Micromonospora lupini sp. nov. and *Micromonospora saelicesensis* sp. nov., isolated from root nodules of *Lupinus angustifolius*

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A study was conducted to determine the taxonomic status of six actinomycete strains isolated from root nodules of *Lupinus angustifolius*. The strains were filamentous, Gram-positive and produced single spores at the tip of the hyphae. Phylogenetic, chemotaxonomic and morphological analyses demonstrated that all six strains belonged to the genus *Micromonospora*. According to the 16S rRNA gene sequence data, the strains were divided into two clusters that are moderately related to *Micromonospora mirobrigensis*, *Micromonospora matsumotoense* and *Micromonospora purpureochromogenes*. Fatty acid patterns also supported the division of the strains, and significant differences between the two groups were found in the amounts of iso-15:0, iso-16:0, iso-16:1 and iso-17:0. Furthermore, the two groups showed physiological differences which included utilization of arabinose, trehalose, alanine and sucrose and xylan hydrolysis. Finally, DNA–DNA hybridization and ribotyping studies confirmed that each group represents a novel species. Based on the genotypic and phenotypic data, the novel species *Micromonospora lupini* sp. nov. (type strain Lupac 14N^T = DSM 44874^T = LMG 24055^T) and *Micromonospora saelicesensis* sp. nov. (type strain Lupac 09^T = DSM 44871^T = LMG 24056^T) are proposed.

The genus *Micromonospora* Ørskov 1923, a member of the family *Micromonosporaceae* (Stackebrandt *et al.*, 1997), forms a stable group based on phylogenetic, chemotaxonomic and morphological data (Lechevalier & Lechevalier, 1970; Kroppenstedt, 1985; Lechevalier *et al.*, 1977; Stackebrandt *et al.*, 1997). Members of *Micromonospora* are widely distributed in aquatic, marine and terrestrial environments (Kawamoto, 1989; Zhao *et al.*, 2004; Maldonado *et al.*, 2005) but, recently, members of this genus have also been recovered from diverse plant tissues, especially nitrogen-fixing root nodules (Valdés *et al.*, 2005; Trujillo *et al.*, 2006b; M. E. Trujillo, unpublished observations). During an ecological study of nitrogen-fixing

bacterial communities from *Lupinus angustifolius* collected in Spain, different orange-pigmented actinomycete colonies were isolated from surface-sterilized nodules on yeast extract-mannitol agar (YMA) (Vincent, 1970). The results of a detailed taxonomic study of six isolates indicate that these strains represent two novel species.

Strains Lupac 06, Lupac 07, Lupac 08, Lupac 09^T, Lupac 13 and Lupac 14N^T were isolated from surface-sterilized nitrogen-fixing nodules of *Lupinus angustifolius* collected in Saelices el Chico (Salamanca, Spain) following the procedure described previously (Trujillo *et al.*, 2006a). Isolation plates were incubated for 2 weeks at 28 °C, after which colonies were picked under a stereoscopic microscope. All strains were Gram-stained using the procedure described by Doetsch (1981). Morphological and cultural characteristics of the strains were determined using various agar media: ISP 2 and ISP 3 (Shirling & Gottlieb, 1966), SA1 (Trujillo *et al.*, 2005), Bennett's (Jones, 1949) and N-Z amine, which contained (l⁻¹) 10.0 g glucose, 20.0 g soluble starch, 5.0 g yeast extract, 5.0 g N-Z amine, 1.0 g CaCO₃, and 15.0 g agar. (DSM medium 554; DSMZ catalogue). Cell morphology and motility were observed by phase-contrast microscopy using 5-day-old cultures on SA1 agar

Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences of isolates Lupac 06, Lupac 07, Lupac 08, Lupac 09^T, Lupac 13 and Lupac 14N^T are respectively AJ783990–AJ783993, AJ783995 and AJ783996.

Scanning electron micrographs of spores of strains Lupac 09^T and Lupac 14N^T, an extended phylogenetic tree, cultural characteristics of the novel strains and detailed fatty acid profiles are available as supplementary material with the online version of this paper.

(Leica; CTR MIC). Isolates Lupac 09^T and Lupac 14N^T were fixed, dehydrated, critical-point-dried and coated with gold before examination by scanning electron microscopy (Zeiss; DSM 940).

All strains grew well on all media tested, except that isolates Lupac 13 and Lupac 14N^T grew poorly or did not grow, respectively, on Bennett's agar. Colonies of all strains ranged from light orange to brown on the different media and were folded and raised, and some of them turned dark brown to black after spore production. Orange–brown and brown diffusible pigments were produced by strains Lupac 09^T, Lupac 06 and Lupac 07 on ISP 3 agar. All strains were Gram-positive, produced branched hyphae (0.3–0.6 µm diameter) with terminal spores and lacked aerial mycelium. Strains Lupac 09^T and Lupac 14N^T formed single spores (0.6–0.8 µm diameter), mainly on long hyphae. The spores had a 'lemon-shaped' form and presented a smooth surface (Supplementary Fig. S1, available in IJSEM Online). Cultural characteristics of the six strains in all tested media are presented in Supplementary Table S1.

DNA extraction and PCR amplification of the 16S rRNA gene were performed using the REDExtract-N-Amp Plant PCR kit (Sigma) following the manufacturer's instructions but with an additional purification step using phenol/chloroform. Amplification and sequencing conditions followed methods described by Rivas *et al.* (2003).

The six sequences obtained were aligned manually and compared with corresponding sequences of all species of the genus *Micromonospora* with validly published names downloaded from GenBank/EMBL. Phylogenetic distances were calculated with Kimura's two-parameter model (Kimura, 1980) and tree topologies were inferred using the maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) methods. Branch support based on 1000 replications was calculated with the bootstrap test. All analyses were carried out using the MEGA3 program (Kumar *et al.*, 2004).

Nearly complete 16S rRNA gene sequences (1460 nt) were obtained for the six isolates and confirmed their phylogenetic affiliation to the genus *Micromonospora*. Identification

of the closest phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Sequence similarities between the new isolates and currently described *Micromonospora* species ranged from 96.7 to 99.0%. Lupac 14N^T showed 98.5% sequence similarity to *Micromonospora mirobrigensis* DSM 44830^T, while Lupac 09^T shared 98.7% sequence similarity with *Micromonospora purpureochromogenes* DSM 43821^T and *Micromonospora matsumotoense* DSM 44110^T. Two different lineages were revealed by the phylogenetic analysis: isolates Lupac 06, Lupac 07 and Lupac 09^T (group A), sharing sequence similarity of 99.6–99.7%, formed a compact cluster, while strains Lupac 08, Lupac 13 and Lupac 14N^T (group B) formed an independent branch and shared sequence similarity of 99.6–100%. Members of the two clusters showed sequence similarity between 98.8 and 99.1% and the clusters were supported by a bootstrap value of 99%. The phylogenetic tree based on the neighbour-joining method (Fig. 1) shows the distribution of the six isolates and the phylogenetically closest *Micromonospora* species. An extended tree including all *Micromonospora* species with validly published names is available as Supplementary Fig. S2.

To investigate the genetic diversity of the six isolates further, automated ribotyping studies were carried out. Strains were grown for 24–48 h at 28 °C on cellulose acetate filters (Microscience) placed over N-Z amine agar (pH 7.2). Automated ribotyping was performed under the conditions recommended by the manufacturer of the Riboprinter microbial characterization system (Qualicon) using the restriction enzyme *PvuII*. Two different riboprint profiles (Fig. 2) were clearly distinguished and divided the strains into two homogeneous clusters which corresponded to the phylogenetic groups (A and B) obtained by 16S rRNA gene sequence analyses.

To determine overall genome similarity, DNA–DNA hybridization experiments were carried out between *M. matsumotoense* DSM 44100^T, the closest neighbour according to the phylogenetic analyses, and strains Lupac 09^T and Lupac 14N^T, respectively representing phylogenetic groups

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships among the six new isolates and the phylogenetically closest *Micromonospora* species. Bootstrap percentages (from 1000 replicates) above 50% are shown at nodes. Bar, 0.005 substitutions per nucleotide position. An extended version of this tree containing all species of *Micromonospora* is available as Supplementary Fig. S2.

Fig. 2. Riboprint patterns of *Micromonospora* isolates generated with the restriction enzyme *PvuII*.

A and B. DNA was isolated and purified on hydroxyapatite (Cashion *et al.*, 1977) and reassociation measurements were obtained by spectrophotometry (De Ley *et al.*, 1970; Huß *et al.*, 1983). These studies revealed low levels of hybridization among the strains tested. Specifically, Lupac 09^T and Lupac 14N^T shared 27.9% and 33.2% relatedness, respectively, with *M. matsumotoense* DSM 44100^T, while Lupac 09^T and Lupac 14N^T showed 26% DNA–DNA relatedness. The G+C contents of strains Lupac 09^T and Lupac 14N^T were 71.6 and 70.9 mol%, respectively, as determined by the thermal melting method (Mandel & Marmur, 1968).

Methyl esters of cellular fatty acids for all study strains were prepared from cells grown for 24 h on trypticase soy agar (Schröder *et al.*, 1997) and analysed using the MIDI system (Microbial ID, Inc.). Diaminopimelic acid (DAP) determination was achieved by cellulose TLC using whole-cell hydrolysates (modified method of Hasegawa *et al.*, 1983; Rhuland *et al.*, 1955). Biomass for additional chemotaxonomic analyses of strains Lupac 09^T and Lupac 14N^T was obtained in tryptic soy broth in flasks on a rotary shaker at 90 r.p.m. and 28 °C. Whole-cell sugars were analysed according to Stanek & Roberts (1974). Phospholipids and menaquinones were prepared according to the method of Minnikin *et al.* (1984) and respectively analysed by two-dimensional TLC and HPLC.

Differences in fatty acid composition divided the strains into two groups which corresponded to those defined by the 16S rRNA gene sequence and riboprint analyses. The main differences between the two groups were in the amounts of iso-15:0, iso-16:0, iso-16:1 and iso-17:0. The detailed fatty acid patterns of the study strains are presented in Supplementary Table S2. All strains contained *meso*-DAP. Whole-cell hydrolysates of Lupac 09^T and Lupac 14N^T contained glucose, mannose, arabinose, xylose and ribose; in addition, Lupac 09^T contained rhamnose. Galactose, which has been reported for *M. matsumotoense* DSM 44100^T and *M. mirobrigensis* DSM 44830^T, was not found in the new isolates. The menaquinones of strain Lupac 09^T were made up of MK-10(H₄) (>90%) and traces

of MK-10(H₆), while Lupac 14N^T contained these two quinones (83 and 10%, respectively) in addition to MK-9(H₄) (7%). The two strains contained phosphatidylglycerol, phosphatidylinositol and phosphatidylethanolamine, which corresponds to phospholipid pattern II. Overall chemotaxonomic differences found between Lupac 09^T and Lupac 14N^T and related species are given in Table 1.

Physiological tests for the six isolates were carried out using standard methods and included catalase and oxidase determination (Trujillo *et al.*, 2006a), degradation of various organic compounds (Trujillo *et al.*, 2005), carbon substrate utilization (Williams *et al.*, 1983) and growth at and tolerance of various temperatures (4–45 °C) and NaCl concentrations (1–5%). Enzyme activities were also determined using API ZYM and API Coryne kits (bioMérieux) for the six isolates and *M. matsumotoense* DSM 44100^T, *M. mirobrigensis* DSM 44830^T and *Micromonospora carbonacea* DSM 43168^T.

All strains were catalase- and oxidase-positive with the exception of Lupac 06, which did not produce oxidase. All strains tolerated 1% NaCl and grew at 20–37 °C. All strains were negative for nitrate reduction and urease production and were sensitive to 0.01% sodium azide. Differentiating metabolic profiles of isolates Lupac 09^T, Lupac 14N^T and phylogenetically related *Micromonospora* type strains are given in Table 1. The two groups represented by strains Lupac 09^T (group A) and Lupac 14N^T (group B) could be differentiated by the use of arabinose, trehalose, alanine and sucrose. Pigment production was also considered an important difference between the two groups. Additional physiological data are presented in Table 1 and the species descriptions.

The 16S rRNA gene sequences, cultural characteristics and chemotaxonomic data clearly demonstrate that the six strains isolated from nitrogen-fixing nodules of *L. angustifolius* belong to the genus *Micromonospora*. Furthermore, riboprint analyses, fatty acid compositions and metabolic profiles indicate that the strains can be divided into two groups which, according to DNA–DNA hybridization data, represent novel species. Thus, we propose to classify strains Lupac 09^T, Lupac 06, and Lupac 07 (group A) within the novel species *Micromonospora saelicesensis* sp. nov. and strains Lupac 14N^T, Lupac 08 and Lupac 13 (group B) as representatives of the species *Micromonospora lupini* sp. nov.

Description of *Micromonospora saelicesensis* sp. nov.

Micromonospora saelicesensis (sae.li.ces.en'sis. N.L. fem. adj. *saelicesensis* pertaining to Saelices, the place where the plants were collected from which the first strains were isolated).

Gram-positive, chemo-organotrophic and aerobic actinomycete. Well-developed branched hyphae (0.3–0.6 µm in diameter). Colonies are orange on ISP 2 agar. Orange–brown to brown diffusible pigments are produced on ISP 3

Table 1. Phenotypic characteristics that distinguish the new isolates and phylogenetically related *Micromonospora* species

Reference strains: 1, *M. matsumotoense* DSM 44100^T; 2, *M. mirobrigensis* DSM 44830^T; 3, *M. carbonacea* DSM 43168^T; 4, *M. siamensis* JCM 12769^T; 5, *M. purpureochromogenes* ATCC 27007^T; 6, *M. halophytica* DSM 43171^T. +, Positive; –, negative; ND, no data available; v (+), variable, type strain positive; v (–), variable, type strain negative; w, weak. All API ZYM and API Coryne data were obtained in this study. Other data were taken from Kawamoto (1989), Thawai *et al.* (2005), Trujillo *et al.* (2005) and Ara & Kudo (2007). Fatty acids, whole-cell sugars, menaquinones and polar lipids are listed in decreasing order of abundance.

Characteristic	<i>M. saelicesensis</i> sp. nov. (n=3)	<i>M. lupini</i> sp. nov. (n=3)	1	2	3	4	5	6
Pigment production	Light orange	–	–	–	–	–	Dark brown	–
Ornamented spores	–	–	ND	–	+	ND	+	ND
NaCl tolerance (%)	2	1	3	3	3	5	1.5	4
Maximum growth temperature (°C)	37	37	40	37	40	40	40	40
Nitrate reduction	–	–	+	–	+	–	–	+
Starch hydrolysis	v (–)	+	+	+	+	+	ND	–
Xylan hydrolysis	–	+	ND	+	ND	ND	–	ND
Oxidase	v (+)	+	–	+	+	ND	ND	ND
Carbon sources								
Arabinose	+	–	+	+	–	+	–	+
Trehalose	–	+	+	+	–	ND	ND	ND
Alanine	–	v (+)	ND	+	ND	ND	ND	ND
Rhamnose	–	–	–	+	–	+	–	–
Histidine	+	–	ND	+	–	ND	ND	ND
Inositol	+	–	–	ND	–	ND	–	ND
Gluconate	–	–	–	ND	ND	ND	ND	ND
Sucrose	–	+	+	+	ND	ND	ND	+
Glutarate	+	v (–)	ND	ND	ND	ND	ND	ND
Mannitol	+	+	–	–	–	–	–	–
Acid production from: (API Coryne)								
Glucose	+	–	–	ND	ND	ND	ND	ND
Maltose	–	–	w	ND	ND	ND	ND	ND
API ZYM results								
Alkaline phosphatase	+	+	–	+	+	ND	ND	ND
Acid phosphatase	+	+	–	+	+	ND	ND	ND
Naphthol-AS-BI-phosphohydrolase	+	+	–	+	+	ND	ND	ND
N-Acetyl-β-glucosaminidase	+	+	–	+	+	ND	ND	ND
α-Mannosidase	v (+)	–	w	–	–	ND	–	–
α-Fucosidase	v (+)	–	–	–	–	ND	ND	ND
Major fatty acids	iso-15:0, <i>cis</i> -8:17:1, iso-16:0	iso-16:0, iso-15:0	iso-16:0, 18:1, 17:1, iso-16:1, iso-15:0	iso-15:0, iso-16:0, iso-17:1, anteiso-17:0	iso-15:0, iso-16:0, <i>cis</i> -9:17:1	iso-16:0, iso-15:0, anteiso-17:0, 17:1	ND	ND
Whole-cell sugars*	Glc, Man, Ara, Xyl, Rib, Rha	Glc, Man, Ara, Xyl, Rib	Ara, Xyl, Gal, Rha	Glc, Gal, Man, Xyl	ND	Xyl, Ara	ND	ND
Menaquinone composition	MK-10(H ₄), MK-10(H ₆)	MK-10(H ₄), MK-10(H ₆), MK-9(H ₄)	MK-10(H ₄), MK-10(H ₆), MK-10(H ₈)	MK-10(H ₄), MK-10(H ₆), MK-9(H ₄)	MK-9(H ₄)	MK-10(H ₄), MK-10(H ₆)	MK-10(H ₄)	MK-9(H ₄)
Major polar lipids†	DPG, PE, PI	DPG, PE, PI	DPG, PI, PG, PE	PI, DPG, PE	ND	PE, DPG, PI	ND	ND

*Ara, Arabinose; Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose.

†DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

agar. Smooth-surfaced spores are produced at the tip of the hyphae. Growth occurs at 20–37 °C but not at 15 or 45 °C; optimum growth at 28 °C. Catalase-positive and oxidase-variable. Nitrate is not reduced. Growth is observed in 2 % NaCl but not in the presence of 0.001 % crystal violet or 0.01 % sodium azide. Arbutin, casein, aesculin, gelatin and xylan are degraded, but not tyrosine. Starch degradation is variable. Carbon sources assimilated include arabinose, cellobiose, galactose, glutarate, histidine, inositol, maltose, mannose, melibiose, raffinose and sucrose. Alanine, arginine, gluconate, lysine, proline, rhamnose, serine, sorbitol, sorbose, sucrose, trehalose, valine and xylitol are not assimilated. Enzyme tests with the API ZYM and API Coryne systems are positive for acid and alkaline phosphatases, esterase (C4), esterase lipase (C8), lipase (C15), leucine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase and pyrazinamidase and negative for β -glucuronidase, pyrrolidonyl arylamidase, urease and β -glucuronidase. Variable reactions are observed for α -mannosidase and α -fucosidase. Acid is produced from glucose. Peptidoglycan contains *meso*-DAP; whole-cell sugars are glucose, mannose, arabinose, xylose, ribose and rhamnose. Major menaquinone is MK-10(H₄). Diagnostic phospholipid is diphosphatidylethanolamine. The G+C content of the type strain is 71.6 mol%.

The type strain, Lupac 09^T (=DSM 44871^T =LMG 24056^T), and two other strains, Lupac 06 (=DSM 44868) and Lupac 07 (=DSM 44869), were isolated from root nodules of *Lupinus angustifolius*.

Description of *Micromonospora lupini* sp. nov.

Micromonospora lupini (lu'pini.i. L. gen. n. *lupini* of a lupin, referring to the isolation of the first strains from *Lupinus angustifolius*).

Gram-positive, chemo-organotrophic and aerobic actinomycete. Well-developed branched hyphae (0.3–0.6 µm in diameter). Colonies are light orange on ISP 2 agar. No diffusible pigments are observed. Smooth-surfaced spores are produced at the tip of the hyphae. Growth occurs at 20–37 °C but not at 15 or 45 °C; optimum growth at 28 °C. Catalase- and oxidase-positive. Nitrate is not reduced. Growth is observed at 1 % NaCl but variable at 2 % NaCl. No growth in 0.001 % crystal violet or 0.01 % sodium azide. Arbutin, casein, aesculin, gelatin, starch and xylan are degraded but not tyrosine. Carbon sources assimilated include alanine, cellobiose, galactose, maltose, mannose, melibiose, raffinose and trehalose. Arginine, histidine, lysine, proline, rhamnose, serine, sorbitol, sorbose, tyrosine, valine and xylitol are not assimilated. Enzyme tests with the API ZYM and API Coryne systems are positive for acid and alkaline phosphatases, esterase (C4), esterase lipase (C8), lipase (C15), leucine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase,

α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase and pyrazinamidase and negative for β -glucuronidase, α -mannosidase, α -fucosidase and urease. Acid is not produced from glucose. Peptidoglycan contains *meso*-DAP; whole-cell sugars are glucose, mannose, arabinose, xylose and rhamnose. Major menaquinone is MK-10(H₄). Diagnostic phospholipid is diphosphatidylethanolamine. The G+C content of the type strain is 70.9 mol%. Strain Lupac 08 produces the antitumoral compounds lupinadins A and B.

The type strain, Lupac 14N^T (=DSM 44874^T =LMG 24055^T), and two other strains, Lupac 08 (=DSM 44870) and Lupac 13 (=DSM 44873), were isolated from root nodules of *Lupinus angustifolius*.

Acknowledgements

We would like to thank Dr J. P. Euzéby for his help with the nomenclature. We also acknowledge the excellent technical assistance of Mrs G. Pötter and other DSMZ staff. M. E. T. and E. M. M. are grateful to the Ministerio de Educación y Ciencia (MEC) for financial support under the project CGL2006-06988. M. E. T. also thanks the European Union for financial support under the BIOREMAT programme.

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