Rhizobium alamii sp. nov., an exopolysaccharide-producing species isolated from legume and non-legume rhizospheres

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A group of exopolysaccharide-producing bacteria was isolated from the root environment of *Arabidopsis thaliana*. The genetic diversity revealed by REP-PCR fingerprinting indicated that the isolates correspond to different strains. 16S rRNA gene sequence analysis showed that the isolates are closely related to the strains *Rhizobium* sp. YAS34 and USDA 1920, respectively isolated from sunflower roots and *Medicago ruthenica* nodules. These bacteria belong to the *Rhizobium* lineage of the *Alphaproteobacteria*, and the closest known species was *Rhizobium sulae*. DNA–DNA hybridization experiments and biochemical analysis demonstrated that the nine strains isolated from *A. thaliana* and *Rhizobium* strains YAS34 and USDA 1920 constitute a novel species within the genus *Rhizobium*, for which the name *Rhizobium alamii* sp. nov. is proposed. The type strain is GBV016^T (=CFBP 7146^T =LMG 24466^T).

Rhizobium species were originally known for their symbiotic association with members of the Fabaceae (Leguminosae), and their nomenclature was shaped by the specificity of their symbiotic plant range. Since the recognition that the Sym plasmid responsible for nodulation and specificity can be lost or transferred between strains related to *Rhizobium*, the nomenclature has been revised, based on phylogenetic and phenotypic data. All *Agrobacterium* and *Allorhizobium* species were included in the genus *Rhizobium* (Young *et al.*, 2001), and the genus consists of 24 species at present. These taxonomic revisions emphasize that the non-symbiotic lifestyle of rhizobia is also of ecological importance (Denison & Kiers, 2004). Free-living *Rhizobium* strains have frequently been isolated from soil (Soberon-Chavez & Najera, 1988; Laguerre *et al.*,

1993; Quigley *et al.*, 1997), rhizospheres and plant roots (Segovia *et al.*, 1991; Sullivan *et al.*, 1996; Yanni *et al.*, 1997; Rosenblueth & Martinez-Romero, 2004) and, recently, three *Rhizobium* species have been described on the basis of non-symbiotic strains isolated from bioreactors (Quan *et al.*, 2005; Hunter *et al.*, 2007) and sawdust of *Populus alba* (Garcia-Fraile *et al.*, 2007). *Rhizobium* species are likely to be involved in a broad range of functions in diverse ecosystems.

Rhizobium sp. YAS34 was selected from the indigenous sunflower taproot microflora for its ability to produce large amounts of gel-forming exopolysaccharides (EPS) (Alami *et al.*, 1998) that play an active role in plant development in a non-symbiotic context (Alami *et al.*, 2000), whereas EPS production by rhizobium populations is usually known to be crucial for establishment of successful symbiosis with legumes. Alami *et al.* (2000) classified strain YAS34 within the genus *Rhizobium* but it was unlike any of the previously described species, on the basis of its complete 16S rRNA gene sequence. Within the framework of a study of bacterial EPS in the rhizosphere of *Arabidopsis thaliana*, a group of nine isolates was assigned to an undescribed *Rhizobium* species closely related to YAS34. In the present

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Abbreviation: EPS, exopolysaccharide.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GBV016^T is AM931436.

A photograph of colonies of one of the novel strains showing EPS production and details of DNA–DNA hybridization results are available as supplementary material with the online version of this paper.

study, the strains were investigated in detail and the results suggest that these strains belong to a novel *Rhizobium* species.

EPS-producing strains were isolated from the indigenous bacterial populations of *A. thaliana* roots and rhizosphere soil by growing plants for 1 month in soil as described by Achouak *et al.* (2004). Pots were kept at 30 °C for seed germination and then shifted to a Phytotron under controlled conditions (8 h light, 16 h dark; 22/18 °C day/night; relative humidity 50 %). Soil was sampled in a eutric cambisol soil from Versailles, France (Derrien *et al.*, 2006). At harvest, following the methods of Amellal *et al.* (1998), macerated, washed roots and samples of soil adhering to roots were diluted and plated on RCV medium containing 20 g glucose l⁻¹ (RCV-Gluc). The composition of RCV medium (l⁻¹) was as follows: MgSO₄·7H₂O, 100 mg; CaCl₂·2H₂O, 100 mg; FeSO₄·7H₂O, 22 mg; EDTA, 20 mg; ZnSO₄·7H₂O, 0.43 mg; MnSO₄·H₂O, 1.30 mg; Na₂MoO₄·2H₂O, 0.75 mg; H₃BO₃, 2.80 mg; CuSO₄·5H₂O, 26 µg; CoSO₄·7H₂O, 70 µg; K₂HPO₄, 5.2 mM; KH₂PO₄, 4.4 mM; yeast extract, 0.1 mg; pH 6.8. After 4–10 days at 30 °C, mucous strains constituted 20–30 % of the cultivable bacteria from the root environment. This represents a fivefold greater proportion than in bulk soil sampled in control pots without plants, showing the effect of the rhizosphere on these mucous bacterial populations. An example of EPS production by these strains on RCV-Gluc medium is shown in Supplementary Fig. S1 (available in IJSEM Online). Eighty-four representative isolates from roots and root-adhering soil were purified on tenfold-diluted trypticase soy agar (0.1 × TSA).

Their role in the function of the *A. thaliana* rhizosphere and their EPS structures are under investigation.

Partial sequences of the 16S rRNA gene (380 bp) were obtained with primers Fd1 and S6 for most of the strains according to Berge *et al.* (2002) to classify them rapidly by using the BLASTN program (Altschul *et al.*, 1997) and sequences available in GenBank. The majority of these culturable mucous strains (>70 %) were affiliated to the genera *Rhizobium* or *Sinorhizobium* (*Ensifer*), showing the prevalence of this group in the rhizosphere of *A. thaliana*. Partial 16S rRNA gene sequences showed that 37 isolates had 99.5 % identity with *Rhizobium* sp. YAS34 and USDA 1920, the single isolate representing one of the three genomic species that form nitrogen-fixing symbioses with *Medicago ruthenica* (van Berkum *et al.*, 1998), suggesting that they belong to the same species. A representative subset of this collection, isolated from three independent experiments and five *A. thaliana* plants, was investigated in this study. Their origin and the reference organisms used in this work are listed in Table 1.

The diversity of strains was assessed by ERIC-PCR and REP-PCR analyses as described by Frey *et al.* (1997). Strains isolated from *A. thaliana* are characterized by different REP- and ERIC-PCR profiles (Table 1), demonstrating that these strains originating from the same soil are not clones. Together with strains YAS34 and USDA 1920, respectively isolated from sunflower and *M. ruthenica*, they represent a bacterial strain collection from different sites and hosts as recommended for the description of novel *Rhizobium* species (Lindström & Martinez-Romero, 2007).

Table 1. Origin and characteristic of strains used in this study

CNCM, Collection Nationale de Culture Microbienne (Institut Pasteur, France); USDA, US Department of Agriculture; P, Patent strains of CEA-CNRS-ARD. ND, Not determined.

Strain	Origin of isolation	PCR profile		Reference
		REP-PCR	ERIC-PCR	
<i>Rhizobium alarii</i> sp. nov.				
GBV016 ^T	Rhizosphere, <i>A. thaliana</i> plant 1	a	A1*	This study
GBV022	Rhizosphere, <i>A. thaliana</i> plant 2	b	A2	This study
GBV023	Rhizosphere, <i>A. thaliana</i> plant 2	c	B1	This study
GBV024	Roots, <i>A. thaliana</i> plant 3	d	C	This study
GBV037	Roots, <i>A. thaliana</i> plant 3	e	D	This study
GBV081	Roots, <i>A. thaliana</i> plant 3	f	B2	This study
GBV044	Roots, <i>A. thaliana</i> plant 4	h	E	This study
ALV118	Roots, <i>A. thaliana</i> plant 5	i	B3	This study
ALV104 (=CNCM I-359 ^P)	Roots, <i>A. thaliana</i> plant 5	j	F	Lodhi-Hassan <i>et al.</i> (2006)
YAS34 (=CNCM I-1809 ^P)	Sunflower roots	k	G	Alami <i>et al.</i> (1998)
USDA 1920	<i>Medicago ruthenica</i>	l	H	van Berkum <i>et al.</i> (1998)
<i>R. sullae</i> USDA 4950 ^T	<i>Hedysarum coronarium</i>	ND	ND	Squartini <i>et al.</i> (2002)
<i>R. mongolense</i> USDA 1844 ^T	<i>Medicago ruthenica</i>	n	J	van Berkum <i>et al.</i> (1998)
<i>R. gallicum</i> R602sp ^T	<i>Phaseolus vulgaris</i>	ND	ND	Amarger <i>et al.</i> (1997)

*Profiles with the same letter shared the majority of bands.

The nearly complete 16S rRNA gene sequence of strain GBV016^T was obtained by sequencing the PCR product obtained using primers Fd1 and S17, according to Berge *et al.* (2002). This new sequence was used for a BLAST search at NCBI and EBI and on a local database (not shown) containing 16S rRNA gene sequences of cultured species only to retrieve the most similar sequences. Among them, the sequence of *Arthrobacter viscosus* LMG 16473^T (GenBank accession no. AJ639832) was found to be closely related to that of GBV016^T. *Arthrobacter viscosus* is known to be an atypical *Arthrobacter* species (Keddie *et al.*, 1986; Heyrman *et al.*, 2005), and its position is uncertain. Moreover, only one 16S rRNA gene sequence of this species is available in GenBank. An eventual close relationship with *Rhizobium* must be investigated in detail, based on authentic strains, in order to allow a final taxonomic conclusion. A taxonomic transfer of this species would therefore be premature and it was removed from our phylogenetic study, as well as every sequence that was not described using a genus name (i.e. *Rhizobium* sp., etc.), providing a dataset of 97 sequences. All retrieved sequences were aligned and the alignment was checked visually and improved if necessary using SeaView (Galtier *et al.*, 1996). A phylogenetic analysis was done using the neighbour-joining (BIONJ) method, which allowed clear positioning of strain GBV016^T within the *Rhizobium* cluster.

From the tree, only 16S rRNA gene sequences for the most closely related type strains, along with those of strains YAS34 and USDA 1920, were retained, providing a dataset of 30 sequences. Alignments were checked again, and phylogenetic analyses were obtained using BIONJ (Kimura's two-parameter correction), DNAML (G option) and DNAPARS

from the PHYLIP package. Positions 45–939 of the sequence of strain GBV016^T were used for these analyses. A strict consensus tree was derived (Fig. 1). Finally, a bootstrap analysis (1000 replications) was obtained using BIONJ as described above.

Phylogenetic analyses of 16S rRNA gene sequences (Fig. 1) confirmed that strains GBV016^T, YAS34 and USDA 1920 form a novel, robust branch in the *Rhizobium* phylogenetic tree, identified by all methods and 93 % of bootstrap replications. *Rhizobium sullae* IS123^T is the closest type strain and, together, they form a separate clade. Apart from *R. sullae* IS123^T, the closest type strains were *Rhizobium indigoferae* CCBAU 71042^T, *R. yanglingense* SH 22623^T, *R. gallicum* R602sp^T and *R. mongolense* USDA 1844^T, and the latter strain was chosen together with *R. sullae* USDA 4950^T (=IS123^T) to be tested in DNA–DNA hybridizations in order to delineate the novel *Rhizobium* species. Actual 16S rRNA gene sequence similarities between GBV016^T and the type strains of these closely related species were 98.9 % (*R. sullae* IS123^T) and 98.5 % (*R. mongolense* USDA 1844^T).

DNA–DNA hybridization experiments were carried out in microtitre plates by using biotinylated probe DNA, as described by Ezaki *et al.* (1989) and improved by Willems *et al.* (2001). Hybridization was performed with denatured, sonicated, counterpart DNA labelled with photobiotin, for 3 h at 42 °C in 50 % formamide. Cross-hybridization was done systematically and means were calculated (Supplementary Table S1). One main DNA hybridization group was clearly delineated, including the nine strains isolated from *A. thaliana*, as well as strains YAS34 and USDA 1920 (Supplementary Table S1). DNA–DNA

Fig. 1. Combined final dendrogram, displaying BIONJ topology based on nearly complete 16S rRNA gene sequences of strains GBV016^T, YAS34 and USDA 1920 and closely related type strains (see text). Bootstrap percentages (1000 replications) are shown only for branches that were also retrieved using the maximum-likelihood and parsimony analyses. Bar, 1 substitution per 100 nucleotide positions.

hybridization values between these strains were higher than the recommended threshold value of 70 % (except for the value of 66 % relatedness obtained between strains GBV022 and GBV044), demonstrating that they belong to the same genomic species. DNA–DNA hybridization values between four strains of this group (including GBV016^T) and the type strains *R. sulae* USDA 4950^T and *R. mongolense* USDA 1844^T were respectively 14–22 % and 10–16 %, indicating a genetic distance that supports the conclusion that these strains constitute a novel species within the

genus *Rhizobium*. This result is in agreement with those of van Berkum *et al.* (1998) showing that strain USDA 1920 does not belong to the species *R. mongolense*.

Phenotypic characterization of the strains from this study was based on metabolism of carbon sources, resistance to antibiotics and tolerance to salt. The strains were grown on 0.1 × TSA for 24 h and characterized with Biolog GN2 and GP2 microplates. Antibiotic resistance and NaCl tolerance were assessed by growing the strains in microplates in

Table 2. Differential characteristics of *Rhizobium alarii* sp. nov. and type strains of related species

Strains: 1, *R. alarii* sp. nov. GBV016^T (numbers in parentheses indicate the number of the 11 strains that tested positive); 2, *R. sulae* USDA 4950^T; 3, *R. mongolense* USDA 1844^T; 4, *R. gallicum* R602sp^T. Unless indicated, data were obtained in this study. NA, No data available.

Characteristic	1	2	3	4
Utilization of carbon source in Biolog GN2 or GP2 microplate				
D-Alanine (GN2)	– (1)	+	+	+
L-Leucine (GN2)	– (2)	+	+	+
L-Phenylalanine (GN2)	– (0)	–	+	–
Maltose (GN2/GP2)	+	–	+	–
DL-Carnitine (GN2)	+	–	–	–
α-Ketobutyric acid (GN2)	– (0)	+	+	+
Glucuronamide (GN2)	– (1)	+	+	+
α-Ketovaleric acid (GN2/GP2)	– (0)	+	+	+
Succinamic acid (GN2/GP2)	– (1)	+	+	+
L-Ornithine (GN2)	– (0)	+	+	–
γ-Hydroxybutyric acid (GN2/GP2)	– (0)	+	+	–
α-Ketoglutaric acid (GN2/GP2)	– (0)	+	+	–
Putrescine (GP2)	– (1)	–	+	+
L-Pyrogutamic acid (GN2)	– (1)	–	+	+
Xylitol (GN2)	+	–	+	+
D-Galactonic acid lactone (GN2)	+	–	+	+
myo-Inositol (GP2)	– (0)	–	+	–
p-Hydroxyphenylacetic acid (GP2)	– (1)	–	+	–
N-Acetyl-L-glutamic acid (GP2)	– (0)	–	+	–
2,3-Butanediol (GP2)	– (1)	–	+	–
2,3-Butanediol (GN2)	– (1)	–	–	+
Glucose 1-phosphate (GP2)	– (0)	–	+	–
Glucose 6-phosphate (GP2)	– (0)	–	+	–
Formic acid (GN2)	+	+	+	–
D-Ribose (GP2)	+	+	+	–
Methyl pyruvate (GN2)	– (1)	–	–	+
Growth in:				
0.1 % NaCl	– (0)	+	–	NA
0.5 % NaCl	– (0)	+	–	+
1 % NaCl	– (0)	+	–	–
2 % NaCl	– (0)	–	–	–
Antibiotic resistance				
Streptomycin (3 µg ml ^{–1})	– (0)†	NA	+	NA
Kanamycin (10 µg ml ^{–1})	– (0)†	+	NA	+
Nalidixic acid (40 µg ml ^{–1})	+	+	NA	+

*Data taken from: a, Amarger *et al.* (1997); b, Struffi *et al.* (1998).

†Only four strains were tested, including the type strain.

tenfold-diluted tryptic soy broth ($0.1 \times$ TSB) containing the following: streptomycin ($3 \mu\text{g ml}^{-1}$), kanamycin ($10 \mu\text{g ml}^{-1}$), nalidixic acid ($40 \mu\text{g ml}^{-1}$) or NaCl (0.1, 0.5, 1 and 2 %).

Slight differences in Biolog metabolic fingerprints were observed between the 11 strains belonging to the novel species, but they showed several differences compared with their closest relatives *R. sullae* USDA 4950^T, *R. mongolense* USDA 1844^T and *R. gallicum* R602sp^T (Table 2). They all differ clearly from *R. sullae* USDA 4950^T in metabolism of α -ketobutyric acid, α -ketovaleric acid, L-ornithine, γ -hydroxybutyric acid and α -ketoglutaric acid, growth in 0.1, 0.5 and 1 % NaCl and kanamycin resistance. They differ from *R. mongolense* USDA 1844^T in metabolism of L-phenylalanine, α -ketobutyric acid, α -ketovaleric acid, L-ornithine, γ -hydroxybutyric acid, α -ketoglutaric acid, myo-inositol, N-acetyl L-glutamic acid, glucose 1-phosphate and glucose 6-phosphate and streptomycin resistance. They differ from *R. gallicum* R 602sp^T in metabolism of α -ketobutyric acid, α -ketovaleric acid and D-ribose and growth in 0.5 % NaCl (Table 2).

Nodulation was tested by van Berkum *et al.* (1998) only in a symbiotic context, by inoculating strain USDA 1920 to *M. ruthenica* and *Medicago sativa* (alfalfa). These authors showed that strain USDA1920 nodulates the two *Medicago* species; however, it was only capable of nitrogen fixation with *M. ruthenica*, since alfalfa plants were small and chlorotic.

In conclusion, our data based on a polyphasic taxonomic study demonstrate that the EPS-producing *Rhizobium* isolates analysed in the present study represent a novel species that can be differentiated genotypically and phenotypically from their nearest phylogenetic neighbours. We therefore propose the name *Rhizobium alamii* sp. nov. to accommodate these isolates. The examined isolates produced large amounts of EPS and, except USDA 1920, they were associated with non-legumes.

Description of *Rhizobium alamii* sp. nov.

Rhizobium alamii (a.la'mi.i. N.L. gen. masc. n. *alamii* of Alami, named in honour of Younes Alami, who isolated strain YAS34 and demonstrated its effect on non-legume plant growth).

Gram-negative rods, $0.7 \times 1.0 \mu\text{m}$, motile, aerobic, catalase-positive and oxidase-negative. Colonies are white, circular or irregular, raised and mucous, 3–4 mm in diameter within 3 days on $0.1 \times$ TSA at 30°C . Cells grow in $0.1 \times$ TSB or tenfold-diluted Luria–Bertani (LB) broth, but not in undiluted TSB or LB broth. No growth is observed at NaCl concentrations of 0.1 % or above. Grows on glucose, sucrose or mannitol, leading to the production of large amounts of EPS slime. No growth with fructose or succinate as carbon and energy sources. Carbon sources used are reported in Table 2. All known strains use L-fucose, lactulose, D-xylose and D-malic acid but not

itaconic acid, L-ornithine, L-phenylalanine, D-serine, γ -aminobutyric acid, thymidine, phenylethylamine, methyl α -D-glucoside, lactamide, L-alaninamide, 2'-deoxyadenosine or DL- α -glycerol phosphate. Strains are resistant to nalidixic acid ($40 \mu\text{g ml}^{-1}$) but susceptible to streptomycin ($3 \mu\text{g ml}^{-1}$) and kanamycin ($10 \mu\text{g ml}^{-1}$). The strains can be distinguished by their 16S rRNA gene sequence and by DNA hybridization.

The type strain, GBV016^T (=CFBP 7146^T =LMG 24466^T), was isolated from rhizosphere soil of *Arabidopsis thaliana*. Strains YAS34, USDA 1920, GBV022, GBV023, GBV024, GBV037, GBV044, GBV081, ALV118 and ALV104, also assigned to the species, were isolated from the rhizosphere and roots of *A. thaliana* plantlets, from sunflower roots or from *Medicago ruthenica* nodules.

Acknowledgements

We thank G. Laguerre (INRA, Dijon, France) for providing *R. gallicum* R602sp^T, P. van Berkum and P. Elia (USDA *Rhizobium* Germplasm Resource Center, Beltsville, MD, USA) for providing *Rhizobium* sp. USDA 1920, *R. mongolense* USDA 1844^T and *R. sullae* USDA 4950^T, J. Euzéby for his help in correct naming of the species and P. De Vos for his expertise on the taxonomic position of *A. viscosus*. We are grateful to J. Maurel, J. Thomas, S. Fochesato and A. Hertz for their helpful assistance. Plants were grown in our institute with the help of the platform GRAP, IBEB/SBVME, Cadarache, France.

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