Micromonospora cremea sp. nov. and Micromonospora zamorensis sp. nov., isolated from the rhizosphere of Pisum sativum

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Three actinobacterial strains, CR30T, CR36 and CR38T, were isolated from rhizosphere soil of Pisum sativum plants collected in Spain. The strains were filamentous, Gram-stain-positive and produced single spores. Phylogenetic, chemotaxonomic and morphological analyses confirmed that the three strains belonged to the genus Micromonospora. 16S rRNA gene sequence analysis of strains CR30T and CR36 showed a close relationship to Micromonospora coriariae NAR01T (99.3 % similarity) while strain CR38T had a similarity of 99.0 % with Micromonospora saelicesensis Lupac 09T. In addition, gyrB gene phylogeny clearly differentiated the novel isolates from recognized Micromonospora species. DNA–DNA hybridization, BOX-PCR and ARDRA profiles confirmed that these strains represent novel genomic species. The cell-wall peptidoglycan of strains CR30T and CR38T contained meso-diaminopimelic acid. Both strains had MK-10(H4) as the main menaquinone and a phospholipid type II pattern. An array of physiological tests also differentiated the isolates from their closest neighbours. Considering all the data obtained, it is proposed that strains CR30T and CR36 represent a novel species under the name Micromonospora cremea sp. nov. (type strain CR30T = CECT 7891T = DSM 45599T), whereas CR38T represents a second novel species, for which the name Micromonospora zamorensis sp. nov. is proposed, with CR38T (= CECT 7892T = DSM 45600T) as the type strain.

Recent reports on the isolation of novel members of the genus Micromonospora from diverse habitats (de Menezes et al., 2008; Kirby & Meyers, 2010; Maldonado et al., 2008; Thawai et al., 2005, 2007; Tanasupawat et al., 2010; Trujillo et al., 2010; Wang et al., 2011) indicate the ability of these micro-organisms to adapt to very different niches, highlighting their metabolic diversity. The genus Micromonospora has moved from a ‘forgotten-status’ with 14 species described in 2000 (Kasai et al., 2000) to 43 at the present time (Euzéby, 2011). The results of a detailed taxonomic study of three novel isolates support the conclusion that these strains represent two novel species of the genus Micromonospora.

Abbreviation: ARDRA, amplified rDNA restriction analysis.

The GenBank/EMBL/DDJB accession numbers for the 16S rRNA/gyrB gene sequences of strains CR30T, CR36 and CR38T are FN658654/FN662533, FN658655/FN662534 and FN658656/FN662535, respectively.

Two supplementary tables and three supplementary figures are available with the online version of this paper.
non-motile and spherical to oval spores (0.7–1.0 μm).

The colony colour of isolates CR30^T and CR36 was cream and grew well on Bennett’s, SA1 and ISP 2 (1981) using 4-day-old cultures grown on SA1 agar. The three isolates grew well on Bennett’s, SA1, Bennett’s (Jones, 1949) and yeast extract-mannitol (YMA) agars (Vincent, 1970). Cell morphology and spore motility were observed by phase-contrast microscopy (Leica; CTR MIC). The Gram stain reaction was performed following the protocol of Doetsch (1981) using 4-day-old cultures grown on SA1 agar.

The three isolates grew well on Bennett’s, SA1 and ISP 2 agar, but moderately to poorly on ISP 3 and YMA agar. The colony colour of isolates CR30^T and CR36 was cream on SA1 agar and cream to orange on the remaining media tested. Strain CR38^T was orange on all media tested and also produced an orange diffusible pigment. A summary of the cultural characteristics of all strains is given in Table S1 (available in IJSEM Online). All strains produced extensively branched, non-fragmenting substrate hyphae (0.3–0.5 μm in width), but no aerial mycelium was seen. Single, non-motile and spherical to oval spores (0.7–1.0 μm) were observed in strains CR30^T and CR38^T. Spore surface ornamentation was warty (CR30^T) and smooth (CR38^T). The Gram stain reaction was positive.

DNA extraction, PCR amplification and sequencing of the 16S rRNA and gyrB genes were performed as described previously (Garcia et al., 2010). The sequences obtained were compared with those deposited in the public databases and pairwise distances were calculated with the EzTaxon server (Kim et al., 2012). The sequences of the three isolates and those of their most closely related taxa retrieved from GenBank were aligned using the CLUSTAL X program (Thompson et al., 1997) and checked for alignment inconsistencies. Phylogenetic distances were calculated with Kimura’s two-parameter model (Kimura, 1980) and evolutionary trees were inferred using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. Phylogenetic analyses were performed using the software packages MEGA4 (Tamura et al., 2007) and ARB (Ludwig et al., 2004). Branch support based on 1000 replications was calculated to evaluate the various tree topologies.

Nearly complete 16S rRNA gene sequences (1450–1452 bp) were obtained for the novel strains. Identification of the closest phylogenetic neighbours confirmed their phylogenetic affiliation to the genus Micromonospora. Pairwise sequence similarities calculated via the EzTaxon server (Kim et al., 2012) between the novel isolates and the type strains of recognized Micromonospora species ranged from 96.8 to 99.3 %. Strains CR30^T and CR36, which exhibited 100 % 16S rRNA gene sequence similarity, had 99.3, 99 and 98.9 % 16S rRNA gene sequence similarities with Micromonospora coriariae NAR01^T, Micromonospora endolithica DSM 44398^T and Micromonospora chersina DSM 44151^T, respectively. Strain CR38^T had sequence similarities of 99, 98.5 and 98.3 % with Micromonospora saelicesensis Lupac 09^T, Micromonospora chokoriensis 2-19(6)^T and Micromonospora lupini Lupac 14N^T, respectively. The phylogenetic position of the three isolates in relation to the 43 recognized Micromonospora species based on the neighbour-joining method is shown in Fig. 1. A bootstrap value of 98 % supported the clade in which strains CR30^T, CR36 and M. coriariae DSM 44875^T were recovered. In the case of isolate CR38^T, it formed a cluster with M. saelicesensis Lupac 09^T with a bootstrap value of 80 %. The results of phylogenetic analyses using other tree-making algorithms were very similar (data not shown) and common branches are marked in Fig. 1.

The gyrB gene is proving to be a useful marker for the delineation of Micromonospora genomic species, as shown by recent reports (Garcia et al., 2010; Wang et al., 2011; Zhang et al., 2012). Partial sequences of this gene were obtained (1051–1112 bp) and the gyrB gene sequence similarities between the novel isolates and available gyrB gene sequences for Micromonospora type strains ranged from 89.1 to 96.9 %. Strains CR30^T and CR36 (100 %) had a gyrB gene sequence similarity of 95.1 % with M. chokoriensis 2-19(6)^T; strain CR38^T and M. lupini Lupac 14N^T and M. saelicesensis Lupac 09^T shared gyrB gene sequence similarities of 96.9 and 96.5 %, respectively. The gyrB gene phylogenetic tree constructed using the neighbour-joining method shows the strains forming independent clades from the rest of the type strains analysed (Fig. S1).

The phylogenetic tree based on the gyrB gene sequences confirmed the relationship between strain CR30^T and M. saelicesensis Lupac 09^T, but indicated that isolates CR30^T and CR36 were more closely related to M. chokoriensis 2-19(6)^T than to M. coriariae NAR01^T. Other studies have also reported similar results, i.e. that the topology of phylogenetic trees based on gyrB gene sequences differed from those based on 16S rRNA gene sequence data (Kasai et al., 2000; Kirby & Meyers, 2010; Wang et al., 2011). Recently, a multilocus sequence analysis scheme was developed to address the phylogeny of the genus Micromonospora using the genes 16S rRNA, atpD, gyrB, recA and rpoB. This study, which included isolates CR30^T and CR36, clearly supported the close relationship of the two strains to M. coriariae NAR01^T. In all cases (except for the gyrB gene), single and concatenated gene sequence analyses produced phylogenetic trees in which strains CR30^T and CR36 were more closely related to M. coriariae NAR01^T than to M. chokoriensis 2-19(6)^T (Carro et al., 2012).

To investigate the genetic diversity of the three isolates, BOX-PCR and ARDRA (amplified rDNA restriction analysis) were carried out. DNA was extracted using the REDExtract-N-Amp Plant PCR kit (Sigma). BOX-PCR was performed as described previously (Trujillo et al., 2010). For ARDRA profiles, samples (4 μl) of amplified 16S rRNA gene products were separately digested overnight with the endonucleases PstI (5’-CTGCAG-3’), Hin6I (5’-GGCC-3’), Bsh1285I (5’-CGPUPyCG-3’) and FspBI (5’-CTAG-3’) according to the manufacturer’s protocols (Thermo Scientific). Digested samples were loaded on a 2 % agarose gel containing 0.5 μg ethidium bromide ml⁻¹ and electrophoresis was run in 1 x TBE buffer at 75 V for 2 h (Bio-Rad powerPac 300 power supply). The four restriction profiles were compared with those deposited in the public databases, and the relationships of the novel isolates to other Micromonospora species were determined. BOX-PCR and ARDRA profiles were separately digested overnight with the endonucleases PstI, Hin6I, Bsh1285I and FspBI. Digested samples were loaded on a 2 % agarose gel and the results were compared with those deposited in the public databases.
Ley measurements were obtained by spectrophotometry (De et al., 1970; Huß et al., 1977). Hybridization was done extracted and purified according to Minnikin et al. (1983). Hybridization was done

Cluster analysis of ARDRA profiles revealed two groups at the 75 % similarity level with strains CR30T and CR36 sharing 97 % identity (Fig. S2a), while strain CR38T formed an independent branch. The BOX-PCR profiles differentiated the isolates at the strain level, indicating that they were not clones (Fig. S2b).

DNA–DNA hybridization experiments were carried out between isolate CR30T and M. cortiariae NAR01T and M. endolithica DSM 44398T, and between isolate CR38T and M. saelicesensis Lupac 09T. DNA was isolated and purified on hydroxypatite (Cashion et al., 1977) and reassocation measurements were obtained by spectrophotometry (De Ley et al., 1970; Huß et al., 1983). Hybridization was done in 2 × SCC and 10 % formamide at 70 °C. The DNA G+C contents of all isolates were determined by the thermal melting method (Mandel & Marmur, 1968).

Values of 19.3 ± 6 and 53 ± 0.6 % DNA–DNA relatedness were obtained between strain CR30T and M. endolithica DSM 44398T and M. cortiariae NAR01T, respectively; strain CR38T and M. saelicesensis Lupac 09T showed a value of 41.6 ± 8 %. In both cases, the values warrant the proposal of novel genomic species. The DNA G+C contents of strains CR30T and CR38T were 72.4 and 71.8 mol%, respectively.

Biomass for chemotaxonomic analyses was obtained in ISP 2 broth in flasks shaken 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 Staneck & Roberts (1974). Menaquiones and polar lipids were extracted and purified according to Minnkin et al. (1984).

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strains CR30T, CR36 and CR38T and current species recognized in the genus Micromonospora. A total of 1371 nt was analysed. Asterisks indicate that the corresponding nodes were also recovered in the maximum-likelihood tree. Bar, 0.002 substitutions per nucleotide position.
Menaquinones were analysed by HPLC and polar lipids were separated and identified by two-dimensional TLC. TLC plates were developed with anisaldehyde, ninhydrin and molybdenum blue reagents. Methyl esters of cellular fatty acids for the new isolates and *M. coriariae* NAR01<sup>T</sup> and *M. saelicesensis* Lupac 09<sup>T</sup> were prepared from cells grown for 3–6 days in trypticase soy broth at 28 °C under shaking conditions until good growth was obtained (Sasser, 1990). Samples were analysed by GLC using the standard MIDI system (Sherlock version 4.5) (Schröder *et al.*, 1997) and peaks were named using the database ACTINO (http://www.actinobase.in/).

The chemotaxonomic markers analysed confirmed the phylogenetic identification of the novel strains in the genus *Micromonospora*. The fatty acid pattern of the isolates was composed of saturated and unsaturated iso- and anteiso-branched fatty acids, which are characteristic of other species of the genus. However, an important difference was found between *M. coriariae* NAR01<sup>T</sup> and the other strains in the amount of 10-methyl C<sub>17:0</sub> (20.09 %); a similar value was reported previously for the same strain (Trujillo *et al.*, 2006). The amount of iso-C<sub>16:0</sub> in strain CR38<sup>T</sup> was significantly lower (4.87%) compared with the rest of the strains (12.79–36.48%). Fatty acid profiles are given in Table S2.

The major menaquinone found in strains CR30<sup>T</sup> and CR38<sup>T</sup> was MK-10(H<sub>4</sub>) in various amounts: 34 % in strain CR30<sup>T</sup> and 69 % in strain CR38<sup>T</sup>. In addition, strain CR30<sup>T</sup> contained MK-9(H<sub>4</sub>) (30 %), MK-9(H<sub>6</sub>) (18 %) and MK-10(H<sub>4</sub>) (18 %). Strain CR38<sup>T</sup> included MK-10(H<sub>4</sub>) (20 %), MK-9(H<sub>4</sub>) (5 %), MK-10(H<sub>2</sub>) (3 %) and MK-10(H<sub>6</sub>) (3 %). MK-10(H<sub>4</sub>) was also present in large amounts in *M. coriariae* NAR01<sup>T</sup> and *M. saelicesensis* Lupac 09<sup>T</sup>. The overall menaquinone compositions of the two novel strains allowed a clear differentiation between them and their closest phylogenetic relatives, *M. coriariae* and *M. saelicesensis* (Table 1).

The cell wall of strains CR30<sup>T</sup> and CR38<sup>T</sup> contained meso-diaminopimelic acid and the whole-cell sugars glucose, mannose, ribose and xylose. In addition, strain CR30<sup>T</sup> contained galactose and rhamnose. The phospholipids detected in both strains were diphosphatidylglycerol, phosphatidylinositol and phosphatidylethanolamine (Fig. S3), the last being the diagnostic phospholipid for microorganisms with a phospholipid type II (Lechevalier *et al.*, 1977). This pattern is typical of the genus *Micromonospora*. Unidentified phosphoglycolipids were also detected.

The three novel isolates and the type strains of *M. chersina*, *M. coriariae*, *M. endolitica*, *M. lupini* and *M. saelicesensis* were studied for the following tests: oxidation and catalase production (Trujillo *et al.*, 2006); degradation of various organic compounds (Trujillo *et al.*, 2005); carbon substrate utilization (Williams *et al.*, 1983); growth at different temperatures (4, 10, 20, 28, 37 and 45 °C), NaCl concentrations (1, 3, 5, 7 and 9 %, w/v) and pH values (4.5, 5.5, 6.5, 8.0 and 9.0). Where appropriate, SA1 agar was used as the basal medium. In the case of pH measurement, the medium was adjusted using appropriate buffer solutions (McIlvaine, 1921; Bates & Bower, 1956). API ZYM and API Coryne kits (bioMérieux) were used according to the manufacturer’s recommendations to determine various enzyme reactions.

Table 1 lists several tests that can be used to differentiate the three novel strains and their closest relatives. NaCl tolerance can be considered an important test to differentiate the novel isolates and their closest phylogenetic neighbours. In addition, arbutin was not degraded by strain CR30<sup>T</sup>, while only a weak reaction was detected for strain CR36 and complete degradation was carried out by *M. coriariae*, the closest related species. Strains CR30<sup>T</sup> and CR36 were also characterized by the ability to use L-lysine and m-erythritol as sole carbon sources. Strains CR38<sup>T</sup> and *M. saelicesensis* Lupac 09<sup>T</sup> could also be differentiated by their ability to grow at 37 and 45 °C, respectively, and by the production of urease by *M. saelicesensis*. The carbon sources gluconate, propionic acid, rhamnose and trehalose were used by isolate CR38<sup>T</sup> but not by *M. saelicesensis*.

The overall results presented strongly support the proposal that strains CR30<sup>T</sup>, CR36 and CR38<sup>T</sup> represent two novel species in the genus *Micromonospora*. We propose the names *Micromonospora cremea* sp. nov., represented by strains CR30<sup>T</sup> and CR36, and *Micromonospora zamorensis* sp. nov., represented by strain CR38<sup>T</sup>.

**Description of *Micromonospora cremea* sp. nov.**

*Micromonospora cremea* (cre’me.a. N.L. fem. adj. cremea cream-coloured, referring to the cream colour of the substrate mycelium).

Gram-stain-positive, chemo-organotrophic, aerobic actinobacterium. Produces extensively branched, non-fragmenting substrate mycelium (0.3–0.5 μm in width), but no aerial hyphae. Single, non-motile and spherical to oval spores (0.7–1.0 μm) with a warty surface are produced. Colonies are cream on SA1 agar and cream to orange on ISP 2 agar; diffusible pigments are not observed. Colonies are raised and folded. Growth occurs at 10–37 °C, at pH 7–8 and with 1 % (w/v) NaCl. Catalase- and oxidase-positive. Degrades gelatin and Tween 80. Variable for the degradation of casein, starch, urea, xylan (type strain is positive), Tween 20 and L-tyrosine (type strain is negative). Urea is not degraded. L-Arginine, fructose, glucose, maltose, mannose, raffinose, sucrose, salicin, L-serine and starch are used as carbon sources, but not galacturonic acid, glutaric acid, quinic acid, L-histidine, sorbitol, L-tyrosine, xylitol or xylose. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, β-glucoamidase and N-acetyl-β-glucosaminidase. Other characteristics are given in Table 1. Contains *meso*-diaminopimelic acid in its cell wall. Whole-cell sugars are galactose, glucose, mannose, rhamnose, ribose and xylose. Major fatty acids are iso-C<sub>15:0</sub> 3-oxo, iso-C<sub>16:0</sub>, anteiso-C<sub>15:0</sub> and anteiso-C<sub>17:0</sub>. Menaquinone composition includes MK-10(H<sub>4</sub>), MK-9(H<sub>4</sub>), MK-9(H<sub>6</sub>).
Table 1. Differential characteristics of strains CR30\textsuperscript{T}, CR36 and CR38\textsuperscript{T} and their closest phylogenetic relatives in the genus *Micromonospora*

| Strains: 1, CR30\textsuperscript{T}; 2, CR36; 3, *M. coriariae* NAR01\textsuperscript{T}; 4, *M. endolithica* DSM 44398\textsuperscript{T}; 5, *M. chersina* DSM 44151\textsuperscript{T}; 6, CR38\textsuperscript{T}; 7, *M. saelicesensis* Lupac 09\textsuperscript{T}; 8, *M. lupini* Lupac 14N\textsuperscript{T}. Data are from the present study, except for menaquinone composition for *M. coriariae* NAR01\textsuperscript{T} (Trujillo et al., 2006), *M. endolithica* DSM 44398\textsuperscript{T} (Hirsch et al., 2004), and *M. lupini* Lupac 14N\textsuperscript{T} and *M. saelicesensis* Lupac 09\textsuperscript{T} (Trujillo et al., 2007). +, Positive; −, negative; w, weak; ND, not determined.

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<td>−</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Sorbose</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>l-Valine</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Main menaquinones</td>
<td>MK-10(H\textsubscript{4}), MK-9(H\textsubscript{4}), MK-9(H\textsubscript{6}), MK-10(H\textsubscript{6}), ND</td>
<td>MK-10(H\textsubscript{4}), MK-9(H\textsubscript{4}), MK-9(H\textsubscript{6}), MK-10(H\textsubscript{6})</td>
<td>MK-10(H\textsubscript{4}), MK-9(H\textsubscript{4}), MK-9(H\textsubscript{6}), MK-10(H\textsubscript{6})</td>
<td>MK-10(H\textsubscript{4}), MK-9(H\textsubscript{6}), MK-10(H\textsubscript{6})</td>
<td>MK-10(H\textsubscript{4}), MK-9(H\textsubscript{6}), MK-10(H\textsubscript{6})</td>
<td>MK-10(H\textsubscript{4}), MK-9(H\textsubscript{6}), MK-10(H\textsubscript{6})</td>
<td>MK-10(H\textsubscript{4}), MK-9(H\textsubscript{6}), MK-10(H\textsubscript{6})</td>
<td>MK-10(H\textsubscript{4}), MK-9(H\textsubscript{6}), MK-10(H\textsubscript{6})</td>
</tr>
</tbody>
</table>
and MK-10(H₈). Presents a type II polar lipid pattern with phosphatidylethanolamine as the diagnostic lipid.

The type strain, CR30T (=CECT 7891T = DSM 45599T), and an additional isolate, CR36, were isolated from the rhizosphere of a Pisum sativum plant collected in Zamora, Spain. The DNA G+C content of the type strain is 72.4 mol%.

**Description of *Micromonospora zamorensis* sp. nov.**

*Micromonospora zamorensis* (za.mo.ren’sis. N.L. fem. adj. zamorensis of or belonging to Zamora, from where the type strain was isolated).

Gram-stain-positive, chemo-organotrophic, aerobic actinobacterium. Produces extensively branched, non-fragmenting substrate mycelium (0.3–0.5 μm in width), but no aerial hyphae. Single, non-motile and spherical to oval spores (0.7–1.0 μm) with a smooth surface are produced. Colonies are orange to bright orange on ISP 2, ISP 3 and SA1 agars; an orange pigment is also produced in SA1 medium. Colonies are raised and folded. Growth occurs at 10–37 °C, at pH 6.5–9.0 and with 3 % (w/v) NaCl. Catalase- and oxidase-positive. Degradates starch, casein, gelatin, Tween 20, Tween 80, L-tyrosine and xylan, but not urea; arbutin is weakly degraded. L-Arabinose, L-arginine, fructose, glucose, maltose, mannose, L-proline, raffinose, sucrose, salicin, L-serine and starch are used as carbon sources, but not galacturonic acid, glutaric acid, quinic acid, L-histidine, sorbitol, L-tyrosine, xylitol or xylose. Positive for alkaline phosphatase, phosphomonoesterase (C4), esterase lipase (C8), lipase, leucine arylamidase and β-glucosidase and N-acetyl-β-glucosaminidase. Other characteristics are given in Table 1. Contains meso-diaminopimelic acid in its cell wall. Whole-cell sugars are glucose, mannose, ribose and xylose. Major fatty acids are iso-C₁₅:0, anteiso-C₁₅:0 and C₁₇:0 3-OH. Menaquinone composition includes MK-10(H₄), MK-10(H₈), MK-9(H₄), MK-10(H₂) and MK-10(H₈). Presents a type II lipid pattern with phosphatidylethanolamine as the diagnostic lipid.

The type strain, CR38T (=CECT 7291T = DSM 45600T), was isolated from the rhizosphere of a Pisum sativum plant collected in Zamora, Spain. The DNA G+C content of the type strain is 71.8 mol%.

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**References**


