**Cohnella lupini** sp. nov., an endophytic bacterium isolated from root nodules of *Lupinus albus*

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A bacterial strain designated RLAHU4BT was isolated from root nodules of *Lupinus albus* in León (Spain). The 16S rRNA gene sequence of this strain showed similarities lower than 97 % with respect to the species of the genus *Cohnella*. The strain was a Gram-variable, sporulating rod, motile by means of peritrichous flagella, and facultatively anaerobic. It was positive for oxidase, catalase and β-galactosidase production but negative for urease, amylase and gelatinase. Strain RLAHU4BT grew in the presence of 5 % NaCl. MK-7 was the predominant menaquinone and meso-diaminopimelic acid was present in the peptidoglycan. anteiso-C15 : 0, iso-C16 : 0, iso-C15 : 0 and C16 : 0 were the major fatty acids. Major polar lipids of strain RLAHU4BT were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, three unknown phospholipids, two unknown aminophospholipids and one unknown lipid. The DNA G+C content was 57.8 mol%. Strain RLAHU4BT presented phenotypic differences from all recognized species of the genus *Cohnella*. The phylogenetic, chemotaxonomic and phenotypic data indicated that strain RLAHU4BT belongs to a novel species of the genus *Cohnella*, for which the name *Cohnella lupini* sp. nov. is proposed, with strain RLAHU4BT (=LMG 27416T=CTC 8236T) as the type strain.

The genus *Lupinus* is one of the most diverse and widespread legume genera in the world, with *Lupinus albus* (white lupin) being one of the three most widely cultivated species (Wolko *et al.*, 2011). It has great potential in biocropping with cereals (Azo *et al.*, 2012) and, in Spain, debittered varieties are consumed by man. This legume forms nitrogen-fixing nodules with slowly growing rhizobia of the genus *Bradyrhizobium* in Spain (Velázquez *et al.*, 2010). Nevertheless, rhizobia are not the only inhabitants of legume nodules and, in some species of *Lupinus*, several endophytes from the genera *Micromonospora*, *Microvirga* and *Bosea* have been found (Ardley *et al.*, 2012; De Meyer & Willems, 2012; Trujillo *et al.*, 2007, 2010). Nevertheless, to our knowledge, there are no reports on the endophytic bacteria present in nodules of the species *L. albus*.

The strain identified in this study as a member of genus *Cohnella*, RLAHU4BT, was isolated together with strains of the genus *Bradyrhizobium* (Velázquez *et al.*, 2010) from nodules of *L. albus* in Spain. The genus *Cohnella*, described by Kämpfer *et al.* (2006), comprises Gram-positive, endospore-forming, aerobic, non-motile, rod-shaped bacteria. The main menaquinone is MK-7 and diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine are the predominant polar lipids (García-Fraile *et al.*, 2008). The major fatty acids are iso-C16 : 0, anteiso-C15 : 0 and C16 : 0 (Kämpfer *et al.*, 2006). Most species of the genus *Cohnella* have been isolated from soil, but there are also some endoge endophytes in this genus such as *Cohnella phaseoli*, isolated from *Phaseolus coccineus* (García-Fraile *et al.*, 2008). Strain RLAHU4BT was isolated from a nodule of *L. albus* growing in Riego de la Vega (León, Spain) during a study of rhizobia and nodular endophytes present in different legumes. To sterilize the root nodules, they were washed several times with sterile distilled water and then surface-sterilized in 2.5 % (w/v) HgCl2 for 2 min. The nodules
were rinsed five times with sterile distilled water and then crushed using a sterile pestle. Homogenized nodule tissue was inoculated on modified yeast extract mannitol agar (YMA; Vincent, 1970) (1 l: 10 g mannitol, 1 g yeast extract, 0.2 g K$_2$HPO$_4$, 0.2 g MgSO$_4$$\cdot$7H$_2$O, 0.5 g NaCl, 20 g agar) and the plates were incubated at 28 °C for 4 days. In parallel, some disinfected entire nodules were incubated on the same medium in order to confirm their complete external disinfection; no growth was observed around these nodules. The cultures used in further phenotypic and molecular studies were purified from a single colony after 2 days of incubation at 28 °C on YMA. The colonies were white, mucoid, translucent and convex on this medium. Strain RLAHU4B$^T$ was unable to produce nodules on $L$. albus. This strain, as well as those used as references in this study, Cohnella luojiensis DSM 24270$^T$, C. arctica CCTCC AB 2010228$^T$, C. soli DSM 25951$^T$, C. suwonensis DSM 25950$^T$ and C. thermotolerans DSM 17683$^T$, were maintained on TSA plates (Becton Dickinson, BBL).

Strain RLAHU4B$^T$ was grown on nutrient agar for 48 h at 22 °C to check for motility by phase-contrast microscopy with the hanging drop method. Gram staining was carried out by using the procedure described by Doetsch (1981). The flagellation type was determined by electron microscopy after 48 h of incubation on nutrient agar at 22 °C. Cells were gently suspended in sterile water and then stained with 0.2 % uranyl acetate and examined at 80 kV with a Zeiss EM 209 transmission electron microscope. Strain RLAHU4B$^T$ was Gram-variable, rod-shaped, sporulating and motile by means of peritrichous flagella (Fig. S1a, available in IJSEM Online). Oval endospores were formed in swollen sporangia, and they were positioned centrally or subterminally in the cells (Fig. S1b).

Amplification and sequencing of the 16S rRNA gene were performed according to Rivas et al. (2007). The sequence obtained was compared with those from GenBank using BLASTN (Altschul et al., 1990) and the EzTaxon-e server (Kim et al., 2012). Sequences were aligned using the CLUSTAL_X software (Thompson et al., 1997) and distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). The phylogenetic tree was inferred using the neighbour-joining and maximum-likelihood (ML) models (Saitou & Nei, 1987; Rogers & Swofford, 1998), which yielded similar results; only the results of ML analysis are shown. The MEGA5 package (Tamura et al., 2011) was used for all analyses.

Comparison of the 16S rRNA gene sequence of strain RLAHU4B$^T$ (1492 nt) against the sequences of type strains held in the EzTaxon-e database indicated its affiliation with the genus Cohnella. Strain RLAHU4B$^T$ was equidistant from several Cohnella species, with similarities lower than 97 % according to the results from the EzTaxon-e server. The ML phylogenetic tree (Fig. 1) including species of the genus Cohnella with validly published names showed that strain RLAHU4B$^T$ formed a cluster with C. arctica M9-62$^T$ (96.8 % similarity), C. luojiensis HY-22RT (96.7 %), C.

![Fig. 1. Maximum-likelihood phylogenetic tree based on the nearly complete 16S rRNA gene sequence (1492 nt) of Cohnella lupini sp. nov. RLAHU4B$^T$ and related members of the genus Cohnella. The significance of each branch is indicated by a bootstrap percentage calculated for 1000 subsets. Bar, 2 substitutions per 100 nucleotide positions. Nodes marked with filled circles were also obtained with the neighbour-joining algorithm.](image-url)
suwonensis WD2-19T (96.7 %) and C. soli YM2-7T (95.7 %).

DNA for the determination of G+C content was prepared according to Chun & Goodfellow (1995) and the value was obtained by using the thermal denaturation method (Mandel & Marmur, 1968). The DNA G+C content of strain RLAHU4B T was 57.8 mol%; this value is similar to those reported for species closely related to strain RLAHU4B T such as C. arctica, C. suwonensis and C. soli (Kim et al., 2011; Jiang et al., 2012), and higher than that of C. luojensis (Cai et al., 2010).

Chemotaxonomic analyses were carried out by the Identification Service of the DSMZ (Braunschweig, Germany). Strain RLAHU4B T was cultivated on TSA (Becton Dickinson, BBL) for 48 h at 28 °C. Respiratory quinones and polar lipids were analysed as described by Tindall (1990) and cellular fatty acids were analysed according to the instructions of the Microbial Identification System (MIDI, Microbial ID; TSBA40 4.10 library). For analysis of the peptidoglycan, whole cells of strain RLAHU4B T were hydrolysed with HCl at 100 °C for 15 h. The hydrolysate was subjected to TLC on cellulose plates using the solvent system of Rhuland et al. (1955).

MK-7 was the predominant quinine (90 %) found in strain RLAHU4B T; MK-6 was also detected (10 %). This quinone profile is characteristic of species of the genus Cohnella (Kämpfer et al., 2006). meso-Diaminopimelic acid was detected in the peptidoglycan, which corresponds to type A17 (Schumann, 2011). The major fatty acids detected in strain RLAHU4B T were anteiso-C15:0 (42.4 %), C16:0 (14.4 %) and iso-C15:0 (14.3 %), followed by iso-C15:0 (7.6 %); this profile is consistent with those described for the genus Cohnella and its type species, C. thermotolerans (Kämpfer et al., 2006). The fatty acid profile of strain RLAHU4B T differed slightly in the proportions of some fatty acids with respect to the type strains of phylogenetically related Cohnella species (see Table S1). The polar lipid profile of strain RLAHU4B T (Fig. S2) consisted predominantly of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine, as described for the genus Cohnella (Garcia-Fraile et al., 2008). Three unknown phospholipids, two unknown aminophospholipids and one unknown lipid were also detected (Fig. S2). In strain RLAHU4B T, lysylphosphatidylglycerol was not detected, in contrast with closely related species of the genus Cohnella and the type species C. thermotolerans, in which this polar lipid was detected in moderate amounts (Kämpfer et al., 2006; Cai et al., 2010; Kim et al., 2011; Khianngam et al., 2012).

Phenotypic characterization included the characteristics recommended in the minimal standards for aerobic endospore-forming bacteria (Logan et al., 2009) and was performed according to the standard methods described by Claus & Berkeley (1986) and by using the API 50CH (with CHB/E medium), API 20NE, API 20E and API ZYM systems (bioMérieux) according to the manufacturer’s instructions. Tween 80 hydrolysis was analysed on Sierra basal medium (Sierra, 1957) containing 1 g Tween 80 l−1, which was autoclaved separately. Acid production from D-glucose, D-xylene, mannitol and L-arabinose, gas production from D-glucose, acetoin production, the ability to grow in the presence of 2, 5 and 7 % NaCl, nitrate reduction, anaerobic growth and phenylalanine deaminase and amylase activities were analysed as described by Claus & Berkeley (1986). Catalase activity was determined by observing bubble formation in a culture after the addition of 3 % hydrogen peroxide. Oxidase activity was determined by oxidation of 1 % tetramethyl p-phenylenediamine. Growth at 4–50 °C was determined in YED medium (0.5 % yeast extract, 0.7 % glucose and 2 % agar). Growth at pH 5.7 and 6.8 was tested as described by Claus & Berkeley (1986), growth at pH 7–8 was tested in YED medium containing 100 mM Na2HPO4/NaH2PO4 and growth at pH 9 and 10 was tested in the same medium containing 100 mM NaHCO3/Na2CO3. The characteristics determined are given in the species description. Strain RLAHU4B T differed from its closest relatives in the genus Cohnella in acid production from several carbohydrates (Table 1).

In summary, the phylogenetic, phenotypic and chemotaxonomic results obtained showed that strain RLAHU4B T represents a novel species of the genus Cohnella distinguishable from the phylogenetically most closely related species in the 16S rRNA gene sequence as well as in chemotaxonomic features and phenotypic characteristics.

Table 1. Differential phenotypic characteristics of strain RLAHU4B T and type strains of phylogenetically related species of the genus Cohnella

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>4</th>
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<tr>
<td>Colony colour*</td>
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<td>WH</td>
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<td>WH</td>
<td>WH</td>
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<td>Growth at 55 °C</td>
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<td>Growth in the presence of 2 %</td>
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<td>NaCl</td>
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<td>Acid from (API 50CH):</td>
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<tr>
<td>d-Glucose</td>
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<td>1-L-Arabinose</td>
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<td>+</td>
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<tr>
<td>d-Mannose</td>
<td>+</td>
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<td>Mannitol</td>
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<tr>
<td>N-Acetylgalcosamine</td>
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<tr>
<td>d-Ribose</td>
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<td>1-Rhamnose</td>
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*O, Orange; WH, white; WHC, white–cream.
mainly in acid production from several carbon sources. The name Cohnella lupini sp. nov. is proposed for the novel species.

**Description of Cohnella lupini sp. nov.**

*Cohnella lupini* (lu.pi’ni. L. n. lupinus a lupin and also a botanical genus name; L. gen. n. lupin of Lupinus, referring to the isolation source of the strain, nodules of *Lupinus albus*).

Endospore-forming rods, 0.8–1.0 μm wide and 2.5–3.5 μm long. Gram-variable. Motile by means of peritrichous flagella. Oval endospores are formed in swollen sporangia, positioned centrally or subterminally in cells. Colonies on TSA are circular, flat, white–cream, opaque and usually 1–3 mm in diameter after 48 h of growth at 28 °C. Growth occurs at 10–38 °C (optimal growth at 28 °C) and at pH 6.5–8.0 (optimal growth at pH 7.0). Facultatively anaerobic. Oxidase- and catalase-positive. Grows in the presence of 5% NaCl. Amylase, caseinase, gelatinase and phenylalanine deaminase are not produced and Tween 80 is not hydrolysed. Nitrate is not reduced to nitrite. Arginine dihydrolase, indole production, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase and hydrogen sulfide production are negative. Acetoin production is weak in the API 20E system. β-Galactosidase is produced in the API 20NE and API ZYM systems, and aesculins is hydrolysed in the API 20NE and API 50CH systems. D-Glucose, D-mannose and maltose are assimilated in the API 20NE system but L-arabinose, mannitol, N-acetylglucosamine, malate, gluconate, caproate, adipate, citrate and phenylacetate are not. In API 50CH, assimilation with acid production is observed from methyl β-D-glucose, aesculin, salicin, cellobiose, maltose, lactose, melibiose, starch and gentiobiose and is weak from D-xylene, D-glucose, D-fructose and arbutin. Assimilation with acid production is negative from glycerol, erythritol, D- and L-arabinose, D-ribose, L-xyllose, adonitol, mannose, L-sorbose, L-rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, sucrose, trehalose, inulin, melezitose, raffinose, glycerogen, xylitol, turanose, D-lyxose, D-tagatose, D- and L-fucose and D- and L-arabitol. Assimilation of gluconate and 2- and 5-ketogluconate in API 50CH is negative. In the API ZYM system, acid and alkaline phosphatases, α- and β-glucosidases, α-galactosidase and α-fucosidase are produced, in addition to β-galactosidase. Production of leucine and valine arylamidases, esterase and trypsin is weak. Production of esterase lipase, lipase, cystine arylamidase, chymotrypsin, phosphohydrolase, glucuronidase, N-acetylglucosaminidase and α-mannosidase is negative. The polar lipid profile consists predominantly of diphostatidyglycerol, phosphatidylglycerol, phosphatidylethanolamine, three unknown phospholipids, two unknown aminophospholipids and one unknown lipid. The major quinone is MK-7. The major fatty acids are anteiso-C₁₅:₀, C₁₆:₀ and iso-C₁₆:₀.

The type strain, RLAHU4Bᵀ (LMG 27416ᵀ = CECT 8236ᵀ), was isolated from root nodules of *Lupinus albus* in León (Spain). The DNA G+C content of the type strain is 57.8 mol%.

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**References**


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