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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 (ChimEc512^T) was isolated from 56 host seedlings of the hyperaccumulating *Anthyllis vulneraria* legume, which was on an old zinc mining site at Les Avinières, Saint-Laurent-Le-Minier, Gard, South of France. On the basis of 16S rRNA gene sequence similarities, strain ChimEc512^T was shown to belong to the genus *Rhizobium* and to be most closely related to *Rhizobium endophyticum* CCGE 2052^T (98.4%), *Rhizobium tibeticum* CCBAU 85039^T (98.1%), *Rhizobium grahamii* CCGE 502^T (98.0%) and *Rhizobium mesoamericanum* CCGE 501^T (98.0%). The phylogenetic relationships of ChimEc512^T were confirmed by sequencing and analyses of *recA* and *atpD* genes. DNA–DNA relatedness values of strain ChimEc512^T with *R. endophyticum* CCGE 2052^T, *R. tibeticum* CCBAU 85039^T, *R. mesoamericanum* CCGE 52^T, *Rhizobium grahamii* CCGE 502^T, *Rhizobium etli* CCBAU 85039^T and *Rhizobium radiobacter* KL09-16-8-2^T were 27, 22, 16, 18, 19 and 11%, respectively. The DNA G+C content of strain ChimEc512^T was 58.9 mol%. The major cellular fatty acid was C18:1 ω 7c, characteristic of the genus *Rhizobium*. The polar lipid profile included phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol and phosphatidylcholine and moderate amounts of aminolipids, phospholipid and sulfoquinovosyl diacylglycerol. Although ChimEc512^T 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 ChimEc512^T from species of the genus *Rhizobium* with validly published names. Strain ChimEc512^T, therefore, represents a novel species, for which the name *Rhizobium metallidurans* sp. nov. is proposed, with the type strain ChimEc512^T (=DSM 26575=CIP 110550^T).

In Gard County (South of France), the zinc mining site of Les Avinières was exploited for centuries and closed in 1991. Such past mining activities led to serious land degradation and environmental pollution. One of the few plant species naturally growing on the heavy metal enriched soil is

Anthyllis vulneraria (Escarré *et al.*, 2010). It is one of the rare legumes able to hyperaccumulate heavy metals, such as Zn in high concentrations: 154, 202 mg Zn kg⁻¹ soil (Grison *et al.*, 2010).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA, *atpD* and *recA* gene sequences of strain ChimEc512^T are JX678769, KF863911 and KF863912, respectively; of strain ChimEc712 they are KM387391, KM393189 and KM393191, respectively; and of strain ChimEc313 they are KM387390, KM393188 and KM393190, respectively.

As with every plant of the *Leguminosae* family, *A. vulneraria* can live in symbiosis with a nitrogen-fixing *Rhizobium*. The genus *Rhizobium* was first described by Franck in 1889, and the knowledge of this genus has largely been improved as half of the 40 species with validly published names have been discovered in the past 10 years.

Four supplementary figures and two supplementary tables are available with the online Supplementary Material.

In 2009, at the site of Les Avinières, a novel bacterial species, *Mesorhizobium metallidurans*, was discovered living in

association with *A. vulneraria* (Vidal *et al.*, 2009). However further investigation showed that strain ChimEc512^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⁻¹ soil) and on an intermediately polluted area (9.5 g of Zn kg⁻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), K₂PO₄ (0.5 g/L), MgSO₄ (0.2 g/L), NaCl (0.1 g/L), CaCO₃ (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^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^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'-AGAGTTT-GATCMTGGCTCAG-3') and 1492r (5'-ACCTTGTTACG-ACTT-3') and cloned before sequencing (Kämpfer, *et al.*, 2003). The sequenced full-length 16S rRNA genes reached 1475 bp nucleotides 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^T (NR_116477) (98.4%), *Rhizobium tibeticum* CCBAU 85039^T (NR_116254) (98.1%), *Rhizobium grahamii* CCGE 502^T (JF424608) (98.0%) and *Rhizobium mesoamericanum* CCGE 501^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 recombinase 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 *atpD* and *recA* phylogeny. The maximum-likelihood analyses for *atpD* gene sequences displayed *R. grahamii* CCGE 502^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^T (HQ735075.1), *Rhizobium etli* CCBAU 85039^T (EU288694.1), and *Rhizobium radiobacter* KL09-16-8-2^T (AB738671). Housekeeping genes analyses clearly showed that ChimEc512^T belongs to a novel species of the genus *Rhizobium*.

The DNA G+C content of ChimEc512^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^T, *R. tibeticum* CCBAU 85039^T, *R. grahamii* CCGE 502^T, *R. mesoamericanum* CCGE 501^T, *R. etli* CCBAU 85039^T, and *R. radiobacter* KL09-16-8-2^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^T had low DNA–DNA relatedness with the type strains *R. endophyticum* CCGE 2052^T (27%), *R. tibeticum* CCBAU 85039^T (22%), *R. grahamii* CCGE 502^T (18%), *R. mesoamericanum* CCGE 501^T (16%), *R. etli* CCBAU 85039^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

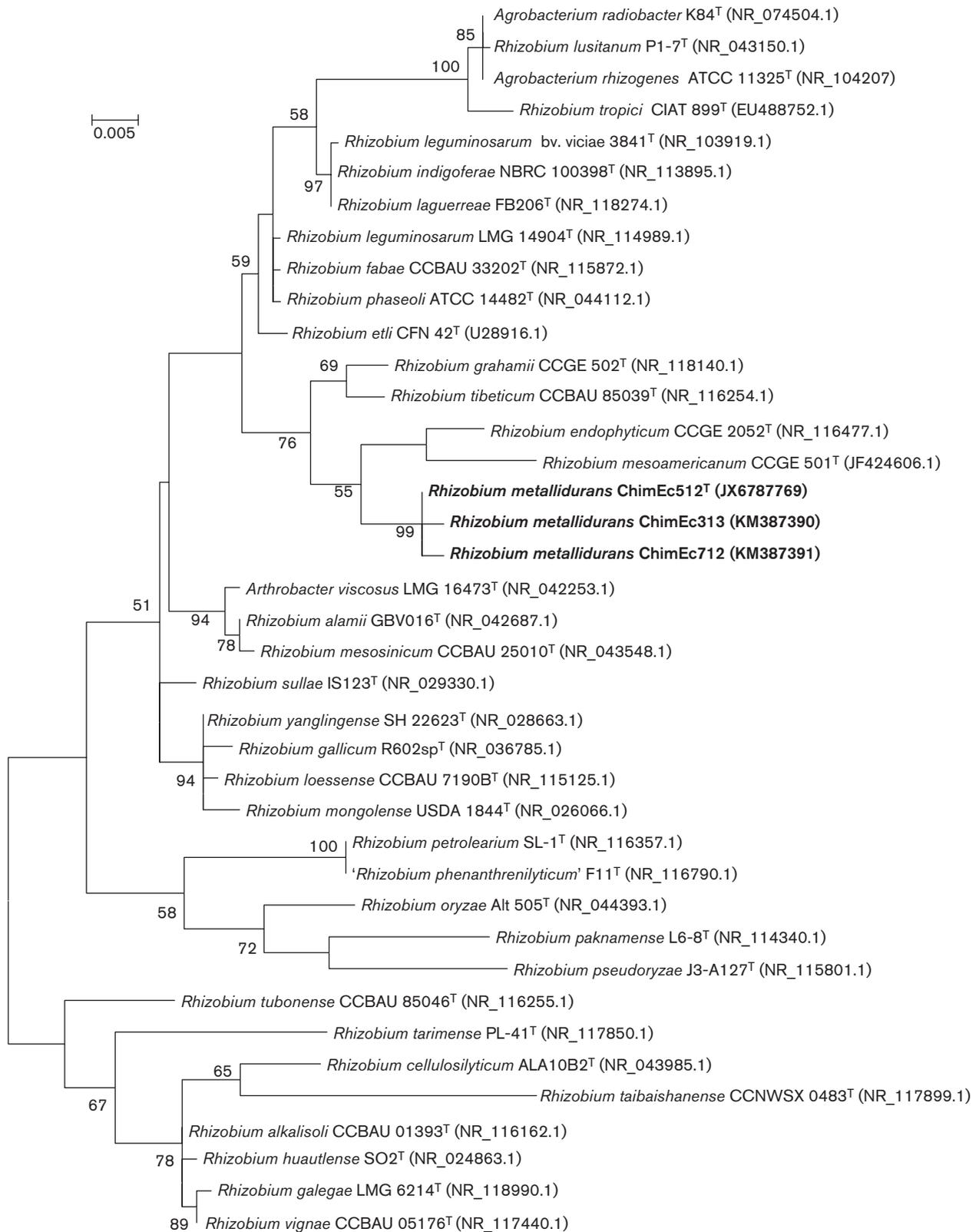


Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain ChimEc512^T and the most closely related reference strains. The analysis was based on 1330 nt. GenBank accession numbers are given in parentheses. The significance of each branch is 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.

Table 1. DNA–DNA hybridizations of ChimEc512^T with type strains of closely related species of the genus *Rhizobium*

Strains (accession number): 1. ChimEc512^T (JX678769); 2, *R. endophyticum* CCGE 2052^T (NR_116477); 3, *R. tibeticum* CCBAU 85039^T (NR_116254); 4, *R. grahamii* CCGE 502^T (JF424608); 5, *R. mesoamericanum* CCGE 52^T (JF424606); 6, *R. etli* CCBAU 85039^T (EU288694.1); 7, *R. radiobacter* KL09-16-8-2^T (AB738671). All data were obtained in the present study.

Strain of related species	DNA–DNA relatedness with DNA of strain (%)						
	1	2	3	4	5	6	
1	100						
2	27	100					
3	22	12	100				
4	16	17	23	100			
5	18	21	12	9	100		
6	19	24	26	21	18	100	
7	11	7	14	12	11	9	100

from *A. vulneraria* should be considered to represent a novel species.

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 fatty acid composition of strain ChimEc512^T were carried out by the Identification Service, Leibniz-Intsitut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. After three days of incubation at 28 °C on YEM plates, the cells were harvested and fatty acids were saponificated, methylated and extracted. The fatty acid methyl ester mixtures were separated using the Sherlock Microbial Identification 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 ChimEc512^T 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 (5.9%), 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 Service, Leibniz-Intsitut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The predominant polar lipids were phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol and phosphatidylcholine. Additionally, moderate amounts of aminolipids, phospholipid and sulfoquinovosyl diacylglycerol were detected (Fig. S3).

The phenotypic characteristics of the novel rhizobial lineage were compared with those of the closest related species, *R. endophyticum* CCGE 2052^T, *R. tibeticum* CCBAU 85039^T, *R. grahamii* CCGE 502^T, *R. mesoamericanum* CCGE 501^T, *R. etli* CCBAU 85039^T and *R. radiobacter* KL09-16-8-2^T. 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 mannitol broth (YMB) (Yadav & Vyas, 1973; Vidal *et al.*, 2009).

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 (10 mg ml⁻¹), gentamicin (10 mg ml⁻¹), kanamycin (50 mg ml⁻¹) and tetracycline (30 mg ml⁻¹). The novel strains also differed from species of the genus *Rhizobium* with validly published names with respect to the assimilation of carbon sources.

Since the new rhizobial species had been found on a Zn mining site, the impact of metals on growth kinetics was tested. Heavy metal resistant clones were first selected on YEM medium artificially enriched with solid Zn and Cd. The positive clones were transferred into YMB medium. A 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 ChimEc512^T aggregated in liquid medium due to the production of extracellular polysaccharides (Dorken *et al.*, 2012). Bacterial dry mass increased with time in up to 35 mM zinc (Fig. S4). ChimEc512^T growth was not affected by these concentrations of ZnSO₄. As shown in Table S2, specific growth rates during exponential growth were all similar and confirmed that zinc, within certain limits, had no impact on the growth of this strain. This was good evidence that this novel rhizobial strain tolerated high concentrations of zinc up to a maximum of 35 mM and that its growth kinetics were unaffected. However, Zn was not accumulated within cells, as the Zn mass introduced to the medium was not found in the bacterial mass.

From these data, the heavy metal resistant strain from *A. vulneraria* root nodules, ChimEc512^T, phenotypically and

Table 2. Differential phenotypic characteristics between the novel strains isolated from *Anthyllis vulneraria* and strains of closely related species of the genus *Rhizobium*

Strains (accession number): 1, ChimEc512^T (JX678769), ChimEc712 (KM387391) and ChimEc313 (KM387390); 2, *R. endophyticum* CCGE 2052^T (NR_116477); 3, *R. tibeticum* CCBAU 85039^T (NR_116254); 4, *R. grahamii* CCGE 502^T (JF424608); 5, *R. mesoamericanum* CCGE 52^T (JF424606); 6, *R. etli* 85039^T (EU288694.1); 7, *R. radiobacter* KL09-16-8-2^T (AB738671); +, growth; -, no growth; w, weak growth; ND, not determined. All data were obtained in the present study.

Characteristic	1	2	3	4	5	6	7
Growth in:							
Phenyl acetic acid	-	-	+	+	-	+	-
L-Arabinose	+	+	+	+	+	-	-
Citric acid	-	-	-	-	+	-	-
Fumaric acid	+	+	+	+	+	+	ND
D-Gluconate	+	+	+	+	+	-	+
Glutamic acid	-	-	-	+	-	ND	+
Malic acid	+	-	+	-	+	+	-
D-Mannose	+	+	+	+	+	ND	+
N-Acetylglucosamine	+	ND	-	-	ND	+	+
Growth in LB	+	+	-	-	-	-	+
Growth in YEM							
at 37 °C	w	-	-	w	+	+	w
at pH 4	w	+	-	w	w	-	-
with ZnSO ₄ (35 mM)	+	-	-	-	-	-	-
with CdCl ₂ (0.5 mM)	+	-	-	-	-	-	-
Resistance to antibiotics (mg ml ⁻¹)							
Kanamycin (50)	-	-	-	+	-	+	+
Tetracycline (30)	-	-	-	-	+	+	-

genotypically diverged from closely related strains of species of the genus *Rhizobium* and, therefore, was considered to represent a single novel species of the genus *Rhizobium*.

Description of *Rhizobium metallidurans* sp. nov.

Rhizobium metallidurans (me.ta.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 phosphatidylcholine. Additionally, moderate amounts of aminolipids, phospholipids and sulfoquinovosyl diacylglycerol are detected. The fatty acid profile is largely composed of C_{18:1}ω7c. 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, maltose, D-mannose, D-mannitol, pyruvate, D-ribose, sorbitol, N-acetylglucosamine, but not capric acid, adipic acid, meso-erythritol, galactitol, sorbose, soluble starch, DL-asparagine, glycine, L-methionine, phenylacetic acid, L-proline as sole carbon sources. Can use most amino acids but not L-arginine, L-aspartic acid, L-glutamic acid, or D-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, ChimEc512^T (DSM 26575=CIP 110550^T), was isolated from the root nodules of *Anthyllis vulneraria* growing on a soil contaminated with heavy metals at the mining site of Les Avinières (Saint-Laurent-Le-Minier, Gard County, France). The genomic DNA G + C content of the type strain is 58.9 mol%.

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