Micromonospora marina sp. nov., isolated from sea sand

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Two actinomycete strains, JSM1-1^T and JSM1-3, were isolated from sea sand collected in Thailand. Their taxonomic position was determined using a polyphasic approach. The chemotaxonomic characteristics of these strains coincided with those of the genus *Micromonospora*, i.e. the presence of meso-diaminopimelic acid and *N*-glycolyl muramic acid in the peptidoglycan, whole cell sugar pattern D, phospholipids type II, and cellular fatty acid type 3b. Phylogenetic analysis of 16S rRNA gene sequences revealed a close relationship between strains JSM1-1^T and JSM1-3 (99.8%), and between JSM1-1^T and *Micromonospora aurantiaca* JCM 10878^T (99.3%), *Micromonospora chalybeata* JCM 3031^T (99.0%), and *Micromonospora coxensis* JCM 13248^T (99.0%). However, strains JSM1-1^T and JSM1-3 could be clearly distinguished from these type strains by a low DNA–DNA relatedness and by phenotypic differences. On the basis of the data presented, a new species, *Micromonospora marina* sp. nov., is proposed. The type strain is JSM1-1^T (=JCM 12870^T =PCU 269^T =TISTR 1566^T).

The actinomycete genus *Micromonospora* was proposed by Ørskov (1923) on the basis of morphological properties, which include the absence of a true aerial mycelium and the presence of spores borne singly on the substrate mycelium. Strains belonging to the genus *Micromonospora* are distributed widely in different environments, such as soils, water, sandstone and root nodules (Hirsch *et al.*, 2004; Kawamoto, 1989; Trujillo *et al.*, 2005, 2006; Ara & Kudo, 2007). The genus *Micromonospora* was redefined to 14 species by Kasai *et al.* (2000) based on *gyrB* and 16S rRNA gene sequence analyses and DNA–DNA relatedness. More recently, the species *Micromonospora auratinigra*, *M. eburnea*, *M. endolithica*, *M. mirobrigensis*, *M. siamensis*, *M. citrea*, *M. echinaurantiaca*, *M. echinofusca*, *M. fulviviridis*, *M. inyonensis*, *M. peucetia*, *M. sagamiensis*, *M. viridifaciens*, *M. coriariae*, *M. chokoriensis*, *M. coxensis*, *M. lupini*, *M. saelicesensis*, *M. chaiyaphumensis*, *M. krabiensis* and *M. narathiwatensis* were reported (Hirsch *et al.*, 2004; Trujillo *et al.*, 2005, 2006,

2007; Thawai *et al.*, 2004, 2005a, b, 2008; Kroppenstedt *et al.*, 2005; Ara & Kudo, 2007; Jongrungruangchok *et al.*, 2008a, b).

During the course of an investigation of rare actinomycetes from sea sand in Thailand, we isolated two actinomycetes, JSM1-1^T and JSM1-3, which showed the typical morphological characteristics of the genus *Micromonospora*. In this paper we report the taxonomic characterization and classification of these isolates and propose that they represent a new species, *Micromonospora marina* sp. nov.

Strains JSM1-1^T and JSM1-3 were isolated from sand collected along the sea shore in Hua-Hin, Prachuabkirikhun province, Thailand, by using starch-casein nitrate agar [10 g starch, 0.3 g sodium caseinate (Difco), 2 g KNO₃ and 15 g agar per litre; pH 7.0–7.4] incubated at 30 °C for 21 days. They were purified and maintained on yeast extract-malt extract agar, ISP 2 (The International *Streptomyces* Project medium 2), as described by Shirling & Gottlieb (1966). The isolates were grown on ISP 2 agar and oatmeal agar, ISP 3 (Shirling & Gottlieb, 1966), for 2 weeks at 30 °C and were observed by light and scanning electron microscopy (model JSM-5410 LV; JEOL). Samples for scanning electron microscopy were prepared as described previously (Itoh *et al.*, 1989). The Hucker–Conn method was used for the Gram stain (Hucker & Conn, 1923).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strains JSM1-1^T and JSM1-3 are AB196712 and AB196713, respectively.

A scanning electron micrograph of strain JSM1-1T, a maximum-parsimony tree based on 16S rRNA gene sequences and tables containing cultural characteristics, cellular fatty acid profiles, and DNA G+C content and DNA–DNA relatedness of strains JSM1-1T and JSM1-3 are available with the online version of this paper.

Cultural characteristics were determined by using 14-day-old cultures grown at 30 °C on ISP media, as described by Shirling & Gottlieb (1966). The Colour Harmony Manual (Jacobson *et al.*, 1958) was used for determining colours of colonies. Gelatin liquefaction, milk peptonization, nitrate reduction and starch hydrolysis were determined as described by Arai (1975) and Williams & Cross (1971). The decomposition of various compounds was examined by using the basal media recommended by Gordon *et al.* (1974). The utilization of carbohydrates as sole carbon sources was tested by using neutralized yeast nitrogen base without amino acids (Difco) as a basal medium, supplemented with 1% (w/v) carbon sources and 0.05% (w/v) amino acids according to the method of Stevenson (1967). Tolerance to sodium chloride and the effect of temperature and pH on growth were determined on Sucrose Bennett's Agar (Arai, 1975). In addition, tyrosine agar (ISP 7) and peptone-iron agar (ISP 6) supplemented with 0.1% (w/v) yeast extract were used to determine melanoid pigment production.

Freeze-dried cells required for chemotaxonomic analyses were obtained from cultures grown in ISP 2 broth incubated on a rotary shaker at 30 °C for 14 days. Cell wall peptidoglycan was prepared and hydrolysed as described by Kawamoto *et al.* (1981). The isomers of diaminopimelic acid in the cell walls were determined as described by Stanek & Roberts (1974). Reducing sugars from whole cell hydrolysates were analysed by the HPLC method of Mikami & Ishida (1983). The acyl group of muramic acid in the peptidoglycan was determined using the method of Uchida & Aida (1984). Phospholipids in cells were extracted and identified as described by Minnikin *et al.* (1984). Methyl esters of cellular fatty acids were prepared by the direct transmethylation method with methanolic hydrochloride (Suzuki & Komagata, 1983) and were analysed by using GLC according to the instructions of the Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996) as described previously (Kudo *et al.*, 1993). The identification of each peak was carried out by comparison with commercially available standards and by using GC-MS. Isoprenoid quinones were extracted by the method of Collins *et al.* (1977) and were analysed by HPLC with a Cosmosil 5C₁₈ column (4.6 by 150 mm; Nacalai Tesque). Methanol/2-propanol (2:1, v/v) was used as an eluent.

DNA was extracted from cells grown in ISP 2 broth according to the method of Tamaoka (1994). The G+C content of the DNA was determined using HPLC as described by Tamaoka & Komagata (1984). DNA-DNA relatedness was carried out in 2×SSC (saline trisodium citrate) and 50% formamide solution at 53 °C for 5 h (Ezaki *et al.*, 1989). Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA genes, and sequencing of the PCR products were carried out as described by Nakajima *et al.* (1999). The sequences of the two strains were multiply aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases by

using the CLUSTAL_X program (Thompson *et al.*, 1997). The phylogenetic trees were reconstructed using the Kimura two-parameter model in the neighbour-joining method (Saitou & Nei, 1987) and the maximum-parsimony method using MEGA version 3.1 (Kumar *et al.*, 2004). The confidence values of branches of the phylogenetic trees were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Gaps and ambiguous nucleotides were eliminated from the calculations.

Strains JSM1-1^T and JSM1-3 had morphological, cultural and chemotaxonomic properties consistent with their classification in the genus *Micromonospora*. They formed well-developed and branched substrate hyphae. No aerial mycelium was produced. Spores at maturity were spherical to oval, appeared to be smooth and were non-motile (Supplementary Fig. S1, available in IJSEM Online). The phenotypic characteristics of strains JSM1-1^T and JSM1-3 are presented in the species description and in Table 1 and Supplementary Tables S1 and S2. The strains contained *meso*-diaminopimelic acid in the cell wall. The acyl type of the cell wall in the peptidoglycan was determined to be the glycolyl type. Whole-cell sugars were detected as glucose, xylose, mannose, ribose, galactose and arabinose [pattern D of Lechevalier & Lechevalier (1970)]. The polar lipid profile contained diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and phosphatidylethanolamine [type PII pattern of Lechevalier *et al.* (1977)]. Significant fatty acids of strains JSM1-1^T and JSM1-3 were iso-C_{15:0} (20.1–25.1%), iso-C_{16:0} (16.4–21.6%), iso-C_{17:0} (5.4–7.9%), anteiso-C_{17:0} (6.9–7.7%), iso-C_{17:1ω9c} (6.4–6.9%) and 10-methyl C_{17:0} (1.2–5.7%) (Supplementary Table S2). This pattern corresponded to fatty acid type 3b of Kroppenstedt (1985). The predominant menaquinones were MK-10(H₄) (52.0–55.0%), MK-10(H₆) (18.3–22.2%), MK-10(H₈) (3.7–6.1%), MK-10(H₂) (2.5–2.8%), MK-9(H₄) (5.9–6.0%) and MK-9(H₆) (2.1–2.3%). Trace amounts of MK-9(H₂) and MK-10(H₀) were

Table 1. Differential characteristics of strains JSM1-1^T and JSM1-3 and related species of the genus *Micromonospora*

Strains: 1, JSM1-1^T; 2, JSM1-3; 3, *M. aurantiaca* JCM 10878^T; 4, *M. chalcone* JCM 3031^T. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4
Decomposition of tyrosine	+	+	+	–
Growth at pH 5	+	+	+	–
Maximum NaCl tolerance (%)	7	7	4	5
Utilization of:				
L-Arabinose	+	w	+	w
Glycerol	w	w	–	–
Lactose	–	–	+	+
D-Mannitol	w	w	–	–
Raffinose	+	+	w	+
D-Ribose	+	+	–	–

also detected. The DNA G+C contents of JSM1-1^T and JSM1-3 were 72.0 and 71.9 mol%, respectively.

Analysis of the almost complete 16S rRNA gene sequences (1436 nt) of strains JSM1-1^T and JSM1-3 indicated that they were 99.8% related to each other and that they formed a monophyletic clade with the closely related species *Micromonospora aurantiaca* JCM 10878^T (99.3%), *Micromonospora chalcone* JCM 3031^T (99.0%), *Micromonospora coxensis* JCM 13248^T (99.0%), *Micromonospora halophytica* DSM 43171^T (98.8%) and *Micromonospora purpureochromogenes* DSM 43821^T (98.8%), as shown in Fig. 1 and Supplementary Fig. S2 (tree reconstructed by the maximum-parsimony method). Comparison with the descriptions of previously characterized species of the genus *Micromonospora* showed that our

novel isolates were distinguishable from the related species *M. aurantiaca* JCM 10878^T and *M. chalcone* JCM 3031^T by a combination of biochemical and physiological properties, in particular decomposition of tyrosine, utilization of L-arabinose, glycerol (weak), lactose, D-mannitol, raffinose and D-ribose, growth at pH 5 and maximum NaCl tolerance (7%) (Table 1).

The level of DNA–DNA relatedness between strains JSM1-1^T and JSM1-3 was 92–97%, while the levels of DNA–DNA relatedness between these strains and the type strains of closely related species *M. aurantiaca* JCM 10878^T, *M. chalcone* JCM 3031^T and *M. coxensis* JCM 13248^T were less than 44–49% (Supplementary Table S3). These values were obtained from independent determinations and are below the threshold value of 70% for distinguishing genomic

Fig. 1. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing the phylogenetic relationships between strains JSM1-1^T and JSM1-3, all type strains of the genus *Micromonospora*, and related species of the genus *Actinoplanes*. Based on 1000 replications, bootstrap percentages above 50% are shown. Bar, 0.005 substitutions per nucleotide position.

species (Wayne *et al.*, 1987). It is evident from the genotypic and phenotypic data that strains JSM1-1^T and JSM1-3 form a new taxonomic position in the genus *Micromonospora*. Consequently, we propose a novel species, *Micromonospora marina* sp. nov., to accommodate strains JSM1-1^T and JSM1-3.

Description of *Micromonospora marina* sp. nov.

Micromonospora marina sp. nov. (ma. ri' na. L. fem. adj. *marina* of the sea, marine, pertaining to sea sand, the source from which the type strain was isolated).

Aerobic, Gram-positive, mesophilic actinomycete that forms a well-developed and extensively branched substrate mycelium. No aerial mycelium is produced. The vegetative mycelium on ISP 2 is brownish orange, turning to brownish black upon sporulation. A pale yellow diffusible pigment is produced on ISP 2 and a pale brown to brown pigment on ISP 7. Spores are spherical to oval, appear to be smooth and are non-motile. Nitrate reduction is negative. Starch hydrolysis, gelatin liquefaction and milk peptonization are positive. Decomposes L-tyrosine, but not adenine, hypoxanthine or xanthine. Utilizes L-arabinose, D-fructose, D-galactose, D-glucose, glycerol (weakly), D-mannitol (weakly), melibiose, raffinose, D-ribose and D-xylose, but not lactose, L-rhamnose or salicin as sole carbon sources for energy. The optimal temperature for growth is 25–30 °C. Grows at 15–45 °C, at pH 5 to 9, and in the presence of 7 % NaCl. The cell wall peptidoglycan contains glutamic acid, alanine and *meso*-diaminopimelic acid. The acyl type of the cell wall is glycolyl. The predominant menaquinones are MK-10(H₄) and MK-10(H₆). Major cellular fatty acids are iso-C_{15:0} and iso-C_{16:0}. The DNA G+C content of the type strain is 72.0 mol%. Habitat is sea sand.

The type strain is JSM1-1^T (=JCM 12870^T =PCU 269^T =TISTR 1566^T), isolated from sea sand in Thailand.

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