Rhizobium skierniewicense sp. nov., isolated from tumours on chrysanthemum and cherry plum

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Three isolates of Gram-negative, rod-shaped, non-spore-forming bacteria were recovered from galls on chrysanthemum (*Chrysanthemum* L.; Ch11^T, Ch12) and cherry plum (*Prunus cerasifera* var. *divaricata*; AL9.3). All three isolates were able to cause crown galls on various plant species. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the three isolates were probably identical (100% sequence similarity) and closely related to *Rhizobium rubi* (99.6%), *Rhizobium radiobacter* (98.7%) and *Rhizobium larrymoorei* (98.1%). Similar analysis based on the housekeeping genes *glnA*, *gyrB* and *rpoB* also indicated that the novel isolates were identical and closely related to *R. rubi*. The major cellular fatty acids of strain Ch11^T were C_{18:1ω7c} (62.1%), summed feature 2 (comprising C_{12:0} aldehyde, iso-C_{16:1} I and/or C_{14:0} 3-OH; 10.8%), summed feature 3 (comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; 7.7%) and C_{10:0} 3-OH (7.5%). However, the DNA–DNA relatedness between Ch11^T and *R. rubi* LMG 156^T was only 48% and, unlike phylogenetically related established *Rhizobium* species, the novel isolates were able to utilize β-hydroxybutyric acid but not L-fucose. Based on the phylogenetic and phenotypic evidence, the isolates are considered to represent a single novel species of the genus *Rhizobium*, for which the name *Rhizobium skierniewicense* sp. nov. is proposed; the type strain is Ch11^T (=LMG 26191^T=CFBP 7420^T).

For many years, the genus *Agrobacterium* contained species of bacteria that caused tumours or hairy roots in over 800 plant species. A few years ago, however, the genus *Agrobacterium* was merged with the genus *Rhizobium*. At the time of writing, 41 *Rhizobium* species have been described (<http://www.bacterio.cict.fr>), including five plant-pathogenic species: *Rhizobium radiobacter*, *Rhizobium rhizogenes*, *Rhizobium rubi*, *Rhizobium vitis* and *Rhizobium larrymoorei* (Young *et al.*, 2001; Young, 2004).

In this study, a polyphasic approach was used for the taxonomic characterization of three bacterial strains: two

(Ch11^T and Ch12) isolated from tumours on chrysanthemum plants (*Chrysanthemum* L.) and one (AL9.3) isolated from a gall on cherry plum (*Prunus cerasifera* var. *divaricata*). The media used to recover strains Ch11^T, Ch12 and AL9.3, with incubation at 27 °C for 3 days, were Patel medium (Patel, 1926), King B medium (King *et al.*, 1954) and tellurite-amended mannitol-glutamate medium (Mougel *et al.*, 2001), respectively. All three strains were pathogenic and caused crown galls on various plants, including sunflower, chrysanthemum, *Cerasus* species and *Rosa* species (unpublished results).

Almost the entire 16S rRNA gene sequence of each of the three novel isolates was amplified by PCR and sequenced using primers fd1 and rP2 (Weisburg *et al.*, 1991). The sequences were then aligned using the CLUSTAL W algorithm (Thompson *et al.*, 1994), with overhangs trimmed to give a final length of 1285 bp. The genetic distances between the sequences were estimated by using the Kimura two-parameter correction method (Kimura, 1980), before neighbour-joining and maximum-parsimony trees were generated in MEGA4.0 (Tamura *et al.*, 2007). The significance of each internal branch of the phylogenetic trees was estimated with 1000 bootstrap replicates. (Fig. 1; Supplementary Fig. S1, available in IJSEM Online, shows extended trees containing a larger number of reference sequences). In terms of their 16S rRNA gene

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene sequences of strains Ch11^T, Ch12 and AL9.3 are HQ823551–HQ823553, respectively. Accession numbers for the partial *glnA* gene sequences of strains Ch11^T, Ch12, AL9.3, *R. vitis* LMG 8750^T, *R. undicola* LMG 11875^T and *R. larrymoorei* LMG 21410^T are FR754362, FR754364, FR754366, HQ823558, FR754361 and FR754359, respectively. Accession numbers for the partial *gyrB* gene sequences of strains Ch11^T, Ch12, AL9.3 and *R. borbori* LMG 23925^T are HQ438226, HQ823554, HQ823555 and HQ823556, respectively. Accession numbers for the partial *rpoB* gene sequences of strains Ch11^T, Ch12, AL9.3, *R. borbori* LMG 23925^T and *R. larrymoorei* LMG 21410^T are FR754363, FR754365, FR754367, HQ823557 and FR754360, respectively.

A supplementary table and four supplementary figures are available with the online version of this paper.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the relationships between strains Ch11^T, Ch12 and AL9.3 and members of related species. Bootstrap values (expressed as percentages of 1000 replications) are given at the nodes. Bar, 0.01 substitutions per nucleotide position.

sequences, strains Ch11^T, Ch12 and AL9.3 were identical (100% sequence similarity) and appeared most closely related to *R. rubi* LMG 156^T (99.6%) and *R. radiobacter* LMG 140^T (98.7%).

The *glnA* gene sequences of the novel isolates and some reference strains were amplified with the primers described by Martens *et al.* (2007). The *glnA* gene sequence of *R. vitis* LMG 8750^T was, however, amplified with the primers *glnA.vitisF* (5'-GTCATGTTTCGACGGYTCYTCT-3') and *glnA.vitisR* (5'-TGGAKCTTGTTCTTGATGCCA-3'). The *gyrB* gene sequences of strains Ch11^T, Ch12 and AL9.3 were amplified by using the universal primers UP-1 and UP-2r (Yamamoto & Harayama, 1995). Since the *parE* gene coding for topoisomerase IV was also probably amplified in the PCR based on these primers (Yamamoto & Harayama, 1995), the amplicons were cloned into pGEM-T vectors, to separate the *parE* inserts from the *gyrB* inserts, before sequencing of the *gyrB* gene. The *gyrB* sequences of the reference strains and the *rpoB* gene sequences of the novel isolates and reference strains were determined as described previously (Martens *et al.*, 2008). Phylogenetic analyses were performed on the individual gene sequences (896, 699 and 930 bp for *glnA*, *gyrB* and *rpoB*, respectively) as well as on the concatenated dataset of 2525 bp, with neighbour-joining and maximum-parsimony trees generated as for the 16S rRNA gene sequences. The novel isolates Ch11^T, Ch12 and AL9.3 possessed identical *glnA*, *gyrB* and *rpoB* gene sequences and

formed a phylogenetic lineage that was separate from established species (Fig. 2; Supplementary Figs S2, S3 and S4, available in IJSEM Online). In terms of their *glnA*, *gyrB* and *rpoB* gene sequences, the novel isolates appeared most closely related to *R. rubi*, with sequence similarities of 91.2, 91.4 and 89.0%, respectively. These values are lower than the between-strain levels of *gyrB* gene sequence similarity seen in *R. rubi*, which were all found to exceed 94.9% (J. Puławska & M. Kałużna, unpublished results).

For fatty acid analysis, strains were grown for 24 h on trypticase soy agar (LMG medium 185) at 28 °C. Harvesting of cells, extraction and analysis were performed by following the recommendations of the manufacturer of the MIDI identification system (Microbial Identification System) and peaks were identified using version 5.0 of the Sherlock MIDI Library. The fatty acid profiles of the novel isolates were very similar to that of *R. rubi*, with C_{18:1}ω7c as predominant fatty acid; other fatty acids present in large amounts in the cells of the novel isolates and *R. rubi* were C_{16:0}, C_{16:0} 3-OH, summed feature 2 (comprising C_{12:0} aldehyde, iso-C_{16:1} I and/or C_{14:0} 3-OH) and summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), whereas 11-methyl-C_{18:1}ω7c and 10-methyl-C_{19:0} are minor components (Supplementary Table S1, available in IJSEM Online). The presence of C_{10:0} 3-OH and 11-methyl-C_{18:1}ω7c in the novel isolates distinguishes them from their close phylogenetic relatives *R. larrymoorei* and *R. radiobacter*.

Fig. 2. Neighbour-joining tree based on the concatenated sequences of genes *glnA*, *gyrB* and *rpoB* showing the relationships between strains Ch11^T, Ch12 and AL9.3 and members of related species. Bootstrap values (expressed as percentages of 1000 replications) are given at the nodes. Bar, 0.02 substitutions per nucleotide position.

DNA–DNA hybridizations were performed at 42 °C with photobiotin-labelled probes in microplate wells, as described by Goris *et al.* (1998), using an HTS7000 bio-assay reader (PE Applied Biosystems) for the fluorescent measurements. The level of DNA–DNA relatedness between Ch11^T and *R. rubi* LMG 156^T was 48 %. The genomic DNA G+C content of strain Ch11^T, which was determined by HPLC as described previously (Mesbah *et al.*, 1989), was 57.2 mol%.

Physiological and biochemical tests were performed using API 50CH strips (bioMérieux) and GN2 MicroPlates (Biolog) according to the manufacturers' instructions. Production of 3-ketolactose, citrate utilization, growth and pigmentation in ferric ammonium citrate broth, acid production from erythritol and (+)-D-melezitose, action on litmus milk, ability to grow in the presence of 2 % (w/v) NaCl, and oxidase reaction were also investigated (Moore *et al.*, 2001). The GN2 plates were read 24 h after inoculation. Other tests were observed for 6 days, although most reactions occurred within 3 days. The three novel isolates could be differentiated from *R. larrymoorei* LMG 21410^T, *R. vitis* LMG 8750^T, *R. radiobacter* LMG 140^T, *R. rhizogenes* ATCC 11325^T and *R. rubi* LMG 156^T by their inability to utilize L-fucose and ability to utilize β -hydroxybutyric acid.

The main discriminatory phenotypic characteristics are summarized in Table 1.

Based on the phylogenetic and phenotypic evidence, strains Ch11^T, Ch12 and AL9.3 belong to the same novel species of *Rhizobium*, for which the name *Rhizobium skierniewicense* sp. nov. is proposed.

Description of *Rhizobium skierniewicense* sp. nov.

Rhizobium skierniewicense (ski.er.nie.wi.cen'se. N.L. neut. adj. *skierniewicense* of or belonging to Skierniewice, the town in Poland where the type strain was isolated).

Cells are Gram-negative, oxidase-positive, strictly aerobic, non-spore-forming rods (0.5 × 2.5–3.0 μ m). Good growth occurs on King B medium, nutrient agar and mannitol-glutamate medium with and without tellurite (at 70 mg l⁻¹; Mougél *et al.*, 2001). After 48–72 h of growth at 26 °C, colonies on King B medium are small, beige and round, with a diameter of approximately 1 mm. Gives alkaline reaction, with proteolysis, in litmus milk. Does not produce 3-ketolactose from lactose or utilize citrate. Grows in the presence of 2 % NaCl and, with pigmentation, in ferric ammonium citrate broth. Able to utilize adonitol,

Table 1. Phenotypic characters that differentiate *Rhizobium skierniewicense* sp. nov. from other closely related members of the genus *Rhizobium*

Taxa: 1, *R. skierniewicense* sp. nov. (strains Ch11^T, Ch12 and AL9.3); 2, *R. rubi* (strains LMG 156^T, LMG 159 and LMG 294); 3, *R. larrymoorei* LMG 21410^T; 4, *R. vitis* LMG 8750^T; 5, *R. radiobacter* LMG 140^T. Data from this study.

Characteristic	1	2	3	4	5
Utilization of:					
L-Arabinose	+	–	+	+	+
Fructose	+	–	+	+	+
Sorbose	–	–	–	–	+
Sucrose	+	–	+	+	+
Citrate	–	–	–	+	–
β -Hydroxybutyric acid	+	–	–	–	–
L-Histidine	+	–	+	+	+
L-Pyrogutamic acid	–	+	–	+	+
L-Fucose	–	+	+	+	+
3-Ketolactose production	–	–	–	–	+
Growth and pigmentation on ferric ammonium citrate	+	–	–	–	+
Acid from (+)-D-melezitose	–	+	–	–	+

L-arabinose, D-arabitol, aesculin, D-fucose, D-fructose, D-galactose, gentiobiose, α -D-glucose, *myo*-inositol, α -D-lactose, lactulose, D-mannitol, D-mannose, D-melibiose, methyl β -D-glucoside, L-rhamnose, sucrose, trehalose, turanose, D-xylose, acetic acid, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, β -hydroxybutyric acid, malonic acid, quinic acid, succinic acid, bromosuccinic acid, L-alanyl glycine, L-asparagine, L-glutamic acid, L-ornithine, L-proline, inosine, uridine, and glycerol. Unable to utilize glycerol, erythritol, D-arabinose, ribose, methyl β -D-xyloside, sorbose, dulcitol, methyl α -D-mannoside, methyl α -D-glucoside, *N*-acetyl-D-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-tagatose, L-fucose, L-arabitol, gluconate, 2-ketogluconate, 5-ketogluconate, α -cyclodextrin, glycogen, Tween 40, i-erythritol, *cis*-aconitic acid, citric acid, formic acid, D-galacturonic acid, D-glucuronic acid, α -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, propionic acid, D-saccharic acid, sebacic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-pyrogutamic acid, D-serine, L-serine, DL-carnitine, γ -aminobutyric acid, urocanic acid, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, DL- α -glycerol phosphate or D-glucose 6-phosphate. The predominant fatty acids are C_{18:1} ω 7c, summed feature 2 (comprising C_{12:0} aldehyde, iso-C_{16:1} I and/or C_{14:0} 3-OH) and summed feature 3 (comprising C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH).

The type strain, Ch11^T (=LMG 26191^T=CFBP 7420^T), was isolated from gall on chrysanthemum in Skierniewice,

Poland. The DNA G+C content of the type strain is 57.2 mol%. The two reference strains, Ch12 and AL9.3 were isolated from galls on chrysanthemum and cherry plum, respectively.

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