Rhizobium metallidurans sp. nov., a symbiotic heavy metal resistant bacterium isolated from the Anthyllis vulneraria Zn-hyperaccumulator

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A Gram-stain-negative, aerobic, rod-shaped, non-spore-forming bacterium (ChimEc512T) was isolated from 56 host seedlings of the hyperaccumulating Anthyllis vulneraria legume, which was on an old zinc mining site at Les Avinieres, Saint-Laurent-Le-Minier, Gard, South of France. On the basis of 16S rRNA gene sequence similarities, strain ChimEc512T was shown to belong to the genus Rhizobium and to be most closely related to Rhizobium endophyticum CCGE 2052T (98.4 %), Rhizobium tibeticum CCBAU 85039T (98.1 %), Rhizobium grahamii CCGE 502T (98.0 %) and Rhizobium mesoamericanum CCGE 501T (98.0 %). The phylogenetic relationships of ChimEc512T were confirmed by sequencing and analyses of recA and atpD genes. DNA–DNA relatedness values of strain ChimEc512T with R. endophyticum CCGE 2052T, R. tibeticum CCBAU 85039T, R. mesoamericanum CCGE 502T, Rhizobium grahamii CCGE 502T, Rhizobium etli CCBAU 85039T and Rhizobium radiobacter KL09-16-8-2T were 27, 22, 16, 18, 19 and 11 %, respectively. The DNA G+C content of strain ChimEc512T was 58.9 mol%. The major cellular fatty acid was C18 : 1 v 7 c, characteristic of the genus Rhizobium. The polar lipid profile included phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol and phosphatidylcholine and moderate amounts of aminolipids, phospholipid and sulfoquinovosyldiacylglycerol. Although ChimEc512T was able to nodulate A. vulneraria, the nodC and nifH genes were not detected by PCR. The rhizobial strain was tolerant to high concentrations of heavy metals: up to 35 mM Zn and up to 0.5 mM Cd and its growth kinetics was not impacted by Zn. The results of DNA–DNA hybridizations and physiological tests allowed genotypic and phenotypic differentiation of strain ChimEc512T from species of the genus Rhizobium with validly published names. Strain ChimEc512T, therefore, represents a novel species, for which the name Rhizobium metallidurans sp. nov. is proposed, with the type strain ChimEc512T ( = DSM 26575 = CIP 110550T ).
association with A. vulneraria (Vidal et al., 2009). However further investigation showed that strain ChimEc512\textsuperscript{T}, ChimEc712 and ChimEc313 were associated with the same plant species, A. vulneraria, at the same mining site.

Fifty-six Antyllis vulneraria seedlings were collected at different strategic spots of the mining site of Les Avinières: on three different tailing basins (160 g of Zn kg\textsuperscript{-1} soil) and on an immediately polluted area (9.5 g of Zn kg\textsuperscript{-1} soil). A. vulneraria was extracted by digging a large hole to obtain the roots safely. Roots were hand-washed with water. By cutting the roots 0.5 cm from the nodule, 103 nodules were extracted. They were surface-sterilized with sodium hypochlorite (3 % v/v) for 3 min. Then they were rinsed five times with sterile water and were crushed in sterile water. The suspension was spread on yeast extract mannitol (YEM) medium [yeast extract (1 g/L), mannitol (10 g/L), K\textsubscript{2}PO\textsubscript{4} (0.5 g/L), MgSO\textsubscript{4} (0.2 g/L), NaCl (0.1 g/L), CaCO\textsubscript{3} (1.0 g/L), agar (15 g/L)]. The first colonies appeared after 32 h of incubation at 28 °C under aerobic conditions. Three subcultivations were carried out on YEM agar to obtain pure clones. All strains (ChimEc512\textsuperscript{T}, ChimEc712 and ChimEc313) were kept in 20 % glycerol (v/v) at 80 °C.

The nodulating ability of the rhizobial isolates was verified by inoculating A. vulneraria seeds according to the protocol described by Vidal et al. (2009). The inoculating ability of strain ChimEc512\textsuperscript{T} is currently being used on a large scale to develop a phytoremediation programme at the mining site of Saint-Laurent-Le-Minier (Grison et al., 2014).

In order to find the taxonomic position of the rhizobia, a region of approximately 1500 bp from their 16S rRNA genes was amplified using the primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-ACGGCTACCTTGTTACGACTT-3′) and cloned before sequencing (Kämpfer et al., 2003). The sequenced full-length 16S rRNA genes reached 1475 bp nuleotides and were well suited for phylogenetic analysis. The analyses were based on 1330 nt. Similarity searches for sequences were carried out with the BLASTN (Altschul et al., 1990) program of the National Center of Biotechnology Information, MD, USA. Multiple nucleotide sequence alignments were generated by using the MEGA 5.10 program with the closest related 16S rRNA sequences obtained from GenBank (Kumar et al., 2004). Fig. 1 shows a phylogenetic tree that was reconstructed by using the maximum-likelihood method (Tamura et al., 2011) via the program MEGA 5.10.

Surprisingly M. metallidurans, the previous strain found in symbiosis with A. vulneraria on the same mining site, was not found in the 103 nodules sampled.

Moreover and interestingly, sequence similarity calculations after maximum-likelihood analysis indicated that the 103 isolates from A. vulneraria nodules clustered together with 99.9 % similarity, but clearly diverged from M. metallidurans. They did not even belong to the same genus of Mesorhizobium, but to the genus of Rhizobium, which was confirmed by the naïve Bayesian classifier available at the Ribosomal Database Project site (http://rdp.cme.msu.edu/classifier). Although the bootstrap values were low from the branch, the rhizobia isolated from A. vulneraria clustered together and were clearly distinct from Rhizobium endophyticum CCGE 2052\textsuperscript{T} (NR_116477) (98.4 %), Rhizobium tibeticum CCGE 85039\textsuperscript{T} (NR_116254) (98.1 %), Rhizobium grahamii CCGE 502\textsuperscript{T} (JF424608) (98.0 %) and Rhizobium mesoamericanum CCGE 501\textsuperscript{T} (JF424606) (98.0 %), the most closely related strains.

Phylogenetic relationships of the novel Rhizobium lineage were further explored by sequence analysis of two housekeeping genes usually used in taxonomic studies of species of the genus Rhizobium. Fragments of recA and atpD genes encoding, respectively, the recombinate A protein and ATP synthase beta subunit, were amplified and sequenced, as previously described (Gaunt et al., 2001).

Sequence alignments and maximum-likelihood phylogenetic analyses were performed as described for the 16S rRNA gene. As expected, the rhizobia isolated from A. vulneraria clustered together for recA and atpD phylogeny. The maximum-likelihood analyses for atpD gene sequences displayed R. grahamii CCGE 502\textsuperscript{T} (JF424608) as the closest related species (Fig. S1, available in the online Supplementary Material), which is consistent with the 16S rRNA phylogeny. The maximum-likelihood analyses of recA gene sequences displayed similarities of less than 90 % with the closest related species (Fig. S2): R. tibeticum LMG24453\textsuperscript{T} (HQ735075.1), Rhizobium etli CCBAU 85039\textsuperscript{T} (EU288694.1), and Rhizobium radiobacter KL09-16-8-2\textsuperscript{T} (AB738671). Housekeeping genes analyses clearly showed that ChimEc512\textsuperscript{T} belongs to a novel species of the genus Rhizobium.

The DNA G+C content of ChimEc512\textsuperscript{T} was measured by the De Ley thermal denaturation method (De Ley, 1970) and reached 58.9 mol%, which is within the DNA G+C content range (57–66 mol%) for the genus Rhizobium.

DNA–DNA hybridization (De Ley et al., 1970) was performed with R. endophyticum CCGE 2052\textsuperscript{T}, R. tibeticum CCBAU 85039\textsuperscript{T}, R. grahamii CCGE 502\textsuperscript{T}, R. mesoamericanum CCGE 501\textsuperscript{T}, R. etli CCBAU 85039\textsuperscript{T}, and R. radiobacter KL09-16-8-2\textsuperscript{T}, as these were found to be closely related species in the recA phylogeny. This was carried out by labelling DNA probes with photo-activable biotin (PAB) (Ezaki et al., 1989). The mixture of labelled probe and target DNA was heated to 95 °C for 10 min and held at 67 °C. The assays were performed in triplicate and self-hybridization of the labelled probe with homologous target DNA was set at 100 %. As shown in Table 1, strain ChimEc512\textsuperscript{T} had low DNA–DNA relatedness with the type strains R. endophyticum CCGE 2052\textsuperscript{T} (27 %), R. tibeticum CCBAU 85039\textsuperscript{T} (22 %), R. grahamii CCGE 502\textsuperscript{T} (18 %), R. mesoamericanum CCGE 501\textsuperscript{T} (16 %), R. etli CCBAU 85039\textsuperscript{T} (19 %) and R. radiobacter KL09-16-8-2 (11 %). The recommended threshold value of DNA–DNA relatedness is 70 % for the definition of a species (Wayne et al., 1987). Therefore, these results indicate that the strains isolated...
Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain ChimEc512^T and the most closely related reference strains. The analysis was based on 1330 nt. GenBank accession numbers are given in parentheses. The significance of each branch is indicated by a bootstrap value calculated for 1000 replicates. Bootstrap values greater than 50% are indicated. *M. metallidurans* STM2683^T was used as an outgroup (data not shown). Bar, Mean number of substitutions per site.
fatty acid composition of strain ChimEc512T were carried
bacterial species (Tighe et al., 2000). Analyses of the cellular
fatty acid composition of strain ChimEc512T were carried
out by the Identification Service, Leibniz-Intsitut DSMZ –
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bation at 28° C on YEM plates, the cells were harvested and
fatty acids were saponified, methylated and extracted. The
fatty acid methyl ester mixtures were separated using the
Sherlock Microbial Indentification System (MIS) (MIDI,
Microbial ID, Newark, DE 19711 USA), which consisted of an
Agilent model (6890N) gas chromatograph, and were
determined with the TSBA40 4.10 database. The cellular
fatty acid profile of strain ChimEc512T is presented in Table
S1. It seems that C_{18:1}ω7c (67.4 %), 11-methyl C_{18:1}ω7c
(6.7 %), C_{12:0}-aldehyde and an unknown ECL 10.928 (3.9 %),
C_{18:0} (5.6 %), C_{16:0} (5.6 %), C_{14:0} 3-OH and C_{16:1} ISO 1
(5.5 %) are the major fatty acids common to all species of the
genus Rhizobium, although the proportions vary in different
species. In addition, other fatty acids, C_{18:0} 3-OH (2.1 %),
C_{20:1}ω7c (0.2 %) and C_{14:0} (0.07 %) were also detected.

The polar lipid profile was determined by the Identification
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dyethanolamine, phosphatidylmonomethylethanolamine,
phosphatidylglycerol and phosphatidylcholine. Additionally,
morden amounts of aminolipids, phospholipid and sulfo-
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The phenotypic characteristics of the novel rhizobial lineage
were compared with those of the closest related species, R.
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Classical phenotype features and the resistance to heavy
metals were tested.

Assimilation of carbon and nitrogen sources was determined
by using the API 20NE and the API 50CH systems
(bioMérieux). Tests for antibiotic resistance, tolerance to
high concentration of heavy metals, and temperature and
pH ranges for growth were performed in YEM and yeast
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As shown in Table 2, the novel strains were resistant to
penicillin G (10 mg ml⁻¹), erythromycin (15 mg ml⁻¹) and
cefuroxime (30 mg ml⁻¹); they were sensitive to ampicillin
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Since the new rhizobial species had been found on a Zn
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YEM medium artificially enriched with solid Zn and Cd.
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range of enriched YMB was tested: 0 mM ZnSO₄, 15 mM
ZnSO₄, 25 mM ZnSO₄, 35 mM ZnSO₄ and 40 mM ZnSO₄.
Bacterial population evolution had been determined from
dry mass measurements as ChimEc512TT aggregated in
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from A. vulneraria should be considered to represent a novel

Profiles of cellular fatty acids have been used to
discriminate species of Rhizobium and to describe novel
bacterial species (Tighe et al., 2000). Analyses of the cellular
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Table 2. Differential phenotypic characteristics between the novel strains isolated from Anthyllis vulneraria and strains of closely related species of the genus Rhizobium

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<td>Resistance to antibiotics (mg ml⁻¹)</td>
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Rhizobium metallidurans (metal.li.du’ rans. L. n. metallum metal; L. part. adj. durans enduring; N.L. part.adj. metallidurans enduring metal, referring to metal resistance).

Cells are non-motile, non-spore-forming rods (approximately 2 μm in length). Gram-stain-negative, aerobic showing oxidative metabolism. Colonies appear on YEM agar within 32 h of incubation at 28 °C with a diameter of approximately 2 mm and are circular, opaque, convex and cream. Optimum growth temperature is 25 to 30 °C; can grow at 18–37 °C. Optimum pH is 7.0–8.0; can grow at pH 5–9. Predominant polar lipids are phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol and phosphatidylincholine. Additionally, moderate amounts of aminolipids, phospholipids and sulfoquinovosyl diacylglycerol are detected. The fatty acid profile is largely composed of C₁₈:₁ω7c. Resistant to high concentrations of heavy metals up to 35 mM Zn and 0.5 mM Cd. Produces extracellular polysaccharides on YEM and assimilates acetic acid, D-fructose, fumaric acid, D-galactose, D-gluconate, D-glucose, lactose, malic acid, threonine as sole nitrogen sources. It can be distinguished from closely related species by DDH and by sequence analysis of 16S rRNA. Able to nodulate A. vulneraria roots.

The type strain, ChimEc512T (DSM 26575= CIP 110550T), was isolated from the root nodules of Anthyllis vulneraria growing on a soil contaminated with heavy metals at the mining site of Les Avinieres (Saint-Laurent-Le-Minier, Gard County, France). The genomic DNA G+C content of the type strain is 58.9 mol%.

Aknowledgements

The authors would like to thank Dr. Jacques Biton and Jocelyn Méré for their encouragement and ANR (11ECOT 011 01) program and Science Foundation Ireland for financial support.

References


