Kribbella lupini sp. nov., isolated from the roots of *Lupinus angustifolius*

Martha E. Trujillo,¹ Reiner M. Kroppenstedt,² Peter Schumann²
and Eustoquio Martínez-Molina¹

Correspondence
Martha E. Trujillo
mett@usales

¹Departamento de Microbiología y Genética, Edificio Departamental, Lab. 209, Campus Miguel de Unamuno, Universidad de Salamanca, 37007 Salamanca, Spain

²DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany

Strain LU14^T, isolated from the roots of *Lupinus angustifolius*, was characterized using a polyphasic approach. 16S rRNA gene sequence studies showed a similarity of 98.7 % to the corresponding sequence of *Kribbella sandramycini* DSM 15626^T. Chemotaxonomic data gathered for fatty acids, phospholipids, cell-wall peptidoglycan and menaquinones strongly supported the classification of this strain in the genus *Kribbella* and DNA–DNA hybridization studies suggested that it may represent a novel species. Many physiological features were found that clearly distinguished isolate LU14^T from other *Kribbella* species. Based on the above data, a novel species of the genus *Kribbella*, *Kribbella lupini* sp. nov., is proposed with the type strain LU14^T (= DSM 16683^T = LMG 22957^T).

The genus *Kribbella* (Park *et al.*, 1999) includes six species that form a homogeneous group within the family *Nocardioidaceae*. While most *Kribbella* species have been isolated from soil, the recent description of *Kribbella solani* (Song *et al.*, 2004) recovered from potato tubers suggests that soil is not the only ecological niche where these bacteria may be found. In the present paper, we report on the isolation of a novel actinomycete strain, LU14^T, from the root nodules of *Lupinus angustifolius*, a native plant from the Mediterranean.

Strain LU14^T was isolated from root nodules of *L. angustifolius* growing near a former uranium mine in Salamanca, Spain. The root samples were washed twice with sterile distilled water and surface sterilized using HgCl₂ (1 %, w/v) for 2 min. The samples were rinsed five times with sterile distilled water and crushed using a sterile glass rod. The macerate was inoculated on yeast extract-mannitol agar (Vincent, 1970) and the plates were incubated for 3 weeks at 28 °C. Mycelium formation, colony colour and other growth characteristics were examined for 3 weeks on various culture media including SA1 (Trujillo *et al.*, 2005), ISP 2 (Shirling & Gottlieb, 1966) and nutrient agar.

Gram- and acid-fast-staining procedures were performed on 3 day cultures (Doetsch, 1981).

For chemotaxonomic analyses, strain LU14^T was grown in tryptic soy broth in flasks on a rotary shaker at 90 r.p.m. and 28 °C. Biomass was harvested, washed in distilled water and freeze-dried. Cell walls were prepared according to the method of Schleifer (1985). Peptidoglycan structure was studied in whole- and partial-cell-wall hydrolysates using TLC on cellulose (Schleifer & Kandler, 1972). Analysis of sugars in the purified cell walls was carried out as described by Stanek & Roberts (1974). Menaquinones were extracted and purified by the method of Minnikin *et al.* (1984) and analysed by HPLC (1100; Hewlett Packard). Methyl esters of cellular fatty acids for LU14^T and *Kribbella sandramycini* DSM 15626^T were prepared from cells grown for 24 h on trypticase soy agar cultures (28 °C) and analysed by GLC (Schröder *et al.*, 1997). Polar lipids were extracted and identified by two-dimensional TLC (Minnikin *et al.*, 1984).

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. Hydrolysis of arbutin, aesculin, starch, tyrosine and xylan was determined according to Trujillo *et al.* (2005). Tests for the utilization of various substrates as sole carbon and energy sources were performed as described by Williams *et al.* (1983). Antibiotic resistance was examined as described by Trujillo *et al.* (2005). Determination of temperature and pH growth rates and tolerance of NaCl were performed on nutrient agar.

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Abbreviation: LL-Dpm, LL-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Kribbella lupini* sp. nov. strain LU14^T is AJ811962.

A supplementary table detailing cellular fatty acid profiles for strain LU14^T and *Kribbella sandramycini* is available in IJSEM Online.

Strain LU14^T was also characterized using miniaturized API 20NE, API Coryne and API ZYM tests (bioMérieux) following the manufacturer's instructions. The reference strains *Kribbella antibiotica* DSM 15501^T, *Kribbella flavida* KACC 20248^T, *Kribbella koreensis* IMSNU 50530^T and *K. sandramycini* DSM 15626^T were also included in the physiological studies for comparison.

Genomic DNA extraction, PCR amplification of the 16S rRNA gene and sequencing parameters were as described previously (Rivas *et al.*, 2003). An almost-complete sequence was obtained and aligned against members of the family *Nocardioideae* using CLUSTAL_X (Thompson *et al.*, 1997). A phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) using the Kimura two-parameter distance matrix and compared with an additional tree obtained using the maximum-parsimony method (Fitch, 1971). In both cases, 1000 resamplings were used for bootstrap analysis. All analyses were carried out with the MEGA2 program (Kumar *et al.*, 2001).

Genomic DNA for hybridization studies was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization [in 2 × SSC plus 10 % (v/v) DMSO at 67 °C] was performed between strain LU14^T and its closest neighbour, *K. sandramycini* DSM 15626^T, as described by De Ley *et al.* (1970) and modified by Huß *et al.* (1983), using a Cary 100 Bio UV/VIS-spectrophotometer (Varian). The DNA G + C content was determined using the thermal melting method (Mandel & Marmur, 1968).

On all media tested, colonies were white to cream coloured with a pasty texture and lichenous shapes. No diffusible pigments were observed on any of the media. Substrate mycelium was extensively branched and penetrated into the agar and fragmented into rods; white aerial mycelium was produced abundantly and fragmented into non-motile rods.

A 16S rRNA gene sequence of 1458 nt was obtained for isolate LU14^T. The results of the phylogenetic analysis (Fig. 1) revealed that the isolate formed a separate line of descent in the phylogenetic cluster of the genus *Kribbella*. The closest neighbour of strain LU14^T was *K. sandramycini*

KACC 20249^T, with 98.7 % 16S rRNA gene sequence similarity, followed by *K. flavida* NBRC 14399^T with 98.6 % similarity. The sequence similarities with other species of the genus were in the range 98.2–97.1 %. A very similar tree was obtained using the maximum-parsimony method (data not shown).

Overall, the chemotaxonomic markers found are in agreement with the affiliation of strain LU14^T to the genus *Kribbella*. The total hydrolysate of the cell-wall peptidoglycan contained LL-diaminopimelic acid (LL-Dpm), glycine, glutamic acid and alanine; the partial hydrolysate contained the peptides L-Ala–D-Glu, LL-Dpm–D-Ala and LL-Dpm–Gly, which corresponded to type A3 γ peptidoglycan (Schleifer & Kandler, 1972). Galactose and one unidentified component, which is neither arabinose, glucose, mannose, rhamnose, ribose nor xylose, were detected in the purified cell wall of isolate LU14^T. Galactose was also reported in the whole cell sugar patterns of the remaining recognized *Kribbella* species (Song *et al.*, 2004; Li *et al.*, 2004), with the exception of *K. antibiotica* DSM 15501^T (Li *et al.*, 2004), which contains glucose, ribose and xylose. In the case of *K. sandramycini* KCTC 9609^T and *K. flavida* KACC 20248^T, glucose, mannose and galactose were reported (Li *et al.*, 2004), while these three sugars and ribose were found in *K. koreensis* IMSNU 50530^T, *Kribbella jejuensis* JCM 12204^T and *Kribbella solani* JCM 12205^T. These data suggest that the cell-wall sugar composition can be used as an important marker to differentiate species of the genus *Kribbella*.

The main menaquinones found were MK-9(H₄) (93 %) and MK-9(H₂) (7 %). The fatty acid profiles of strain LU14^T and its closest phylogenetic neighbour, *K. sandramycini* DSM 15626^T, are presented in Supplementary Table S1 in IJSEM Online. Strain LU14^T differed from *K. sandramycini* DSM 15626^T mainly in the presence of remarkable amounts of iso-16:0, 17:1 ω 8c and 10-methyl branched fatty acids. The phospholipids found in strain LU14^T were diposphatidylglycerol, phosphatidylglycerol, phosphatidylcholine and phosphatidylinositol, which correspond to phospholipid type III according to Lechevalier *et al.* (1977).

Various differentiating physiological characteristics between strain LU14^T and other *Kribbella* type strains are shown in

Fig. 1. Phylogenetic tree showing the relationship between members of the genus *Kribbella* and the novel isolate LU14^T based on 16S rRNA gene sequences. The tree was constructed by neighbour-joining analysis with distances based on the Kimura two-parameter model. Bootstrap values (1000 replicates) are shown as percentages at each node for values above 50 %. GenBank accession numbers are given in parentheses. Bar, 1 substitution per 100 nucleotides.

Table 1. Characteristics that differentiate isolate LU14^T and other *Kribbella* species

Strains: 1, LU14^T; 2, *K. sandramycini* DSM 15626^T; 3, *K. flavida* KACC 20248^T; 4, *K. koreensis* KACC 20250^T; 5, *K. antibiotica* DSM 15501^T; 6, *K. jejuensis* KACC 20266^T; 7, *K. solani* KACC 20196^T. Data for *K. jejuensis* and *K. solani* were taken from Song *et al.* (2004). +, Positive; –, negative; ND, not determined; w, weak reaction. The contradictory results obtained by Park *et al.* (1999) and Lee *et al.* (2000) are shown in parentheses.

Characteristic	1	2	3	4	5	6	7
Enzyme activities:							
Casein hydrolysis	+	+	+	+	+	W	–
Cystine arylamidase	W	+	+	+	ND	ND	ND
Esterase (C4)	–	+	+	+	ND	ND	ND
β-Galactosidase	W	+	+	+	ND	ND	ND
Naphthol-AS-BI-phosphohydrolase	–	+	+	–	ND	ND	ND
Nitrate reduction	–	–	+	–	–	–	–
Oxidase	+	+	+	–	+	ND	ND
Pyrrolidonyl arylamidase	–	+	+	+	ND	ND	ND
Starch hydrolysis	–	W	–	+	+	–	–
Urease	–	+	+	+	ND	–	+
Carbon sources:							
L-Arabinose	+	W	–	+	+	+	+
D-Galactose	+	+	–	+	+	+	–
D-Maltose	–	+	+	ND	+	ND	ND
D-Mannose	+	+	–	+	+	–	+
D-Melezitose	–	+	+	+	ND	+	+
L-Rhamnose	+	+	–	+	+	+	+
D-Sorbitol	+	–	–	ND	+	ND	ND
Growth in/at:							
12 °C	+	–	+	+	–	ND	ND
37 °C	+	–(+)	–(+)	–(+)	–	+	–
pH 5·0	–	+	–(+)	–	–	–	–
pH 9·0	+	+	+	+	–	–	–
3 % NaCl	+	+	+	–	–	ND	ND
5 % NaCl	+	–	–	–	–	ND	ND
7 % NaCl	+	–	–	–	–	ND	ND

Table 1. The ability of strain LU14^T to tolerate 7 % NaCl deserves special mention, as the remaining species do not tolerate NaCl concentrations above 3 %. It should also be noted that, in the original and emended descriptions of the genus *Kribbella* (Park *et al.*, 1999; Sohn *et al.*, 2003), production of urease and oxidase are reported as characteristic of the genus, but as more species have been described, the results for these properties seem to vary. The novel isolate showed resistance to ampicillin (2 µg), penicillin G (10 U) and rifampicin (2 µg) after 7 days of incubation and was susceptible to amoxycillin (30 µg), gentamicin (10 µg), neomycin (5 µg), novobiocin (5 µg), oxytetracycline (30 µg), streptomycin (300 µg), tobramycin (10 µg), vancomycin (30 µg) and tetracycline (30 µg). Other results for the novel isolate are given in the species description.

DNA–DNA hybridization experiments revealed a low level of relatedness (38·5 %) between isolate LU14^T and *K. sandramycini* DSM 15626^T. Given the low DNA–DNA

hybridization value obtained for these two strains, further tests were not performed between isolate LU14^T and *K. flavida* NBRC 14399^T, which shared a 16S rRNA gene sequence similarity slightly lower than LU14^T and *K. sandramycini* KACC 20249^T. In the case of *K. sandramycini* KACC 20249^T and *K. flavida* NBRC 14399^T, which share the highest 16S rRNA gene sequence similarity (98·9 %) between all of the recognized *Kribbella* species, the DNA–DNA reassociation value was 43 % (Park *et al.*, 1999). Various authors have reported that certain genera, including actinomycetes (Wink *et al.*, 2003), show less than 70 % DNA–DNA reassociation values at sequence similarities higher than 98·5 %, enabling this correlation between genomic and gene sequence similarities to be used to delineate novel species provided that DNA–DNA reassociation data exist for most of the other species (Romanenko *et al.*, 2004). This indeed, appears to be the case for the presently described species of the genus *Kribbella*. Based on the overall results, we propose that strain LU14^T represents a novel species, *Kribbella lupini* sp. nov.

Description of *Kribbella lupini* sp. nov.

Kribbella lupini (lu'pin.i. L. gen. n. *lupini* of lupin, isolated from *Lupinus angustifolius*).

Gram-positive, non-acid-fast, strictly aerobic actinomycete. Colonies are white to cream, irregular and show lichenous shapes. Well-developed substrate mycelium with hyphae that branch extensively and fragment into coccoid to rod elements. Abundant white aerial mycelium is produced that fragments into rod-shaped elements. No diffusible pigments observed. Grows between 12 and 37 °C, with an optimum temperature of 28 °C. Grows between pH 6–9. Grows in the presence of 7% NaCl. Catalase and oxidase are produced. Arbutin, aesculin, casein and gelatin are degraded, but not starch, urea or xylan. Acid is produced from glucose. The following substrates are used as carbon sources: adipate, L-alanine, L-arabinose, L-arginine, D-cellobiose, D-galactose, gluconate, D-glucose, L-histidine, L-lysine, malate, maltose, D-mannitol, D-mannose, D-melibiose, N-acetylglucosamine, L-proline, D-raffinose, L-rhamnose, D-sorbitol, L-sorbose, sucrose, D-trehalose, L-tyrosine, xylitol and DL-valine. Caprate, citrate, D-melezitose and L-serine are not used as carbon sources. Production of arginine dihydrolase, alkaline phosphatase, esterase lipase (weak), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Does not produce esterase (C4), naphthol-AS-BI phosphohydrolase or β -glucuronidase. Cell wall contains LL-Dpm. Major fatty acids are anteiso-15:0 (28%) and iso-16:0 (21%). The DNA G + C content is 68 mol%.

The type strain, LU14^T (=DSM 16683^T=LMG 22957^T), was isolated from root nodules of *Lupinus angustifolius*.

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