin (10 µg ml⁻¹), gentamicin (10 µg ml⁻¹), polymyxin B (300 µg ml⁻¹) and tetracycline (30 µg ml⁻¹). Assimilates lactulose, L-tryptophan, 4-aminobutyrate, proline, glycerol, arabinose,

Table 2. Differential phenotypic characteristics among five strains isolated from *Anthyllis vulneraria*, the type strains of recognized *Mesorhizobium* species and the type strain of *Rhizobium galegae*

Strains: 1, STM 2683^T, STM 3291, STM 3292, STM 3293 and STM 3294; 2, *M. tianshanense* ORS 2640^T; 3, *M. mediterraneum* ORS 2739^T; 4, *M. amorphae* LMG 18977^T; 5, *M. huakuii* CCBAU 2609^T; 6, *M. chacoense* LMG 19008^T; 7, *M. temperatum* SDW018^T; 8, *M. plurifarium* LMG 11892^T; 9, *M. septentrionale* SDW014^T; 10, *M. albiziae* CCBAU 61158^T (data from Wang *et al.*, 2007); 11, *M. thiogangeticum* SJT^T (Ghosh & Roy, 2006); 12, *M. ciceri* UPM-Ca7^T; 13, *M. loti* NZP 2213^T; 14, *R. galegae* ATCC 43677^T (Nour *et al.*, 1994). +, Positive; –, negative; ±, weakly positive; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Assimilation of:														
Amygdalin, tryptamine, 5-keto-D-gluconate, D-tartrate, meso-tartrate, <i>m</i> -hydroxybenzoate	–	–	–	–	–	–	–	–	–	ND	ND	–	–	+
L-Aspartate, itaconate, maltitol, trigonelline, α-L(–)-fucose	–	+	+	+	+	+	+	+	+	ND	ND	+	+	+
Maltose, fructose	+	+	+	+	+	+	+	+	+	+	–	+	+	+
L(+)-Sorbitol	+	–	+	–	–	–	–	+	–	+	ND	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	–
Lactose	+	+	+	+	+	–	+	+	+	+	–	–	+	ND
Raffinose	–	–	–	+	+	+	+	+	+	+	–	–	ND	–
Melibiose	+	+	+	+	+	+	+	+	+	+	+	–	–	–
L-Malonate	–	+	+	+	+	+	+	–	–	–	ND	+	+	+
Sucrose	±	+	–	+	+	+	+	+	+	+	–	+	+	+
L-Malate	+	–	+	+	+	+	–	+	+	+	–	+	+	+
Erythritol	+	+	+	+	–	–	+	–	+	+	ND	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	–	–	+	+	+
L-Xylose	+	+	–	+	+	+	–	+	+	+	–	+	–	+
L-Arabinose	+	+	+	+	+	+	–	+	+	–	+	+	+	+
Dulcitol	±	+	–	+	–	–	–	–	+	–	ND	–	–	–
Citrate	–	–	–	+	–	–	+	+	–	–	ND	–	–	–

ribose, xylose, sorbose, adonitol, methyl β -D-xylopyranoside, glucose, aesculin, turanose and lyxose. Utilization of galactose, fructose, D-mannose, rhamnose, dulcitol, inositol, mannitol, sorbitol, cellobiose, maltose, lactose, sucrose, trehalose, xylitol, L-alanine, arabinol, methyl galactopyranoside, saccharate, *cis*-aconitate, citrate, glucuronate, galacturonate, quinate, histamine, caprylate, succinate, glutarate, glutamate, propionate and tyrosine as sole carbon source is strain-dependent. No growth is observed with fucose, maltotriose, palatinose, melezitose, maltitol, trigonelline, malonate, methyl D-glucopyranose, mucate, tartrate, L-aspartate, *trans*-aconitate, triscarballylate, protocatechuate, hydroxybenzoate, gentisate, benzoate, 3-phenylpropionate, coumarate, putrescine, lactate, caprate, 5-aminovaleate, ethanolamine, tryptamine, itaconate, D-alanine, L-serine, α -ketoglutarate, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, amygdalin, arbutin, salicin, inulin, melezitose, raffinose, starch, glycogen or potassium ketogluconate.

The type strain, STM 2683^T (=CFBP 7147^T=LMG 24485^T), was isolated from root nodules of *Anthyllus vulneraria* found on heavy metal-enriched spoil heaps at the Saint Laurent le Minier mine (Languedoc, France).

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