Micromonospora coriariae sp. nov., isolated from root nodules of *Coriaria myrtifolia*

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An actinomycete strain, NAR01^T, was isolated from root nodules of a *Coriaria* plant. The 16S rRNA gene sequence of strain NAR01^T showed most similarity to the type strains of *Micromonospora endolithica* (98.94 %) and *Micromonospora chersina* (98.4 %). The chemotaxonomic results obtained confirmed the taxonomic position of the isolate within the genus *Micromonospora*, and revealed differences at the species level. Physiological and biochemical tests showed that strain NAR01^T could be clearly distinguished from its closest phylogenetic neighbours, while DNA–DNA hybridization results indicated that the isolate represents a novel species. On the basis of these results, strain NAR01^T (= DSM 44875^T = LMG 23557^T) is proposed as the type strain of the novel species *Micromonospora coriariae* sp. nov.

The number of novel species of *Micromonospora* Ørskov 1923 described has increased significantly in the last year (Kroppenstedt *et al.*, 2005; Thawai *et al.*, 2005a, b; Trujillo *et al.*, 2005) and the genus currently holds 27 species with validly published names. While most strains have been isolated from soil collected in diverse geographical regions, members of the genus *Micromonospora* also appear to be closely associated with plant roots (Coombs & Franco, 2003; Valdés *et al.*, 2005; M. E. Trujillo, unpublished observations). In the present paper, we report on the isolation of a novel *Micromonospora* strain, NAR01^T, from surface-sterilized, nitrogen-fixing root nodules obtained from the plant *Coriaria myrtifolia*, which was collected in Salamanca, Spain.

The root nodules used for the isolation of strain NAR01^T were washed several times with sterile distilled water and were then surface sterilized in HgCl₂ (2.5 % w/v) for 2 min. The nodules were rinsed several times with sterile distilled water and then crushed using a sterile glass rod. The homogenized plant tissue was inoculated onto yeast extract/mannitol agar (Vincent, 1970) and the plates were incubated at 28 °C for 10 days. The isolation plates were then examined under a stereoscopic microscope: several small orange colonies were readily observed. Strain NAR01^T was selected because of its capacity to degrade xylan, as shown by the

ability of the micro-organism to grow on XED medium (Rivas *et al.*, 2003), which contained this carbohydrate as the sole carbon source.

The cultural characteristics of strain NAR01^T were studied on several media, namely yeast extract-malt extract agar (ISP 2), Bennett's agar (Jones, 1949), oatmeal agar (ISP 3), SA1 agar (Trujillo *et al.*, 2005) and yeast extract/mannitol agar. Abundant growth was observed on Bennett's, ISP 2 and SA1 agars, while moderate growth was obtained on ISP 3 and yeast extract/mannitol agar media. Colonies were an intense orange colour, folded and raised, turning darker after 3 weeks; neither aerial hyphae nor diffusible pigments were produced. Differences in substrate-mycelium colour found between NAR01^T and *Micromonospora* species are presented in Table 1.

Phase-contrast observations (Opti-Phot microscope, × 100; Nikon) were performed using cells obtained after 5 days incubation in liquid medium. The strain produced branched hyphae with terminal spores on both short and long side branches. The shape of the spores produced by strain NAR01^T was very similar to that reported by Hirsch *et al.* (2004) for *Micromonospora endolithica*. Gram staining (Doetsch, 1981) was performed using 3-day-old cultures: strain NAR01^T was Gram-positive.

Extraction of genomic DNA, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were performed as described previously (Rivas *et al.*, 2003). The sequence of strain NAR01^T was manually aligned and compared with other sequences obtained from GenBank/

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NAR01^T is AJ784008.

An extended phylogenetic tree and the cellular fatty acid profile of strain NAR01^T are available as supplementary data in IJSEM Online.

Table 1. Differential characteristics of NAR01^T and phylogenetically related *Micromonospora* species

Reference strains: 1, *M. endolithica* DSM 44398^T; 2, *M. chersina* DSM 44398^T; 3, *M. rosaria* DSM 803^T [data in columns 1–3 are from Hirsch *et al.* (2004), except for API ZYM tests, which were carried out in the present study]; 4, *M. inositol* DSM 43819^T [data from Kawamoto (1989), except for API ZYM tests, which were carried out in the present study]; 5, *M. fulviridis* DSM 43906^T; 6, *M. echinospora* DSM 43816^T; 7, *M. sagamiensis* DSM 43912^T; 8, *M. inyoensis* DSM 46123^T (data in columns 5–8 from Kroppenstedt *et al.*, 2005). With the exception of *M. inositol* DSM 43819^T and *M. fulviridis* DSM 43906^T (no data available), all the strains were positive for alkaline phosphatase, esterase (C8), leucine arylamidase, cystine arylamidase, acid phosphatase and α -glucosidase and negative for α -fucosidase. +, Positive; –, negative; ND, not determined; v, variable; (+), growth better than negative control but less than positive control.

Characteristic	Strain NAR01 ^T	1	2	3	4	5	6	7	8
Colour of substrate mycelium (on ISP 2 medium)	Intense orange	Orange	Light yellow	Orange–brown	Orange	Salmon–orange	Purple	Coral red	Yellow–olive
Diffusible pigments	–	–	Fluorescent yellow	Wine red	–	–	–	–	Yellow
Nitrate reduction	+	–	v	–	–	ND	v	ND	ND
Tyrosine hydrolysis	–	+	–	+	ND	ND	ND	ND	ND
Xylan hydrolysis	+	–	–	ND	–	ND	ND	ND	ND
Carbon sources:									
Fructose	+	+	ND	ND	ND	+	–	–	(+)
Gluconate	+	–	ND	ND	ND	ND	ND	ND	ND
Melibiose	+	+	–	+	+	ND	–	ND	ND
Raffinose	+	+	v	–	ND	+	+	(+)	(+)
Rhamnose	+	–	–	+	–	–	+	–	(+)
Ribose	+	–	–	+	–	ND	–	ND	ND
Sucrose	–	+	+	+	+	+	+	–	(+)
Xylose	+	+	ND	ND	ND	–	–	–	(+)
API ZYM tests									
Esterase (C4)	+	+	+	ND	ND	+	–	–	+
Lipase (C14)	+	–	+	ND	ND	+	+	–	–
Trypsin	+	+	+	ND	ND	–	+	–	+
α -Chymotrypsin	–	–	+	ND	ND	+	+	–	+
α -Galactosidase	+	+	+	ND	+	+	–	+	–
α -Mannosidase	–	–	+	ND	+	–	–	–	–
Maximum NaCl tolerance (% w/v)	1	7	3	ND	1.5	ND	3	ND	ND
Temperature range (°C)	12–37	8–39	18–49	18–42	25–40	ND	27–37	ND	ND
Main menaquinone	MK-10(H ₄)	MK-10(H ₄)	MK-9(H ₄)	MK-10(H ₄)	MK-10(H ₄)	MK-9(H ₄)	MK-10(H ₄)	MK-9(H ₄)	MK-9(H ₄)
Whole-cell sugars*	Glc, Xyl, Ara, Man, Rib	Gal, Ara, Xyl, Rib, Rha	Glc, Man, Xyl, Ara	Ara, Xyl, Gal	ND	Xyl, Glc	Xyl, Glc	Xyl, Glc	Xyl, Glc, Ara

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

EMBL. Phylogenetic distances were calculated with the Kimura two-parameter model, and the tree topologies were inferred using the least-squares (De Soete, 1983), maximum-parsimony (Fitch, 1971) and neighbour-joining methods (Saitou & Nei, 1987). Bootstrap replications (1000) were performed using the MEGA program as described by Kumar *et al.* (2001).

An almost-complete 16S rRNA gene sequence (1451 nt) was obtained for NAR01^T and compared with those deposited in the public databases. The highest levels of similarity were with *M. endolithica* DSM 44398^T (98.94 %) and

Micromonospora chersina DSM 44151^T (98.4 %). The results of the phylogenetic analysis using various tree-making algorithms were very similar (data not shown). A subset of the closest phylogenetic relatives of strain NAR01^T based on the neighbour-joining method are presented in Fig. 1. A phylogenetic tree that includes all *Micromonospora* species with validly published names is available as Supplementary Fig. S1 in IJSEM Online.

For chemotaxonomic analyses, strain NAR01^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

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships among phylogenetically related members of the genus *Micromonospora* and the novel isolate NAR01^T. The tree was constructed by using a neighbour-joining analysis with distances based on the Kimura two-parameter model. Bootstrap percentages (of 1000 replicates) above 50% are shown at nodes. Bar, 5 nucleotide changes per 1000 nt. An extended version of this tree containing all species of *Micromonospora* is available as Supplementary Fig. S1 in IJSEM Online.

freeze-dried. The chemotaxonomic analyses performed included diaminopimelic acid determination in whole-cell hydrolysates, using 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 using the method of Minnikin *et al.* (1984) and then analysed by HPLC (model 1100; Hewlett Packard). Polar lipids were extracted and identified by two-dimensional TLC (Minnikin *et al.*, 1984). Cellular fatty acid methyl esters were prepared from cells grown for 24 h on trypticase soy agar (Schröder *et al.*, 1997).

The chemotaxonomic markers found were in agreement with the results of phylogenetic analyses, confirming the classification of strain NAR01^T within the genus *Micromonospora*, and also revealing differences at the species level. The novel isolate contained *meso*-diaminopimelic acid, while the whole-cell sugars detected were arabinose, glucose, mannose, ribose and xylose. Specifically, the presence of glucose and mannose and the absence of galactose and rhamnose distinguished the novel isolate from *M. endolithica* DSM 44398^T. Other differences in sugar composition between NAR01^T and its closest phylogenetic neighbours are given in Table 1. Large amounts of menaquinone MK-10(H₄) (>60%) and smaller amounts of MK-9(H₄) and MK-10(H₆) were detected. The polar lipid composition included phosphatidylglycerol, phosphatidylinositol and phosphatidylethanolamine, a profile that corresponds to Lechevalier's phospholipid pattern II (Lechevalier *et al.*, 1977). Some unidentified glycolipids were also detected. Significant amounts of iso-15:0 (24%), iso-16:0 (17%), 17:0 (10%) and 10-methyl-17:0 (16%) were detected in the cellular fatty acid profile of NAR01^T. The detailed fatty acid composition of strain NAR01^T is presented in Supplementary Table S1 (available in IJSEM Online). The G+C content, determined using the thermal melting method (Mandel & Marmur, 1968), was found to be 70.2 mol%.

Phenotypic characteristics were studied using several standard methods. Catalase and oxidase activities were determined as described previously (Trujillo *et al.*, 2006). The hydrolysis of arbutin, aesculin, casein, gelatin, Tweens

20 and 80, starch, tyrosine, urea and xylan was investigated as described by Trujillo *et al.* (2005), using SA1 agar as a basal medium. Tests for the utilization of various substrates as sole carbon and energy sources were performed according to Williams *et al.* (1983). The temperature range for growth and the NaCl tolerance were recorded using ISP 2 agar as the basal medium. SA1 agar supplemented with appropriate buffer systems was used for pH-tolerance studies. Enzyme activities for strains NAR01^T, *M. endolithica* DSM 44398^T, *M. chersina* DSM 44151^T and *Micromonospora echinospora* DSM 43816^T were performed using API ZYM and API Coryne kits (bioMérieux) according to the manufacturer's instructions.

The novel isolate, NAR01^T, presented a phenotypic profile that clearly distinguished it from its closest phylogenetic neighbours (Table 1). An important difference found between NAR01^T and its closest neighbour, *M. endolithica* DSM 44398^T, was the capacity of the latter organism to grow in 7% NaCl (NAR01^T tolerated concentrations of only 1%). Xylan degradation also differentiated NAR01^T from *M. endolithica* DSM 44398^T, *M. chersina* DSM 44151^T and *Micromonospora inositol* DSM 43819^T. Furthermore, NAR01^T was negative for tyrosine hydrolysis whereas *M. endolithica* DSM 44398^T and *Micromonospora rosaria* DSM 803^T were positive. Additional phenotypic data are given in the species description.

Finally, DNA–DNA hybridizations between NAR01^T, *M. endolithica* DSM 44398^T and *M. chersina* DSM 44151^T were carried out to confirm the species status of the novel isolate. DNA was isolated and purified on hydroxyapatite according to the method of Cashion *et al.* (1977). Hybridization measurements were obtained according to the spectrophotometric method of De Ley *et al.* (1970), with the modification of Huß *et al.* (1983). DNA–DNA reassociation values of 11.5 and 25% were obtained between NAR01^T and *M. endolithica* DSM 44398^T and *M. chersina* DSM 44151^T, respectively. These percentages fall well below the 70% value recommended for defining genomic species (Wayne *et al.*, 1987). Furthermore, they support the idea that *Micromonospora* strains represent individual genospecies when 16S rRNA gene sequence values are below 99% (Kroppenstedt *et al.*, 2005).

Strain NAR01^T can be clearly differentiated from currently described *Micromonospora* species on the basis of the genotypic and phenotypic data. Thus, isolate NAR01^T represents the type strain of a novel species, for which we propose the name *Micromonospora coriariae* sp. nov.

Description of *Micromonospora coriariae* sp. nov.

Micromonospora coriariae (co.ri.a'ri.ae. N.L. gen. n. *coriariae* of *Coriaria*, pertaining to the isolation of the type strain from root nodules of *Coriaria myrtifolia*).

Gram-positive, chemo-organotrophic, aerobic actinobacterium. Colonies are intensely orange, raised and folded. Well-developed substrate mycelium carrying single spores mostly on the tips of the hyphae. Aerial mycelium is absent. Diffusible pigments are not produced. pH range for growth is 7–9; does not grow below pH 6.5. Optimum growth temperature is 28 °C. Oxidase- and catalase-positive. Arbutin, aesculin, casein, gelatin, starch and Tween 80 are degraded, but Tween 20, tyrosine and urea are not. The following are used as carbon sources: alanine, arabinose, arginine, cellobiose, fructose, gluconate, glucose, histidine, mannose, melibiose, pyruvate, raffinose, rhamnose, salicin, serine, starch, trehalose and xylose. The following substrates are not used as carbon sources: ascorbic acid, *meso*-erythritol, galactose, glutaric acid, lysine, melezitose, proline, propionic acid, quinic acid, sorbitol, sorbose, sucrose, tyrosine, valine and xylitol. Positive enzymic reactions are obtained for the following: acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C8), leucine arylamidase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, pyrazinamidase and valine arylamidase. Negative reactions are obtained for the following enzymes: α -chymotrypsin, α -fucosidase, β -glucuronidase, α -mannoside, naphthol-AS-BI-phosphohydrolase and pyrrolidonyl arylamidase. Other physiological and biochemical characteristics are shown in Table 1. Whole-cell sugars present are glucose, xylose, arabinose, mannose and ribose. The major menaquinone is MK-10(H₄).

The type strain, NAR01^T (=DSM 44875^T=LMG 23557^T), was isolated from root nodules of *Coriaria myrtifolia*.

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