Micromonospora pisi sp. nov., isolated from root nodules of *Pisum sativum*

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A novel actinomycete, designated strain GUI 15^T, isolated from the root nodules of a *Pisum sativum* plant was characterized taxonomically by using a polyphasic approach. The 16S rRNA gene sequence of strain GUI 15^T showed highest similarity to *Micromonospora pattaloongensis* TJ2-2^T (98.7 %) and *Polymorphospora rubra* TT 97-42^T (98.5 %). Phylogenetic analysis based on the gyrase B gene also supported the close relationship of these three strains, but indicated that strain GUI 15^T should be assigned to the genus *Micromonospora*. Chemotaxonomic results confirmed the position of the isolate in the genus *Micromonospora*, but revealed differences at the species level. The novel strain could be distinguished from recognized *Micromonospora* species by using a combination of physiological and biochemical tests. Based on these observations, strain GUI 15^T is considered to represent a novel species of the genus *Micromonospora*, for which the name *Micromonospora pisi* sp. nov. is proposed. The type strain is GUI 15^T (=DSM 45175^T=LMG 24546^T).

The genus *Micromonospora* (Ørskov, 1923) is the type genus of the family *Micromonosporaceae*. At the time of writing, this family comprised 22 genera, including the recently described taxon *Polymorphospora* (Tamura *et al.*, 2006). Phylogenetically, the genus *Polymorphospora* shows a close relationship to the genus *Micromonospora* (Tamura *et al.*, 2006), and also shares various chemotaxonomic markers including cell-wall chemotype II, phospholipid type II and peptidoglycan type A1 γ . The main differences between *Micromonospora* and *Polymorphospora* species are found in the cell-wall sugar composition and fatty acid type. Additionally, *Polymorphospora rubra* is reported to produce short spore chains and polymorphic spores (Tamura *et al.*, 2006).

Many species in the genus *Micromonospora* have been described in the past few years and, at the time of writing, the genus comprised 37 recognized species, including

species isolated from root nodules of *Coriaria* sp. and *Lupinus angustifolius* (Trujillo *et al.*, 2006b, 2007). Here we report the isolation and taxonomic characterization of a novel strain, GUI 15^T, from surface-sterilized, nitrogen-fixing root nodules obtained from a plant of *Pisum sativum* collected in Zamora, Spain.

Root nodules used for the isolation of strain GUI 15^T were processed as described by Trujillo *et al.* (2006b), by using yeast extract-mannitol agar (Vincent, 1970) and incubation at 28 °C for 15 days. Isolation plates were examined routinely under a stereoscopic microscope until bacterial growth was observed. Selected orange colonies were transferred to SA1 agar (Trujillo *et al.*, 2005) to obtain abundant biomass for long-term maintenance as glycerol suspensions (20 %, v/v) at –80 °C. The strain investigated was maintained routinely on SA1 agar. Strain GUI 15^T was selected because of its capacity to degrade xylan, and the colour of its substrate mycelium, which was beige to light yellow on ISP 2 agar (Shirling & Gottlieb, 1966).

Cultural characteristics of strain GUI 15^T were studied on several media, namely Bennett's (Jones, 1949), oatmeal (ISP 3; Shirling & Gottlieb, 1966), SA1 (Trujillo *et al.*, 2005), yeast-extract malt-extract (ISP 2) and yeast extract-mannitol agars. The novel strain showed good growth on all media except oatmeal agar, on which growth was poor and slow; neither aerial hyphae nor diffusible pigments were produced on these test media. The colour of the substrate mycelium was generally pale yellow and colonies were folded and raised, turning brown after 3 weeks. Detailed results of the cultural characteristics of strain

Abbreviation: *gyrB*, gyrase B subunit.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GUI 15^T and those for the *gyrB* gene sequences of strain GUI 15^T, *Micromonospora pattaloongensis* TJ2-2^T and *Polymorphospora rubra* TT 97-42^T are AM944497, FM957540, FM957541 and FM957542, respectively.

A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain GUI 15^T, recognized *Micromonospora* species and other members of the family *Micromonosporaceae*, and tables detailing the cultural characteristics of strain GUI 15^T observed on various growth media and the fatty acid compositions of strain GUI 15^T and *Polymorphospora rubra* TT 97-42^T are available as supplementary material with the online version of this paper.

GUI 15^T on the various test media are given in Supplementary Table S1 in IJSEM Online. Cell morphology and motility were observed by using phase-contrast microscopy (Leica; CTR MIC) of 7-day-old cultures grown on ISP 2 agar. Spore production was examined on 2-week-old cultures on ISP 2 agar by using scanning electron microscopy (DSM 940; Zeiss). Strain GUI 15^T produced extensively branching and non-fragmenting hyphae (0.3–0.5 µm in diameter). No motility was observed. Mainly single, smooth, spherical, non-motile spores (0.6–0.8 µm in diameter) were produced, although in some cases pairs of spores were observed (Fig. 1). This spore morphology corresponds to that described for members of the genus *Micromonospora* but not to that for *Polymorphospora*, which is reported to produce spore chains (Tamura *et al.*, 2006). Gram and acid-fast stains (Doetsch, 1981) were used with 4-day-old cultures. Strain GUI 15^T was Gram-positive and non-acid-fast.

DNA extraction for PCR amplification of the 16S rRNA and gyrase B subunit (*gyrB*) genes was performed as described previously (Trujillo *et al.*, 2007). Amplification and sequencing conditions for the 16S rRNA gene followed the methods described by Rivas *et al.* (2003). The 16S rRNA gene sequence of strain GUI 15^T was aligned manually and compared with other sequences obtained from the GenBank/EMBL/DBJ databases. Phylogenetic distances were calculated with the Kimura two-parameter model (Kimura, 1980) and tree topologies were inferred by using the maximum-parsimony (Fitch, 1971) and neighbour-joining methods (Saitou & Nei, 1987). Bootstrap replications (1000) were performed by using the MEGA4 program (Tamura *et al.*, 2007).

PCR amplification of the *gyrB* gene of strains GUI 15^T, *Micromonospora pattaloongensis* TJ2-2^T and *P. rubra* TT 97-42^T was performed by using the REDExtract-N-Amp Plant PCR kit (Sigma). Each PCR mixture (20 µl final

volume) contained 10 µl REDExtract-N-Amp PCR ReadyMix, 0.4 µl of each primer (20 µM), 2 µl extraction/dilution mixture (50:50 v/v) and 1 µl template DNA. For the design of *gyrB* primers, sequences of the following *Micromonospora* species previously determined by Kasai *et al.* (2000) were used: *M. aurantiaca* (NBRC 16125^T), *M. carbonacea* (NBRC 14108^T), *M. chalicea* (NBRC 13503^T), *M. chersina* (NBRC 15963^T), *M. coerulea* (NBRC 13504^T), *M. echinospora* (NBRC 13149^T), *M. halophytica* (NBRC 14112^T), *M. nigra* (NBRC 16103^T), *M. inositola* (ATCC 21773^T), *M. olivasterospora* (NBRC 14304^T), *M. purpureochromogenes* (NBRC 13324^T) and *M. rosaria* (NBRC 13697^T). These sequences were aligned via the CLUSTAL_X program and were searched for primers similar to those described by Richert *et al.* (2007) but more specific for the genus *Micromonospora*. Two overlapping PCR products were amplified to obtain a final composite sequence of 1110 nt. In the first amplification, a 500-bp fragment was obtained by using primers GYF1 (5'-TCCGGYGGYCT-GCACGGCGT-3'; position 19–38) and GYR1B (5'-CGGAAGCCCTCYTCGTGSGT-3'; position 548–567). The amplification was performed with an initial denaturation at 95 °C for 9 min, followed by 35 cycles with denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 2 min, followed by a step at 72 °C for 7 min. The second fragment of 900 bp was amplified by using primers GYF3 (5'-ACSGTCG-ACTTCGACTTCCA-3', position 220–239) and GYR3B (5'-CAGCACSAAYCTTGTGGTA-3', position 1210–1226). The amplification was performed with an initial denaturation at 95 °C for 9 min, followed by 35 cycles with denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 2 min, followed by a step at 72 °C for 7 min. Sequencing was carried out by using primers GYF1, GYF3, GYF4 (5'-ACCCACGAGGAGGG-CTTCCG-3', position 548–567) GYR1B and GYR3B. The primer positions correspond to the nucleotides in the *gyrB* gene sequence of *Microbacterium imperiale* 33884 (AB074922.1). Phylogenetic analysis was performed as described above.

An almost-complete 16S rRNA gene sequence (1452 nt) was obtained for strain GUI 15^T and was compared with those deposited in the public databases. The highest levels of 16S rRNA gene sequence similarity were with *Micromonospora pattaloongensis* JCM 12833^T (98.7%) and *P. rubra* TT 97-42^T (98.5%). Additionally, identification of its closest phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were performed via the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007), which yielded similar results.

The results of the phylogenetic analysis with various tree-making algorithms were very similar (data not shown). Fig. 2 shows the relationship between strain GUI 15^T and its closest phylogenetic neighbours based on 16S rRNA gene sequences. The selected strains formed two subclades, one of which comprised *P. rubra* TT 97-42^T, *Micromonospora pattaloongensis* TJ2-2^T, *Micromonospora*

Fig. 1. Scanning electron micrograph of cells of strain GUI 15^T producing spores. The strain was grown on ISP 2 agar for 2 weeks at 28 °C. Bar, 2 µm.

olivasterospora DSM 43868^T and strain GUI 15^T. This clustering was supported by bootstrap values which ranged from 62 to 77 %. A tree containing all recognized species of the genus *Micromonospora* at the time of writing and other representatives of the family *Micromonosporaceae* is provided as Supplementary Fig. S1 (in IJSEM Online). This tree showed that the grouping of the above four strains was identical. From these results, the taxonomic affiliation of strain GUI 15^T at the genus level could not be determined.

To clarify the affiliation of strain GUI 15^T to its closest phylogenetic neighbours, the sequences of the *gyrB* gene for strain GUI 15^T, *P. rubra* TT 97-42^T and *Micromonospora pattaloongensis* TJ2-2^T were obtained and compared. Levels of *gyrB* gene sequence similarity between strain GUI 15^T and *Micromonospora pattaloongensis* TJ2-2^T and *P. rubra* TT 97-42^T were 89.6 and 90.5 %, respectively, whereas *P. rubra* TT 97-42^T and *M. pattaloongensis* TJ2-2^T shared 88.1 % *gyrB* gene sequence similarity. Levels of *gyrB* gene sequence similarity between strain GUI 15^T and other available *Micromonospora* species ranged from 90.3 to 93.1 %. The phylogenetic analysis shown in Fig. 3 included all available sequences for the type strains of *Micromonospora* species and other members of the family *Micromonosporaceae*. All *Micromonospora* strains were recovered in one cluster including strain GUI 15^T, *P. rubra* TT 97-42^T and *Micromonospora pattaloongensis* TJ2-2^T,

supported by a 100 % bootstrap value. These results demonstrated that strain GUI 15^T is a member of the genus *Micromonospora*.

Chemotaxonomic analyses were carried out to support the above results. For these analyses strain GUI 15^T was grown in ISP 2 broth in flasks on a rotary shaker at 125 r.p.m. and 28 °C for 1 week. Biomass was harvested, washed in distilled water and freeze-dried. Isomers of diaminopimelic acid in whole-cell hydrolysates were determined by TLC on cellulose (modified method of Hasegawa *et al.*, 1983; Rhuland *et al.*, 1955). Whole-cell sugars were analysed according to Stanek & Roberts (1974). Menaquinones were extracted and purified by using the method of Minnikin *et al.* (1984) and were analysed by HPLC (Hewlett Packard 1100). Methyl esters of cellular fatty acids were prepared from cells grown for 24 h on trypticase soy agar and ISP 2 agar (28 °C) and were analysed by GLC (Schröder *et al.*, 1997). Polar lipids were extracted and identified by using two-dimensional TLC (Minnikin *et al.*, 1984). For whole-cell sugar and fatty acid analyses, *P. rubra* TT 97-42^T was also studied under the same growth conditions. The DNA G+C content of strain GUI 15^T was 71 mol% as determined by using the thermal melting method (Mandel & Marmur, 1968).

Strain GUI 15^T contained *meso*-diaminopimelic acid, which is the characteristic diamino acid of peptidoglycan

Fig. 2. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain GUI 15^T, *P. rubra* TT 97-42^T and phylogenetically related species of the genus *Micromonospora*. Bootstrap values of >50 % are shown at branch points based on 1000 replicates. A total of 1381 nt were analysed. Asterisks indicate that the corresponding nodes were also recovered in the maximum-parsimony tree. Bar, 0.005 substitutions per nucleotide position. An expanded version of this tree, including all recognized *Micromonospora* species at the time of writing and other members of the family *Micromonosporaceae*, is available as Supplementary Fig. S1 (in IJSEM Online).

Fig. 3. Neighbour-joining phylogenetic tree based on the *gyrB* gene sequences of strain GUI 15^T, *Micromonospora pattaloongensis* TJ2-2^T, *P. rubra* TT 97-42^T (=DSM 44947^T) and other members of the family *Micromonosporaceae*. Bootstrap values of >50 % are shown at branch points based on 1000 replicates. A total of 1030 nt were analysed. Asterisks indicate that the corresponding nodes were also recovered in the maximum-parsimony tree. Bar, 0.02 substitutions per nucleotide position.

type A1 γ (Schleifer & Kandler, 1972), as also reported for the genera *Micromonospora* and *Polymorphospora*. Galactose, glucose, mannose, ribose, xylose, rhamnose (trace) and arabinose (trace) were detected as whole-cell sugars in strain GUI 15^T. This sugar profile is identical to that reported for *Micromonospora pattaloongensis* TJ2-2^T (Thawai *et al.*, 2008). The sugar profile of *P. rubra* TT 97-42^T (Tamura *et al.*, 2006) was similar to that of strain GUI 15^T except that arabinose and ribose were absent, and 3-O-methyl mannose was present. In the present sugar analyses, *P. rubra* contained galactose, glucose and xylose but not 3-O-methyl mannose or mannose; instead ribose was detected. A summary of these results is given in Table 1. Strain GUI 15^T contained MK-10(H₄) as the main menaquinone with MK-10(H₆) and MK-10(H₈) as minor components. These compounds were also reported for *Micromonospora pattaloongensis* TJ2-2^T and *P. rubra* TT 97-42^T, in addition to other menaquinones (Table 1). Similarly, the diagnostic lipid phosphatidylethanolamine, which corresponds to pattern II (Lechevalier *et al.*, 1977), was found in strain GUI 15^T, *Micromonospora pattaloongensis* TJ2-2^T and *P. rubra* TT 97-42^T. Additionally, the polar lipid profile of strain GUI 15^T contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unknown phosphoglycolipid; some unidentified

glycolipids and phospholipids were also detected. The major fatty acids of strain GUI 15^T were iso-C_{16:0} (27.4 % of the total), anteiso-C_{15:0} (22.9 %), anteiso-C_{17:0} (15.4 %) and iso-C_{15:0} (13.7 %); a trace of 10-methyl C_{17:0} (0.29 %) was also detected. This profile corresponds to fatty acid type 3b (Kroppenstedt, 1985). The major fatty acids of *P. rubra* TT 97-42^T under the same conditions were iso-C_{16:0} (30.4 %), C_{17:1} ω 8c (23.3 %), C_{17:0} (7.7 %), anteiso-C_{15:0} (7.7 %), anteiso-C_{17:0} (7.6 %) and iso-C_{15:0} (5.7 %); 10-methyl C_{17:0} was also detected (0.3 %). According to our results, the profile of *P. rubra* is also of type 3b (Kroppenstedt, 1985). The full fatty acid profiles of the two strains are given in Supplementary Table S2 (in IJSEM Online). Chemotaxonomic characteristics of strain GUI 15^T, *Micromonospora pattaloongensis* and *P. rubra* are given in Table 1.

Physiological tests were carried out according to standard methods and included determination of catalase and oxidase activities (Trujillo *et al.*, 2006a), degradation of various organic compounds (Trujillo *et al.*, 2005), carbon substrate utilization (Williams *et al.*, 1983), and growth at various temperatures (4–45 °C), pH (4.5–9) and NaCl concentrations (1–5 %). Enzyme activity tests by using API ZYM and API Coryne kits (bioMérieux) were also carried

Table 1. Chemotaxonomic characteristics of strain GUI 15^T and its closest phylogenetic neighbours

All strains contained galactose, glucose and xylose as whole-cell sugars. ND, Not determined.

Characteristic	Strain GUI 15 ^T *	<i>Micromonospora pattaloongensis</i> TJ2-2 ^{T†}	<i>P. rubra</i> TT 97-42 ^{T*}	<i>P. rubra</i> TT 97-42 ^{T‡}
Whole-cell sugars	Ribose, mannose, arabinose	Ribose, mannose, arabinose	Ribose	Mannose, 3-O-methyl-mannose
Major fatty acids	iso-C _{16:0} , anteiso-C _{15:0} , anteiso-C _{17:0} , iso-C _{15:0}	iso-C _{16:0} , anteiso-C _{15:0} , anteiso-C _{17:0} , iso-C _{15:0} , iso-C _{17:1} ω8c	iso-C _{16:0} , C _{17:1} ω8c, C _{17:0} , anteiso-C _{17:0} , iso-C _{15:0} , C _{15:0} , anteiso-C _{15:0}	iso-C _{16:0} , anteiso-C _{15:0} , anteiso-C _{17:0} , iso-C _{17:1}
Fatty acid type	3b	3b	3b	2a
Menaquinone composition	MK-10(H ₄), MK-10(H ₆), MK-10(H ₈)	MK-10(H ₄), MK-10(H ₆), MK-10(H ₈), MK-9(H ₄), MK-9(H ₆), MK-9(H ₂)	ND	MK-10(H ₆), MK-10(H ₄), MK-9(H ₄), MK-9(H ₆)
DNA G + C content (mol%)	71	71.5	ND	70.2

*Data from the present study.

†Data from Thawai *et al.* (2008).

‡Data from Tamura *et al.* (2006).

Table 2. Differential characteristics of strain GUI 15^T and its closest phylogenetic relatives

All strains were positive for alkaline phosphatase, catalase, esterase (C4), esterase lipase (C8), gelatin hydrolysis, α-glucosidase and naphthol-AS-BI-phosphohydrolase. All were negative for α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase, nitrate reduction, urease, and fermentation of glucose, glycogen, lactose, maltose, mannitol, sucrose and xylose. +, Positive; –, negative; w, weakly positive. Data are from the present study unless indicated otherwise.

Characteristic	Strain GUI 15 ^T	<i>P. rubra</i> TT 97-42 ^T	<i>Micromonospora pattaloongensis</i> TJ2-2 ^T	<i>Micromonospora olivasterospora</i> DSM 43868 ^T
Colony colour (ISP 2)	Pale yellow	Reddish orange	Yellowish white	Olive green
API Coryne test results				
Pyrazinamidase	–	+	+	+
Pyrrolidonyl arylamidase	+	–	–	–
Alkaline phosphatase	+	–	+	+
β-Galactosidase	+	–	–	–
N-Acetyl-β-glucosaminidase	–	+	+	+
Aesculin	+	–	+	+
API ZYM test results				
Lipase (C14)	–	+	–	+
Leucine arylamidase	–	+	–	+
Valine arylamidase	–	+	w	+
Cystine arylamidase	–	+	w	+
Trypsin	+	+	–	+
α-Chymotrypsin	–	+	–	–
Acid phosphatase	+	–	+	+
β-Glucosidase	–	+	+	+
Carbon source utilization				
Mannitol	+	+*	–†	–
Rhamnose	+	+*	–	–
Raffinose	–	+	–	–
Maximum NaCl tolerance (%)	<2	<4*	<3†	<4‡
Temperature range for growth (°C)	20–37	20–37*	25–30†	28–38‡

*Data from Tamura *et al.* (2006).

†Data from Thawai *et al.* (2008).

‡Data from Kawamoto *et al.* (1983).

out for strain GUI 15^T, *Micromonospora pattaloongensis* TJ2-2^T, *Micromonospora olivasterospora* DSM 43868^T and *P. rubra* TT 97-42^T.

Strain GUI 15^T presented a phenotypic profile that clearly distinguished it from its closest phylogenetic neighbours (Table 2). In particular, it could be differentiated based on substrate mycelium colour, NaCl tolerance, temperature growth range, and production of pyrazinamidase, β -galactosidase, β -glucosidase and pyrrolidonyl arylamidase. A full description of the physiological properties determined for strain GUI 15^T is provided in the species description.

In summary, the phenotypic and genotypic data presented indicate that strain GUI 15^T represents a novel species of the genus *Micromonospora*, for which we propose the name *Micromonospora pisi* sp. nov. Further investigation will be necessary to determine whether *P. rubra* TT 97-42^T is sufficiently different to warrant representing a species distinct from members of the genus *Micromonospora*.

Description of *Micromonospora pisi* sp. nov.

Micromonospora pisi (pi'si. L. n. *pisum* a species of leguminous plant, *pease* the pea, and also a scientific genus name; L. gen. n. *psi*, of *Pisum*, isolated from *Pisum sativum*).

Gram-positive, non-acid-fast actinobacterium that shows extensive branching, non-fragmenting substrate hyphae. Smooth, non-motile single spores are produced at the tip of the hyphae although pairs of spores are also observed. Strictly aerobic and chemo-organotrophic. Colonies are beige to pale yellow, raised and folded on ISP 2 agar. Aerial mycelium is absent. Diffusible pigments are not produced. Grows at pH 7–9 and 20–37 °C (optimum growth at 28 °C). Oxidase- and catalase-positive. Aesculin, arbutin, casein, gelatin, starch and xylan are degraded, but Tween 20, Tween 80, tyrosine and urea are not. The following substrates are used as carbon sources: L-alanine, (+)-L-arabinose, L-arginine, (+)-D-cellobiose, (–)-D-fructose, (+)-D-galactose, gluconate, (+)-D-glucose, L-histidine, (+)-D-mannose, (+)-D-melibiose, pyruvate, (+)-L-rhamnose, (–)-D-salicin, starch, sucrose and (+)-D-xylose. The following substrates are not used as carbon sources: (+)-D-galacturonic acid, L-lysine, (+)-D-maltose, (+)-D-melezitose, *meso*-erythritol, L-proline, propionic acid, (–)-D-quinic acid, (+)-D-raffinose, sebacic acid, L-serine, sorbitol, L-sorbose, (+)-D-trehalose, L-valine and xylitol. Enzyme profiles based on the API ZYM and API Coryne systems are given in Table 2. Contains *meso*-diaminopimelic acid in its cell wall. Whole-cell sugars present are galactose, mannose, ribose and xylose. Major fatty acids are iso-C_{16:0}, anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{15:0}. The main menaquinone is MK-10(H₄). The DNA G+C content of the type strain is 71 mol%.

The type strain, GUI 15^T (=DSM 45175^T=LMG 24546^T), was isolated from root nodules of *Pisum sativum*.

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