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Nocardioides tritolerans sp. nov., Isolated from Soil in Bigeum Island, Korea

Dastager, Syed G., Jae-Chan Lee, Yoon-Jung Ju, Dong-Jin Park, and Chang-Jin Kim*

Functional Metabolomics Research Center, KRIBB, Daejeon 305-806, Korea

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A Gram-positive strain designated as MSL-14^T isolated from a soil sample collected from Bigeum Island, Korea, was subjected to polyphasic taxonomy. The isolate was strictly aerobic. Cells were short rods and motile. Optimum growth temperature and pH was 28°C and 7.0, respectively. It was characterized chemotaxonomically as having a cell-wall peptidoglycan type based on LL-2,6-diaminopimelic acid and MK-8(H_4) as the predominant menaquinone. The major fatty acids were iso-$C_{16:0}$, $C_{17:1}$ omega8c, and $C_{18:1}$ omega9c. The G+C content was 67.6 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain MSL-14^T is affiliated to the genus *Nocardioides* and formed a distinct lineage within the genus. MSL-14^T showed highest sequence similarity to *Nocardioides aestuarii* JCM 12125^T, having a similarity of 96.5%. Based on the 16S rRNA gene sequence divergence and phenotypic characteristics, it is proposed that strain MSL-14^T should be classified as representing a novel member of the genus *Nocardioides*, for which we propose the name *Nocardioides tritolerans* sp. nov. The type strain is strain MSL-14^T (=KCTC 19289^T= DSM 19320^T).

Keywords: *Nocardioides tritolerans* sp. nov., polyphasic taxonomy, 16S rDNA

The genus *Nocardioides* was proposed by Prauser [16] with a single species, *Nocardioides albus*; currently, it comprises 23 validly published species. Members of the genus *Nocardioides* can be distinguished from other members of the Actinobacteria by using a combination of chemical properties and phylogenetic analysis based on 16S rRNA gene sequences, as well as having LL-2,6-diaminopimelic acid (DAP) and glycine wall chemotype I *sensu* [12] and lack of mycolic acids [16] with a DNA G+C content between 67–74% [24]. Recently, in the course of screening microorganisms from Bigeum Island, Korea, many bacterial strains were isolated and characterized taxonomically. The strain MSL-14^T resembling *Nocardioides* was recovered and characterized by a combination of genotypic and phenotypic methods. It was evident that the isolate was sufficiently distinct from the other species of the genus *Nocardioides* to warrant the description of a novel species, for which the name *Nocardioides tritolerans* sp. nov. is proposed.

Strain MSL-14^T was isolated from a soil sample collected from Bigeum Island, Korea, by serial dilution plating on R2A agar (Difco, 10×diluted) at 30°C after 7 days incubation. Subcultivation was done routinely on R2A agar at 28°C for 3–5 days and maintained as glycerol (20%) stocks at −70°C. Gram staining was performed using a bioMérieux Gram-stain kit according to the manufacturer's instructions. Cell morphology and motility were studied using phase-contrast microscopy and scanning electron microscopy. The physiological characteristics of strain MSL-14^T were examined by growing the isolate on R2A medium. Growth was tested at different pH values (6.0–12.0) and temperatures (4–45°C) on R2A agar. Oxidase activity was tested by assessing the oxidation of 1% (w/v) tetramethyl-phenylenediamine (Merck), and catalase activity was evaluated by assessing the production of O_2 bubbles in a 3% (v/v) aqueous H_2O_2 solution. The hydrolysis of compounds was checked on R2A agar according to methods described previously [11]. Nitrate reduction was performed according to the method of Lanyi [11], and acid production from carbohydrates was tested as described by Leifson [13]. Hydrolysis of various substrates was carried out in an API-ZYM kit according to the manufacturer's instructions (bioMérieux) and evaluated. Biomass for quantitative fatty acid analysis was prepared by scraping growth from R2A agar plates that had been incubated for 5 days at 28°C. The fatty acids were extracted, methylated, and analyzed using the standard MIDI (microbial identification) system [18]. Isoprenoid quinones were extracted with chloroform/methanol (2:1 v/v), evaporated under vacuum condition, and reextracted in *n*-hexane-water (1:1, v/v), and then purified using Sep-Pak Vac Silica Cartridges (Waters) and subsequently analyzed by HPLC, as previously described by Komagata and Suzuki [9].

The genomic DNA of strain MSL-14^T was isolated as described by Hopwood *et al.* [6]. The 16S rRNA gene was

*Corresponding author
Phone: 82-42-860-4332; Fax: 82-42-860-4595;
E-mail: changjin@kribb.re.kr

amplified by PCR using the procedure described by Xu *et al.* [22]. The sequencing and assembly of the 16S rRNA gene sequence were carried out as described by Bakermans and Madsen [1]. The identification of phylogenetic neighbors and comparison of sequence similarity of strain MSL-14^T were achieved using the EzTaxon server (http://www.eztaxon.org/; [2]) to determine the approximate phylogenetic affiliation, and was aligned with close relatives by using CLUSTAL W software [20]. By using the FASTA 3 program in EBI, 16S rRNA gene sequence comparisons for similarity calculations were made between the novel strain and related members of the genus *Nocardioides*. A phylogenetic tree was reconstructed using the neighbor-joining method of Saitou and Nei [17]. A bootstrap analysis was performed using the algorithm of the Kimura two-parameter model [7] of the neighbor-joining method in the PHYLIP package. Phylogenetic trees were constructed using three different methods, namely the neighbor-joining [17], maximum-likelihood [3], and maximum-parsimony [8] algorithms available in PHYLIP, version 3.6 [5]. The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein [4] with 1,000 replicates. DNA for determining the G+C content was obtained by the method of Marmur and Doty [14]. The DNA G+C content was determined as described by Mesbah

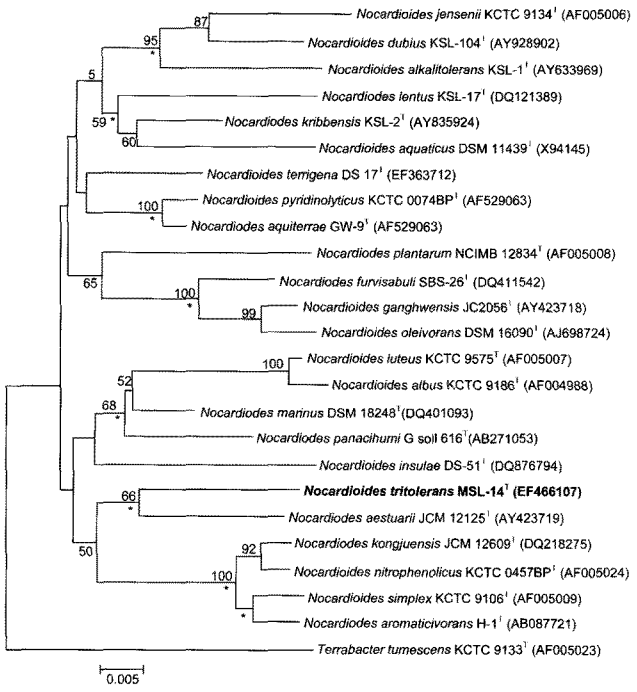

Fig. 1. Phylogenetic dendrogram obtained by neighbor-joining analysis of 16S rRNA gene sequences, showing the position of strain MSL-14^T among its phylogenetic neighbors.
Asterisks indicate branches that were recovered using maximum-likelihood [3] and maximum-parsimony [8] algorithms. Numbers at the branch nodes are bootstrap values, expressed as a percentage of 1,000 replicates (only values above 50% are shown). *Terrabacter tumescens* KCTC 9133^T served as the outgroup. Bar, 0.005 substitutions per nucleotide position.

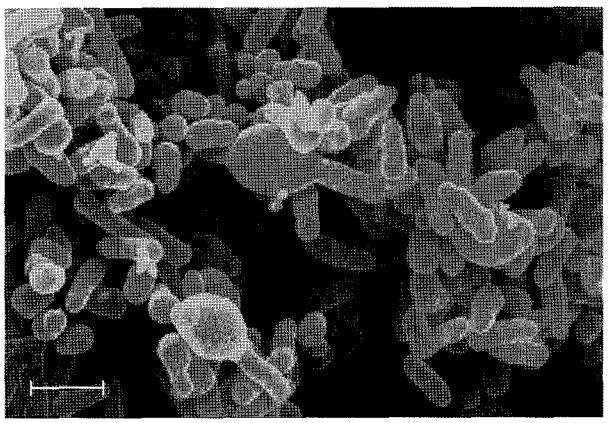

Fig. 2. Scanning electron micrograph of strain MSL-14^T grown on R2A agar for 7 days at 28°C. Bar, 1.0 μm.

et al. [15] using reverse-phase HPLC with a ThermoHypersil-key stone Aquasil c-18 (150×4.6 mm) column. The 16S rRNA gene sequence of strain MSL-14^T determined in this study has been deposited in NCBI GenBank under the accession number EF466107.

Strain MSL-14^T was Gram-positive, motile, short rods (Fig. 2), and formed white to pale yellow, glistening, translucent, slightly sticky, irregular, and slightly raised colonies. Growth was observed at temperatures between 20 and 40°C, and pH 7–12; optimum growth occurred at 28–30°C and pH 7.0–7.5. The cells of the isolate were motile rods (0.9–1.4 μm wide and 2.3–3.4 μm long). The strain MSL-14^T was oxidase-negative, catalase-negative, and did not reduce nitrate to nitrite. Anaerobic growth was not observed under anaerobic conditions for 5 days at 30°C on R2A agar. Growth occurred in the presence of 0–7% NaCl (w/v). The typical phenotypic characteristics of strain MSL-14^T are summarized and compared with those of the type strains of closely related taxa in Table 1. The major respiratory lipoquinone of strain MSL-14^T was MK-8 (H_4). The fatty acid profile of the strain showed a predominance of saturated fatty acids and is briefly indicated in the species description below. The G+C content of the genomic DNA of strain MSL-14^T was 67.6 mol%.

An almost-complete 16S rRNA gene sequence (1,482 nt) of strain MSL-14^T was obtained, and a phylogenetic analysis based on 16S rRNA gene sequences showed that strain MSL-14^T formed a distinct monophyletic cluster with *Nocardioides aestuarii* JCM 12,125^T (AY423719). The topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms also supported the notion that the isolate belongs to the genus *Nocardioides* (Fig. 1). Comparative 16S rRNA gene sequence analyses showed that the isolate was most closely related to *Nocardioides aestuarii* JCM 12125^T, having a similarity of 96.5%. Sequence similarities to other members included in the phylogenetic analysis were 94.6–96.3%. On the basis of phylogenetic

Table 1. Differential phenotypic properties of *Nocardioides tritolerans* sp. nov. and related *Nocardioides* species.

Characteristics	1	2	3	4	5	6
Cell morphology	Rods	Rods	Rods, cocci	Rods, cocci	Rods, cocci	Rods
Motility	+	–	–	+	+	–
Colony color	Cream	Ivory	Yellowish white	Yellowish white	Yellowish white	Milky white
Optimal temp. (°C)	20–40	30	30–37	26–37	30	30
Nitrate reduction	+	–	–	–	–	–
Hydrolysis of						
Aesculin	–	W	–	+	+	+
Starch	+	–	–	W	+	–
Gelatin	–	+	+	+	+	ND
Xanthine	+	–	–	–	–	–
Tyrosine	+	+	–	+	+	–
Carbon sources utilization						
D-Fructose	–	+	–	–	+	+
D-Galactose	+	+	–	–	–	–
m-Inositol	+	–	+	–	–	ND
Lactose	+	+	+	–	–	ND
Maltose	–	ND	–	ND	–	+ or W
D-Mannitol	+	+	+	–	–	ND
D-Mannose	+	–	W	–	W	W
D-Melibiose	+	–	–	ND	ND	ND
D-Raffinose	+	–	–	–	–	ND
L-Rhamnose	+	W	+	–	+	ND
D-Ribose	–	W	–	–	+	ND
API ZYM						
Alkaline phosphatase	+	W	+	+	+	+
Esterase (C4)	+	+	–	–	–	–
Valine arylamidase	+	+	–	W	+	–
Cystine arylamidase	+	–	–	W	W	–
Trypsin	–	+	–	+	+	–
Acid phosphatase	+	W	+	W	+	–
β-Galactosidase	–	+	–	–	–	–
β-Glucosidase	–	–	–	W	W	–
DNA G+C content (mol%)	67.6	70.0	72.1	72.0–74.0	71.0	72.0–72.4
Isolation source	Soil	Tidal flat	Soil	Soil	Industrial waste water	River waste, sediment, soil

Species: 1, MSL-14^T; 2, *Nocardioides aestuarii*; 3, *Nocardioides kongjuensis*; 4, *Nocardioides simplex*; 5, *Nocardioides nitrophenolicus*; 6, *Nocardioides aromaticivorans*. Data are from Yoon *et al.* [10, 23, 25, 26]. +, Positive reaction; –, negative reaction; ND, not determined; W, weakly positive reaction. All species are positive for Gram stain, D-cellobiose, sucrose, D-xylose, casein, Tween 80, D-glucose, leucine arylamidase, esterase lipase (C8), naphthol-AS-BI-phosphohydrolase, and α-glucosidase. All species are negative for urea, L-arabinose, hypoxanthine, lipase (C14), β-glucuronidase, α-chymotrypsin, α-galactosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Type strains (n=1) of each taxon were compared.

distance from established *Nocardioides* species, indicated by relatively low 16S rRNA gene sequence similarity (<97.0%) [19, 21], a marginal differences were observed in phenotypic properties of strain MSL-14^T, which demonstrates that is not affiliated to any species of this genus that are validly described. Therefore, on the basis of data presented, strain MSL-14^T represents a novel species within the genus *Nocardioides*, for which the name *Nocardioides tritolerans* sp. nov. is proposed.

Description of *Nocardioides tritolerans* sp. nov.

Nocardioides tritolerans (tri'to.le.rans.L.pref. triple.three. L. pres. part. tolerans. tolerating; N.L.part. adj. tritolerans referring to the ability of the organism to tolerate high salinity, alkalinity, and temperature).

Cells are aerobic, Gram-positive, appear as short rods of about 0.3–0.8×0.8–4.0 μm. Colonies are smooth, flat, cream in color and 0.9–1.3 μm in diameter after 4–5 days incubation on R2A medium at 30°C. There is no formation of aerial or substrate mycelia. Growth occurs at 20–40°C but not at below 20°C or above 40°C. Optimum pH for growth is 6.0–11.0. Good growth was observed in the presence of 7.0% NaCl (w/v). Cellulose is not hydrolyzed. H_2S production, DNase and citrate are negative. Nitrate is reduced to nitrite. Cell-wall peptidoglycan contains LL-diaminopimelic acid as the diagnostic diamino acid. The Predominant menaquinone is MK-8(H_4), and diphosphatidylglycerol and phosphatidylglycerol are the phospholipids found in the cell wall analysis. The major fatty acids detected are i-$C_{16:0}$

(40.6%), $C_{17:1}$ w8c (7.3%), $C_{18:1}$ w9c (6.1%), $C_{17:1}$ (4.5%), i-$C_{15:0}$ (4.5%), ai-$C_{17:0}$ (4.4%), i-$C_{17:0}$ (4.1%), 10-methyl-$C_{17:0}$ (2.9%), $C_{17:1}$w6c (2.9%), i-$C_{17:1}$ w9c (2.8%), i-$C_{16:1}$ (2.6%), $C_{16:0}$ (2.4%), i-$C_{14:0}$ (1.8%), i-$C_{18:0}$ (1.6%), $C_{18:0}$ (1.6%), and ai-$C_{15:1}$ (1.4%). The DNA G+C content is 67.6 mol%. Other phenotypic properties are listed in Table 1. The type strain MSL-14^T (=KCTC 19289^T=DSM 19320^T) was isolated from a soil sample collected from Bigeum Island in the Republic of Korea.

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