Micromonospora mirobrigensis sp. nov.

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An actinomycete strain was recovered from a pond where radon is known to be dissolved. A polyphasic study was undertaken to identify the new isolate. The 16S rRNA gene sequence of strain WA201^T showed closest similarity to the type strains of *Micromonospora carbonacea* (98.5 %) and *Micromonospora matsumotoense* (98.1 %). The chemotaxonomic results confirmed the taxonomic position of the isolate in the genus *Micromonospora*. DNA–DNA relatedness values supported the classification of this isolate as a novel species. A number of physiological and biochemical tests were able to distinguish strain WA201^T from its closest phylogenetic neighbours. Therefore, it is proposed that isolate WA201^T (= DSM 44830^T = LMG 22229^T) be considered the type strain representing a novel species, *Micromonospora mirobrigensis* sp. nov.

Representatives of the genus *Micromonospora* (Ørskov, 1923) are reported to inhabit soil, water, marine environments and sediments (Lüdemann & Brodsky, 1963; Kawamoto, 1989). *Micromonospora endolithica*, isolated from an extreme Antarctic sandstone environment, has been described recently (Hirsch *et al.*, 2004), showing that this genus is distributed in very different environments. Kasai *et al.* (2000) redefined and reduced the genus to 14 species based on DNA–DNA relatedness, 16S rRNA and *gyrB* phylogenetic data. We report the classification of an organism representing a novel *Micromonospora* species.

Isolate WA201^T was recovered from a water sample of a pond, located on a former uranium mine (Ciudad Rodrigo, Spain) where radon is known to be dissolved in water (Lozano *et al.*, 2002). The isolation procedure using soil extract agar pH 6.5 at 28 °C was described previously by Trujillo *et al.* (2004). Long-term maintenance of strain WA201^T was accomplished by storage in glycerol suspension (20 %, w/v) at –80 °C. The isolate was cultured on Bennett's agar, glucose-yeast extract agar and nutrient agar to check for growth rate. Growth was slow on these media and therefore a basal medium (SA1) was designed to

improve it. SA1 contained glucose, 10 g; yeast extract (Difco), 3 g; tryptone (Difco), 5 g; tryptose (Difco), 2 g; starch (Fluka), 2 g; CaCO₃, 100 mg; CoCl₂, traces; ferric citrate, traces; Bacto-agar (Difco), 18 g; and distilled water, 1 l. The pure culture was then routinely grown on SA1 agar.

Extraction of genomic DNA, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were described previously (Rivas *et al.*, 2003). The sequence of isolate WA201^T was manually aligned and compared with representative sequences of members of the order *Actinomycetales* obtained from GenBank/EMBL. Phylogenetic distances were calculated with the Kimura two-parameter model and tree topologies were inferred using the least-squares (De Soete, 1983), maximum-parsimony (Fitch, 1972) and neighbour-joining methods (Saitou & Nei, 1987). One thousand bootstrap replications were performed using the MEGA program as described by Kumar *et al.* (2001).

Morphological features were studied on glucose-yeast extract agar, nutrient agar and SA1 agar at 28 °C. Cell morphology and motility were observed by phase-contrast microscopy using 5-day-old cultures. Spore production was examined on 3-week-old cultures on SA1 agar using a scanning electron microscope (Zeiss, DSM 940). Agar plugs were fixed overnight in phosphate buffer (pH 7.0) which contained 2 % paraformaldehyde and 0.2 % glutaraldehyde, dehydrated through a graded ethanol series, critical-point dried and sputter-coated with gold. The Gram and acid-fast stains (Doetsch, 1981) were performed using 3-day-old cultures.

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Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WA201^T is AJ626950.

Cellular fatty acid profiles and an extended phylogenetic dendrogram are available as supplementary material in IJSEM Online.

The ability of isolate WA201^T to grow on a range of sole carbon sources at 1% (w/v) was determined according to Williams *et al.* (1983) with agar and a nitrogen base without amino acids (Difco). NaCl tolerance and temperature (4–45 °C) growth ranges were determined on SA1 medium.

Isolate WA201^T and *Micromonospora carbonacea* DSM 43168^T were also characterized using the API 20NE and API ZYM systems (bioMérieux) according to the manufacturer's instructions and incubation of the strips for 48 h at 28 °C. Catalase and oxidase activity were recorded as described previously (Rivas *et al.*, 2003). Hydrolysis of casein (1% skimmed milk), aesculin, arbutin, starch (1% w/v), tyrosine (0.5%) and xylan (0.4%) were tested on SA1 as the basal medium.

Susceptibility to various antibiotics was examined for the isolate and *M. carbonacea* DSM 43168^T using amikacin (30 µg), amoxicillin/clavulanic acid (20/10 µg), ampicillin (2 µg), cefaclor (30 µg), cefazolin (30 µg), ciprofloxacin (5 µg), cloxacillin (1 µg), cefuroxime (30 µg), erythromycin (2 and 15 µg), gentamicin (10 µg), neomycin (5 µg), netilmicin (30 µg), oxacillin (1 µg), oxytetracycline (30 µg), penicillin G (10 U), piperacillin (100 µg), polymyxin B (300 IU), tetracycline (30 µg) and vancomycin (30 µg) disks (Oxoid), and SA1 as the basal medium. Readings were taken at 3, 5 and 10 days.

Isomers of diaminopimelic acid (DAP) 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 the method of Minnikin *et al.* (1984) and analysed by HPLC (Hewlett Packard 1100). Methyl esters of cellular fatty acids 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). The DNA G+C content was determined using the thermal melting method (Mandel & Marmur, 1968).

DNA–DNA relatedness was measured spectrophotometrically between isolate WA201^T and *M. carbonacea* DSM 43168^T following the method of De Ley *et al.* (1970) with the modification of Huß *et al.* (1983). DNA was purified on hydroxyapatite as described by Cashion *et al.* (1977). Renaturation rates were calculated using the TRANSFER.BAS program of Jahnke (1992).

The 16S rRNA gene sequence of isolate WA201^T (1517 nt) showed a close relationship with members of the family *Micromonosporaceae* and fell within the genus *Micromonospora* clade. The highest sequence similarities found were with *M. carbonacea* DSM 43168^T (98.5%) and *Micromonospora matsumotoense* IFO 14550^T (98.1%), which correspond to 22 and 28 nucleotide differences (1473 nucleotides compared), respectively. The various tree-making algorithms yielded similar tree topologies

although some of the branches varied slightly, but isolate WA201^T was always recovered in the same branch as *M. carbonacea* DSM 43168^T. The closest phylogenetic relatives of strain WA201^T are represented on the tree corresponding to the Kimura two-parameter method and the least-squares method (Fig. 1). An extended phylogenetic dendrogram is provided as Supplementary Fig. A in IJSEM Online.

Growth of strain WA201^T was slow on glucose-yeast extract agar and nutrient agar, where small colonies appeared only after 5 days, compared with SA1 agar, where good growth was observed after 2 days. Colonies on the three media were orange, turning dark brown on sporulation after 10–15 days; no diffusible pigments were produced on these media. Colonies on SA1 medium were raised, folded and produced a well-developed substrate mycelium (0.4–0.6 µm diameter), but lacked aerial hyphae. The formation of single, non-motile, spherical spores (0.7–0.9 µm) was observed. The spore surface was slightly warty and most of them occurred as short sporophores.

Strain WA201^T grew at between 20 and 37 °C, but no growth was recorded at 4, 10, 15 or 45 °C after 3 weeks. Tolerates NaCl up to 3%; no growth occurred at 5%. Nitrate was not reduced. The isolate hydrolysed aesculin, arbutin, casein, gelatin, starch and xylan.

WA201^T and *M. carbonacea* DSM 43168^T produced the following enzymes (API ZYM kit): alkaline phosphatase, esterase lipase (C8), lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase.

The above strains produced identical antibiotic profiles, which showed resistance to ampicillin (2 µg) and susceptibility to amikacin (30 µg), amoxicillin/clavulanic acid (20/10 µg), cefaclor (30 µg), cefuroxime (30 µg), cefazolin (30 µg), ciprofloxacin (5 µg), cloxacillin (1 µg), erythromycin (2 and 15 µg), gentamicin (10 µg), neomycin (5 µg),

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences showing the relationship of *M. miobrigensis* sp. nov. WA201^T and related *Micromonospora* species. Numbers at branching points indicate percentages of occurrence using least-squares analysis applying bootstrap values above 60% based on 1000 replications.

netilmicin (30 µg), oxytetracycline (30 µg), penicillin G (10 U), piperacillin (100 µg), polymyxin B (300 IU), oxacillin (1 µg), tetracycline (30 µg) and vancomycin (30 µg).

Various physiological and biochemical results useful to differentiate between isolate WA201^T and its closest phylogenetic neighbours *M. carbonacea* DSM 43168^T and *M. matsumotoense* IFO 14550^T are given in Table 1. Other results are presented in the species description.

Strain WA201^T contained *meso*-DAP, which is the characteristic diamino acid of the peptidoglycan type A1γ (Schleifer & Kandler, 1972). Glucose in large amounts, galactose, mannose and xylose were found as whole-cell sugars. The fatty acid pattern for the isolate was composed of saturated and unsaturated iso/anteiso-branched fatty acids. A significant amount of 10-methyl-branched 17:0 (5.24 %) was found in addition. Straight-chain saturated and unsaturated fatty acids were present only in minor amounts (Supplementary Table A). The main menaquinones were MK-10(H₄) (77 %) and MK-10(H₆) (23 %). A small amount of MK-9(H₄) (5 %) was also found. MK-10(H₂) and MK-9(H₆) occurred only as traces. This menaquinone profile differs from that reported for *M. carbonacea* DSM 43168^T, which contains MK-9(H₄) in large amounts (Kawamoto, 1989), while *M. matsumotoense* IFO 14550^T contains MK-10(H₆), MK-10(H₈) and MK-10(H₄) in a ratio of 1.9:1.8:1 (Lee *et al.*, 1999). The menaquinone composition within the genus *Micromonospora* has been reported to be complex and heterogeneous in comparison to the menaquinone composition found in other actinomycete genera, which show similar high levels of intragenetic relatedness (Koch *et al.*, 1996). The polar lipid pattern was mainly composed of phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and the

diagnostic phosphatidylethanolamine. Some glycolipids of unknown structure were also found. This pattern correlates with phospholipid pattern II *sensu* Lechevalier *et al.* (1977). The G+C content of the DNA was 68.6 mol%. Most chemotaxonomic markers support the placement of isolate WA201^T in the genus *Micromonospora*.

The DNA–DNA relatedness value (36.5 %) clearly indicated that isolate WA201^T does not belong to the species *M. carbonacea*, as this value is well below the threshold value of 70 % for definition of bacterial species according to Wayne *et al.* (1987).

The chemical, morphological and phylogenetic data suggest that strain WA201^T represents a novel species when compared with the type strains of species with validly published names within the genus *Micromonospora*. Thus, on the basis of this polyphasic taxonomic study, strain WA201^T merits classification as a novel species within the genus *Micromonospora* and the name *Micromonospora mirobrigensis* sp. nov. is proposed.

Description of *Micromonospora mirobrigensis* sp. nov.

Micromonospora mirobrigensis (mi.ro.bri.gen'sis. N.L. fem. adj. *mirobrigensis* pertaining to Mirobriga, the region in Spain where the type strain was isolated).

Gram-positive, chemo-organotrophic and strictly aerobic. Colonies on SA1 agar are 2–3 mm in diameter after 2 weeks. Colonies are raised, folded and orange, turning brown to black on sporulation. Well-developed substrate hyphae bearing single slightly warty spores; aerial mycelium is not produced. Optimum pH and temperature for growth are 7 and 28 °C, respectively. Oxidase- and catalase-positive. Nitrate is not reduced; urease-negative. Growth is not observed in the presence of crystal violet 0.001 % or sodium azide 0.01 %. Resistant to ampicillin (2 µg). Acid is produced from glucose. Carbon sources assimilated are L-arabinose, D-cellobiose, D-galactose, D-glucose, D-maltose, D-mannose, D-raffinose, D-sucrose and D-trehalose. Adipate, caprate, citrate, malate, D-mannitol, D-melezitose, phenyl-acetate, L-rhamnose, D-sorbitol, L-sorbose and xylitol are not assimilated. Contains *meso*-DAP in its cell wall; major menaquinone is MK-10(H₄). Major fatty acids are iso-15:0, iso-16:0, iso-17:1 and anteiso-17:0.

The type strain, WA201^T (=DSM 44830^T=LMG 22229^T), was isolated from the region of Mirobriga (Ciudad Rodrigo, Spain).

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Table 1. Characteristics that differentiate strain WA201^T from its closest neighbours

Strains: 1, *M. mirobrigensis* sp. nov. WA201^T; 2, *M. carbonacea* DSM 43168^T; 3, *M. matsumotoense* IFO 14550^T. +, Positive; –, negative; W, weakly positive; ND, not determined.

Characteristic	1	2	3
β-Glucuronidase	+	–	ND
Naphthol-AS-BI-phosphate	+	–	ND
Oxidase	+	+	–
Urease	W	–	+
Nitrate reduction	–	+	+
Assimilation of:			
Gluconate	+	–	–
D-Melibiose	+	–	–
Histidine	+	–	ND
Tolerance of 3 % NaCl	+	+	–
Hydrolysis of tyrosine	–	+	ND
Major menaquinone	MK-10(H ₄)	MK-9(H ₄)	MK-10(H ₆)

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