Pseudonocardia mongoliensis sp. nov. and *Pseudonocardia khuvsgulensis* sp. nov., isolated from soil

Ismet Ara,^{1†} Baljinova Tsetseg,² Damdinsuren Daram,² Manabu Suto¹ and Katsuhiko Ando¹

Correspondence

Ismet Ara
ismetara@yahoo.com

¹Biotechnology Development Center, Department of Biotechnology, National Institute of Technology and Evaluation (NITE), Kazusakamatari 2-5-8, Kisarazu, Chiba 292-0818, Japan

²Institute of Biology, Mongolian Academy of Sciences, Ulaanbaatar-51, Mongolia

Two actinomycetes, designated MN08-A0270^T and MN08-A0297^T, were isolated from soil from the area around Khuvsgul Lake, Khuvsgul province, Mongolia, and subjected to phenotypic and genotypic characterization. They produced well-developed, branched substrate hyphae and, similar to closely related species of the genus *Pseudonocardia*, produced zigzag-shaped aerial hyphae by acropetal budding and blastospores. A comparative analysis of 16S rRNA gene sequences indicated that strains MN08-A0270^T and MN08-A0297^T formed two distinct clades within the genus *Pseudonocardia* and were respectively most closely related to *Pseudonocardia yunnanensis* NBRC 15681^T (97.3 % similarity) and *Pseudonocardia thermophila* IMSNU 20112^T (97.1 %). Chemotaxonomic characteristics, including cell-wall diaminopimelic acid, whole-cell sugars, fatty acid components and major menaquinones, suggested that the two organisms belonged to the genus *Pseudonocardia*. Strains MN08-A0270^T and MN08-A0297^T could be differentiated from each other and from closely related species of the genus *Pseudonocardia* by physiological and biochemical characteristics, predominant fatty acids, menaquinones and whole-cell sugar components. Combined with the results of a broad range of phenotypic tests and DNA–DNA hybridization data and phylogenetic analysis, these results support the conclusion that these strains represent two novel species of the genus *Pseudonocardia*, for which we propose the names *Pseudonocardia mongoliensis* sp. nov. (type strain MN08-A0270^T = NBRC 105885^T = VTCC D9-25^T) and *Pseudonocardia khuvsgulensis* sp. nov. (type strain MN08-A0297^T = NBRC 105886^T = VTCC D9-26^T).

The genus *Pseudonocardia*, originally proposed by Henssen (1957) for mycolateless nocardioform actinomycetes with a type IV cell wall, includes organisms characterized by the presence of *meso*-diaminopimelic acid and the sugars arabinose and galactose (Lechevalier & Lechevalier, 1980); the major menaquinone is MK-8(H₄) and the DNA G + C content is 68–79 mol%. *Pseudonocardia* strains form vegetative and aerial mycelium with spore chains produced by acropetal budding or fragmentation. The description of the genus has been emended repeatedly based on both chemotaxonomic and morphological variations (Henssen & Schäfer, 1971; Warwick *et al.*, 1994; McVeigh *et al.*, 1994; Reichert *et al.*, 1998; Huang *et al.*, 2002; Park *et al.*, 2008).

On the basis of the lack of mycolic acids and the presence of cell-wall type IV, the genera *Amycolata* (Lechevalier *et al.*, 1986) and *Pseudoamycolata* (Akimov *et al.*, 1989) were also proposed for some nocardioform actinomycetes. However, the two genera were found to have chemotaxonomic properties similar to those of the genus *Pseudonocardia* (Kothe *et al.*, 1989; Takeuchi *et al.*, 1992) and were subsequently incorporated in the genus *Pseudonocardia* on the basis of the 16S rRNA gene sequence analysis (Warwick *et al.*, 1994; McVeigh *et al.*, 1994). Subsequently, members of the genus *Actinobispora* (Jiang *et al.*, 1991) were also transferred to the genus *Pseudonocardia* based on identification using specific PCR primers and reanalysis of 16S rRNA gene sequences and the menaquinones of their type species (Huang *et al.*, 2002). At the time of writing, the genus *Pseudonocardia* encompassed 34 species (including *Pseudonocardia spinosa* Henssen and Schäfer 1971) with validly published names listed by Huang *et al.* (2002), Lee *et al.* (2000, 2001, 2002, 2004), Kämpfer & Kroppenstedt (2004), Gu *et al.* (2006), Liu *et al.* (2006), Mahendra &

[†]Present address: Department of Botany and Microbiology, College of Science, King Saud University (KSU), Post Box 22452, Riyadh 11495, Saudi Arabia.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains MN08-A0270^T and MN08-A0297^T are AB521671 and AB521672, respectively.

Alvarez-Cohen (2005), Qin *et al.* (2008, 2010), Park *et al.* (2008), Chen *et al.* (2009), Schäfer *et al.* (2009), Duangmal *et al.* (2009) and Sakiyama *et al.* (2010). Strains of the genus have been isolated from various environmental samples, such as active sludge soils, including those polluted by chemical compounds (Lee *et al.*, 2004; Kämpfer & Kroppenstedt, 2004; Mahendra & Alvarez-Cohen, 2005; Liu *et al.*, 2006; Kämpfer *et al.*, 2006; Park *et al.*, 2008), and plant samples, including stems, leaves, root nodules, tree bark, compost and Chinese medicinal plants (Evtushenko *et al.*, 1989; Reichert *et al.*, 1998; Gu *et al.*, 2006; Chen *et al.*, 2009).

In the course of our research on new actinobacterial sources in Mongolia (Ara *et al.*, 2010a, b), strains MN08-A0270^T and MN08-A0297^T were isolated from soil collected from the area around Khuvsgul Lake, Khuvsgul province. Polyphasic taxonomic analysis demonstrated that the strains represent two novel species of the genus *Pseudonocardia*.

Soil samples were dried at room temperature for 5–7 days and then inoculated using the rehydration-centrifugation method (Hayakawa *et al.*, 2000) on humic acid-vitamin agar (Hayakawa & Nonomura, 1987) containing trimethoprim (20 mg l⁻¹) and nalidixic acid (10 mg l⁻¹). Following isolation, colonies were picked and strains designated MN08-A0270^T and MN08-A0297^T were purified by repeated streaking on yeast extract-soluble starch medium (YS medium; 2 g yeast extract, 10 g soluble starch and 15 g agar l⁻¹, pH 7.3) and Bennett's agar plates (0.1 % yeast extract, 0.1 % beef extract, 0.2 % NZ amine type A, 1.0 % maltose monohydrate and 1.5 % agar; pH 7.3) after incubation for about 3–4 weeks at 28 °C. Purified strains MN08-A0270^T and MN08-A0297^T were maintained on YS slants at 4 °C and as hyphal fragments and spore suspensions in 20 % (v/v) glycerol at –80 °C.

The strains were grown on ISP media 2–7 (Shirling & Gottlieb, 1966), Bennett's agar and YS medium after incubation at 28 °C for 3 weeks and cultural characteristics were observed (Table 1). The colour of the substrate hyphae was determined from the reverse side as described by Shirling & Gottlieb (1966). Cell morphology was observed under a light microscope and by scanning electron microscopy. Light microscope observation of cells suspended in phosphate buffer (pH 7.0, 1 mM) was used to test for spore motility (Sakiyama *et al.*, 2010). For scanning electron microscopy, agar blocks containing the organisms (cultivated on water agar medium for 21 days at 28 °C) were fixed with the vapour of 1 % osmium tetroxide and dried samples were sputter-coated and viewed with a scanning electron microscope (Tamura *et al.*, 1994).

Strains MN08-A0270^T and MN08-A0297^T had morphological properties typical of the genus *Pseudonocardia*. The aerial mycelium was white and the substrate mycelium was pale yellow to yellowish brown on different agar media (Table 1). No diffusible pigment was produced. Typical pseudonocardia-type acropetal budding growth of aerial hyphae and spore chains were observed on water agar medium in both strains (Fig. 1). Spore chains were borne at the ends of branched aerial hyphae, and the spore surface was smooth.

Strain MN08-A0270^T showed very slow growth; colonies become visible after approximately 1 week of incubation and showed moderate to good growth on ISP 2, ISP 3, ISP 6, Bennett's agar and YS agar (Table 1). Growth of strain MN08-A0297^T was relatively fast compared with strain MN08-A0270^T and the former strain showed good growth on most of the agar media tested (Table 1). Strains MN08-A0270^T and MN08-A0297^T and reference strain *Pseudonocardia thermophila* NBRC 15559^T could be differentiated by growth intensity, substrate mycelium colour and production of aerial mycelium on different agar media (Table 1).

Physiological and biochemical characteristics of strains MN08-A0270^T and MN08-A0297^T and the phylogenetically closely related type strain *P. thermophila* NBRC 15559^T were tested after incubation at 28 °C for 3 weeks. Growth was tested at 4, 10, 15, 20, 25, 28, 30, 37, 40, 45 and 50 °C on YS agar medium. The ability of the strains to grow at pH 5.0–10.0 (at intervals of 1.0 pH unit) and 0–8 % (w/v) NaCl (at intervals of 1.0 %) was examined by using YS agar as the basal medium. Media and procedures used for determination of physiological features were those described by Shirling & Gottlieb (1966). For the nitrate reduction test, ISP 8 medium (full strength) (Gordon & Mihm, 1957) was used. Decomposition of urea was determined on Christensen's urea agar containing 2.0 % urea (Gordon *et al.*, 1974). Degradation of casein and other compounds (final concentration 0.5 %) was determined using YS medium as the modified basal medium (Gordon *et al.*, 1974). Catalase activity was detected by dropping 3 % H₂O₂ onto well-developed colonies on agar plates and observing any gas bubbles produced. Other biochemical analyses were performed using modified methods of Kloos *et al.* (1974) and by using the API Coryne commercial biochemical test kit (bioMérieux) according to the manufacturer's instructions. Constitutive enzymic activities were tested using the API ZYM test kit (bioMérieux) according to the manufacturer's instructions (Humble *et al.*, 1977). Detailed and differential results are given in the species descriptions and in Table 2. Each strain showed a characteristic pattern with the API ZYM test system (Table 2). Strain MN08-A0270^T could be differentiated from both strain MN08-A0297^T and *P. thermophila* NBRC 15559^T by several agar-based and traditional biochemical tests, tolerance of NaCl, growth temperature, growth pH and API ZYM test kit results (Table 2). In the API ZYM test strip, strains MN08-A0270^T and MN08-A0297^T were both positive for esterase (C₄), esterase lipase (C₈), leucine arylamidase, α -glucosidase and β -glucosidase, negative for *N*-acetyl- β -glucosaminidase and α -fucosidase and weakly positive for lipase (C₁₄) and naphthol-AS-BI-phosphohydrolase (Table 2).

Genomic DNA was extracted and purified as described by Marmur (1961) and Saito & Miura (1963), but with a slight modification: after lysis, we used 20 % SDS and proteinase K (50 μ l, 4 mg ml⁻¹; Nacalai Tesque) to denature proteins and phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.) to remove denatured proteins. 16S rRNA gene sequences were analysed as described by Tamura & Hatano (2001).

Table 1. Cultural characteristics of strains MN08-A0270^T and MN08-A0297^T and *P. thermophila* NBRC 15559^T

Data were obtained in this study. +, Positive or present; ±, weakly positive or present; –, negative or absent. All strains are negative for production of diffusible pigment on any agar medium tested. Strains were incubated on different agar media at 28 °C for 3 weeks. Colours represented by numbers in parentheses were taken from Rayner (1970). ND, Not done; NA, not applicable. Characteristics highlighted in bold are particularly useful for differentiation of the three strains.

Agar medium	MN08-A0270 ^T			MN07-A0297 ^T			<i>P. thermophila</i> NBRC 15559 ^T		
	Growth	Substrate mycelium	Aerial mycelium	Growth	Substrate mycelium	Aerial mycelium	Growth	Substrate mycelium	Aerial mycelium
Bennett's	Good	Hazel (88), light to moderate yellowish brown	–	Good	Honey (64), moderate yellow	+	Good	Luteus (12), vivid yellow	+
Yeast-starch	Good	Buff (45), pale yellow	–	Good	Buff (45), pale yellow	+	Good	Pale luteus (11), light yellow	+
Water	Poor	Colourless	–	Moderate	White to colourless	±	No growth	NA	NA
ISP 2	Good	Honey (64), moderate yellow	–	Good	Honey (64), moderate yellow	–	Good	Fawn (87), moderate brown	+
ISP 3	Moderate	Buff (45), pale yellow	±	ND	ND	ND	Moderate	Buff (45), pale yellow	+
ISP 4	ND	ND	ND	Good	Buff (45), pale yellow	+	Moderate	Buff (45), pale yellow	+
ISP 5	Poor	Colourless	–	Good	Honey (64), moderate yellow	±	Moderate	Buff (45), pale yellow	±
ISP 6	Moderate	Hazel (88), light to moderate yellowish brown	–	Good	Honey (64), moderate yellow	–	Good	Fawn (87), moderate brown	+
ISP 7	Poor	Colourless	–	Good	Hazel (88), light to moderate yellowish brown	+	Moderate	Buff (45), pale yellow	+

Fig. 1. Scanning electron micrographs of aerial mycelium of strains MN08-A0270^T and MN08-A0297^T grown on water agar medium for 28 days at 28 °C. (a, c) Abundant zigzag-shaped aerial hyphae with developing branches of strains MN08-A0270^T (a) and MN08-A0297^T (c). (b, d) Chains of long, smooth-walled spores of strains MN08-A0270^T (b) and MN08-A0297^T (d) and elongation of hyphae by acropetal budding. Bars, 5 µm (a, c) and 1 µm (b, d).

Sequence analysis was performed with an ABI Prism BigDye Terminator cycle sequencing kit (PE Applied Biosystems) and an automatic DNA sequencer (model 3130 Genetic Analyzer; PE Applied Biosystems). BLAST analysis (Altschul *et al.*, 1990) was used to compare the nearly complete 16S rRNA gene sequences of strains MN08-A0270^T (1479 bp) and MN08-A0297^T (1461 bp) with sequences from the NCBI nucleotide database. Sequence alignments were generated using CLUSTAL_X (Thompson *et al.*, 1997) with corresponding sequences (available in DDBJ/EMBL/GenBank) from the type strains of all *Pseudonocardia* species. Evolutionary distance matrices (Kimura's two-parameter model) were calculated and phylogenetic trees were inferred using the neighbour-joining method in CLUSTAL_X, the minimum-evolution (Rzhetsky & Nei, 1992), maximum-parsimony (Eck & Dayhoff, 1966) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms in MEGA 4 (Tamura *et al.*, 2007) and the maximum-likelihood method using the NUCML program in MOLPHY (Adachi & Hasegawa, 1995; Hasegawa *et al.*, 1985; Mori *et al.*, 2003). Alignment gaps and ambiguous bases were removed prior to phylogenetic analyses by using BioEdit version 7.0.5.3 (Hall, 1999). Stability of the resultant tree topologies was evaluated by

bootstrap analysis (Felsenstein, 1985) based on the neighbour-joining dataset of 1000 resamplings using CLUSTAL_X and MEGA 4. DNA G+C contents were determined by HPLC as described by Mesbah *et al.* (1989). DNA-DNA hybridization between strain MN08-A0297^T and its closest phylogenetic neighbours was carried out by using the method described by Ezaki *et al.* (1989).

A phylogenetic tree was constructed according to the neighbour-joining algorithm based on 16S rRNA gene sequence data from strain MN08-A0297^T and corresponding sequences from type strains of the genus *Pseudonocardia* as well as *Kutzneria kofuensis* NRRL B-24061^T (Fig. 1). The tree topology, supported by high bootstrap values, clearly separated strain MN08-A0297^T within the genus *Pseudonocardia*. It is evident from the phylogenetic tree that strain MN08-A0297^T formed a strong cluster with *P. thermophila* ATCC 19285^T with 97% bootstrap support; the 16S rRNA gene sequence similarity was 97.1%. The 16S rRNA gene sequence similarity of strain MN08-A0297^T to other members of the genus *Pseudonocardia*, such as *Pseudonocardia aurantiaca* AS 4.1537^T (96.8%), *P. chloroethenivorans* SL-1^T (96.6%) and *P. alaniniphila* YIM 16303^T (96.5%), was less

Table 2. Differential physiological and biochemical characteristics of strains MN08-A0270^T and MN08-A0297^T and *P. thermophila* NBRC 15559^T

All data were obtained in this study. +, Positive; ±, weakly positive; –, negative.

Characteristic	MN08-A0270 ^T	MN08-A0297 ^T	<i>P. thermophila</i> NBRC 15559 ^T
Growth temperature (°C)			
Range	15–30	15–30	25–50
Optimum	25–30	25–30	30–45
Growth pH			
Range	4–11	5–11	6–9
Optimum	5–7	6–8	7–8
NaCl tolerance (% w/v)			
Range	0–2	0–3	0–3
Optimum	0–1	0–2	0–2
Calcium malate assimilation	–	+	–
Urea hydrolysis	+	+	±
Starch hydrolysis	–	+	–
Degradation of:			
Arbutin	+	+	–
Hypoxanthine	–	+	–
Tyrosine	–	+	+ (black pigment)
Tween 80	–	–	+
Nitrate reduction	–	–	+
Catalase	–	–	+
API ZYM enzyme assay			
Alkaline phosphatase	–	+	±
Valine arylamidase	±	+	+
Cystine arylamidase	±	+	±
Trypsin	–	±	+
α-Chymotrypsin	–	±	–
Acid phosphatase	–	+	+
α-Galactosidase	–	+	–
β-Galactosidase	–	±	–
β-Glucuronidase	–	±	–
β-Glucosidase	+	+	–
α-Mannosidase	–	+	–

than 97.0 %. In this study, to establish the precise taxonomic position of strain MN08-A0297^T, DNA–DNA hybridization studies were performed. The DNA–DNA relatedness between strain MN08-A0297^T and *P. thermophila* NBRC 15559^T was 11.0 % with both strains (means of three almost equal results). When the recommendation of a threshold value of 70 % DNA–DNA relatedness for the definition of bacterial species (Stackebrandt & Goebel, 1994) is considered, this result suggests strongly that strain MN08-A0297^T represents a novel species of the genus *Pseudonocardia*. Thus, based on the differential physiological and biochemical properties presented in this study (Tables 1 and 2) and DNA–DNA relatedness, strain MN08-A0297^T can be considered to represent a novel member of the genus *Pseudonocardia*.

It is also evident from the phylogenetic tree that strain MN08-A0270^T formed a close cluster with *Pseudonocardia halophobica* DSM 43089^T (96.4 %), *P. ailaonensis* YIM 45505^T (96.4 %) and *P. oroxyli* D10^T (96.2 %), but did not form definite clusters with the most closely related strains,

P. yunnanensis NBRC 15681^T (97.3 %) and *P. alaniniphila* YIM 16303^T (97.2 %). Further, strain MN08-A0270^T could be differentiated from the phylogenetically closely related type strains *P. halophobica* DSM 43089^T, *P. ailaonensis* YIM 45505^T and *P. oroxyli* D10^T by the presence of different proportions of fatty acid components. The complete cellular fatty acid profiles of strain MN08-A0270^T, *P. halophobica* IMSNU 21327^T, *P. ailaonensis* YIM 45505^T and *P. oroxyli* DSM 44984^T are shown in Table 3. Strain MN08-A0270^T was also readily differentiated from its closest relatives *P. yunnanensis* NBRC 15681^T, *P. alaniniphila* YIM 16303^T, *P. halophobica* DSM 43089^T, *P. ailaonensis* YIM 45505^T and *P. oroxyli* D10^T on the basis of physiological and biochemical properties (Table 4). Taking this into consideration, we conclude that the differences between strain MN08-A0270^T and phylogenetically closely related *Pseudonocardia* type strains are large enough to exclude the assignment of the strain to any recognized *Pseudonocardia* species. Therefore, based on its phenotypic and genotypic properties, strain MN08-A0270^T is also considered to represent a novel species of the genus *Pseudonocardia*.

Table 3. Fatty acid compositions of strains MN08-A0270^T and MN08-A0297^T and closely related *Pseudonocardia* type strains

Strains: 1, MN08-A0297^T; 2, MN08-A0270^T; 3, *P. thermophila* NBRC 15559^T; 4, *P. halophobica* IMSNU 21327^T; 5, *P. ailaonensis* YIM 45505^T; 6, *P. oroxyli* DSM 44984^T. Values are percentages of total fatty acids. Data were obtained in this study or taken from Qin *et al.* (2008) (*P. halophobica* IMSNU 21327^T, *P. ailaonensis* YIM 45505^T and *P. oroxyli* DSM 44984^T). –, Not reported or absent.

Fatty acid composition	1	2	3	4	5	6
Saturated straight-chain						
C _{14:0}	–	–	–	0.6	1.8	–
C _{15:0}	0.4	–	0.3	2.0	–	–
C _{16:0}	0.9	0.7	2.7	6.3	8.4	1.7
C _{17:0}	0.8	–	1.3	1.0	–	–
C _{18:0}	–	–	1.2	–	–	–
Saturated branched						
iso-C _{14:0}	0.6	5.7	0.4	0.7	1.6	2.5
iso-C _{15:0}	4.0	3.2	5.0	4.6	7.8	2.9
anteiso-C _{15:0}	–	–	0.4	1.3	1.6	–
iso-C _{16:0}	16.0	27.8	26.4	34.8	35.5	49.5
9-Methyl C _{16:0}	–	–	11.8	–	–	–
10-Methyl C _{16:0}	11.4	6.2	–	7.4	9.0	10.5
10-Methyl C _{17:0}	8.8	1.1	1.0	1.2	–	–
iso-C _{16:0} 2-OH	21.0	12.0	1.9	3.6	10.8	11.5
iso-C _{17:0}	1.6	0.9	23.3	2.6	4.7	0.8
anteiso-C _{17:0}	1.5	–	12.3	6.6	–	–
iso-C _{17:0} 2-OH	–	–	2.0	–	–	–
anteiso-C _{17:0} 2-OH	0.8	–	1.2	–	1.4	–
iso-C _{18:0}	0.7	–	2.8	–	–	–
10-Methyl C _{18:0}	0.4	–	–	–	–	–
Unsaturated						
C _{15:1} B	0.5	–	–	1.5	–	–
iso-C _{16:1} H	18.7	18.5	0.5	5.9	6.9	13.0
C _{16:1} 2-OH	2.2	0.6	2.1	–	–	–
C _{16:1} <i>cis</i> 9	–	–	1.6	14.5	5.2	4.3
anteiso-C _{17:1} C	0.5	–	0.5	0.9	–	–
C _{17:1} <i>cis</i> 9	–	–	0.8	3.5	–	–
C _{18:1} <i>cis</i> 9	–	–	0.5	–	–	–
Unknown 16.048	9.0	23.0	–	–	–	–
Total unidentified	–	–	–	0.4	–	2.2

It is evident from Fig. 2 that strains MN08-A0270^T and MN08-A0297^T formed two distinct clades in the genus *Pseudonocardia* 16S rRNA gene tree with the closely related type strains *P. yunnanensis* IFO 15681^T (97.3%) and *P. thermophila* ATCC 19285^T (97.1%). The binary similarity of the 16S rRNA gene sequences of strains MN08-A0270^T and MN08-A0297^T was 97.0%; this association was supported by the maximum-likelihood (Felsenstein, 1981), minimum-evolution (Rzhetsky & Nei, 1992), maximum-parsimony (Eck & Dayhoff, 1966) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms (Fig. 2). Therefore, strains MN08-A0270^T and MN08-A0297^T were different from closely related type strains of *Pseudonocardia* species. Both strains had DNA G + C contents of 73.0 mol%.

Table 4. Differential physiological and biochemical characteristics of strain MN08-A0270^T and closely related *Pseudonocardia* type strains

Strains: 1, MN08-A0270^T; 2, *P. yunnanensis* NBRC 15681^T; 3, *P. alaniniphila* YIM 16303^T (data in columns 2–3 from Sakiyama *et al.*, 2010; Gu *et al.*, 2006; Huang *et al.*, 2002); 4, *P. halophobica* IMSNU 21327^T; 5, *P. ailaonensis* YIM 45505^T; 6, *P. oroxyli* DSM 44984^T (data in columns 4–6 from Qin *et al.*, 2008). +, Positive; –, negative; ±, weakly positive; ND, not done.

Character	1	2	3	4	5	6
Decomposition of:						
Urea	+	–	–	–	+	–
Hypoxanthine	–	+	+	±	+	+
Adenine	–	–	–	±	–	+
Xanthine	ND	–	–	+	±	+
Tyrosine	–	–	–	±	+	+
Growth on NaCl at:						
3% (w/v)	–	–	–	+	+	+
5% (w/v)	–	–	–	±	+	+
7% (w/v)	–	–	–	±	–	±

Strains MN08-A0270^T and MN08-A0297^T were examined for key chemical markers to determine whether they had a chemotaxonomic profile consistent with their classification in the genus *Pseudonocardia*. Biomass for quantitative fatty acid analysis was collected from fresh agar plates after 2 weeks at 28 °C. Cellular fatty acid profiles were determined by GLC (MIDI) using the standard protocol (Sasser, 1990). Strains MN08-A0270^T and MN08-A0297^T contained large amounts of iso-branched saturated and unsaturated branched fatty acids in comparison with other species of the genus *Pseudonocardia*, predominantly iso-C_{16:0}, iso-C_{16:0} 2-OH, 10-methyl C_{16:0}, iso-C_{16:1} H and a unknown fatty acid. Further, strains MN08-A0270^T and MN08-A0297^T could be differentiated from each other by their complete cellular fatty acid profiles (Table 3).

Biomass for analyses of other chemical markers was harvested using centrifugation of cell mass from cultures grown for 3 weeks in yeast extract-glucose (YG) broth (10 g yeast extract and 10 g glucose l⁻¹, pH 7.0) grown at 28 °C in a shaker incubator. The harvested cell mass was washed twice with sterile distilled water and freeze-dried. Menaquinone analyses were carried out by the method of Minnikin *et al.* (1984). Quinones were extracted from 100 mg freeze-dried cell material using chloroform/methanol and separated by TLC on silica gel, removed from the plate and further analysed by HPLC. The menaquinones were MK-8(H₄) (accounting for 84% of the total), MK-8(H₂) (12%) and MK-8(H₀) (4%) for strain MN08-A0270^T and MK-8(H₄) (92%), MK-8(H₂) (4%), MK-8(H₀) (2%) and MK-8(H₆) (0.8%) for strain MN08-A0297^T. Whole-cell sugars were analysed as described by Stanek & Roberts (1974). The diaminopimelic acid isomer in the peptidoglycan was analysed as described by Nozawa *et al.* (2007). Mycolic acids were analysed as described previously (Tamura *et al.*, 1994).

Fig. 2. Phylogenetic tree constructed by the neighbour-joining method (Saitou & Nei, 1987) using almost-complete 16S rRNA gene sequences, showing relationships of strains MN08-A0270^T and MN08-A0297^T with type strains of all *Pseudonocardia* species. Numbers on branches indicate percentage bootstrap values determined for 1000 replicates (only values above 50 % are indicated). Bar, 0.01 K_{nuc} . Asterisks (*) indicate branches of the tree that were also recovered using the maximum-likelihood tree-making algorithm.

The complete chemotaxonomic profiles of strains MN08-A0270^T and MN08-A0297^T determined in this study can be found in the species descriptions.

Physiological and chemotaxonomic analyses indicated that strains MN08-A0270^T and MN08-A0297^T belong to the genus *Pseudonocardia*; 16S rRNA gene sequence analyses indicated that they represent two novel species. Phenotypic characters and fatty acid analysis are also consistent with novel species status for strains MN08-A0270^T and MN08-A0297^T. Therefore, it is concluded that strains MN08-A0270^T and MN08-A0297^T represents two novel species within the genus *Pseudonocardia*, for which the names *Pseudonocardia mongoliensis* sp. nov. and *Pseudonocardia khuvsgulensis* sp. nov. are proposed.

Description of *Pseudonocardia mongoliensis* sp. nov.

Pseudonocardia mongoliensis (mon.go.li.en'sis. N.L. fem. adj. *mongoliensis* of or belonging to Mongolia, referring to the isolation of the type strain from soil of Mongolia).

Aerobic, Gram-stain-positive. Cells grow well on Bennett's, yeast-starch and ISP 2 agar at 28 °C, forming well-developed, extensively branched and non-fragmented substrate hyphae. Extensive acropetal budding of the white aerial hyphae and the formation of blastospores are observed. Very scanty aerial hyphae are formed, only on the surface of water agar. The substrate mycelium is light to moderate yellowish brown to pale yellow on different agar media. Optimal growth at 25–30 °C; no growth at 37 °C. Grows at pH 4–11 and tolerates up to 2 % NaCl. Catalase is not produced, nitrate is not reduced and gelatin, starch and Tweens 20 and 80 are not hydrolysed. Urea is hydrolysed. Arbutin is decomposed but casein, tyrosine, calcium malate, adenine and hypoxanthine are not. According to the API ZYM enzyme assay, positive for esterase (C_4), esterase lipase (C_8), leucine arylamidase, α -glucosidase and β -glucosidase, weakly positive for lipase (C_{14}), valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase and negative for alkaline phosphatase, trypsin, α -chymotrypsin, acid phosphatase, α -galactosidase, β -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -fucosidase and α -mannosidase. According to the API Coryne enzyme assay, positive for pyrazinamidase, alkaline phosphatase, α -glucosidase, β -glucosidase, urease and hydrolysis of gelatin, weakly positive for utilization of D-glucose, D-ribose, D-xylose, D-mannitol, maltose and sucrose and negative for nitrate reduction, pyrrolidonyl arylamidase, β -glucuronidase, β -galactosidase, *N*-acetyl- β -glucosaminidase and catalase and utilization of lactose and glycogen. Peptidoglycan contains *meso*-diaminopimelic acid; diagnostic sugars are galactose, ribose, glucose, arabinose, mannose and rhamnose (trace). Mycolic acids are absent. The menaquinone system consists of MK-8(H_4), MK-8(H_2) and MK-8(H_0). Major fatty acid methyl esters are iso- $C_{16:0}$, unknown ECL 16.05, iso- $C_{16:1}$ H and iso- $C_{16:0}$ 2-OH. The detailed fatty acid profile of the type strain is given in Table 3. The G + C content of the DNA of the type strain is 73.0 mol%.

The type strain, MN08-A0270^T (=NBRC 105885^T =VTCC D9-25^T), was isolated from soil of Khuvsgul Lake, Khuvsgul province, Mongolia.

Description of *Pseudonocardia khuvsgulensis* sp. nov.

Pseudonocardia khuvsgulensis (khuvs.gul.en'sis. N.L. fem. adj. *khuvsgulensis* of or belonging to Khuvsgul, referring to the isolation of the type strain from soil of Khuvsgul Lake, Khuvsgul province, Mongolia).

Aerobic, non-motile, non-endospore-forming, Gram-stain-positive actinomycete that forms extensively branched substrate and aerial mycelia that carry smooth-surfaced, rod-shaped spores. Extensive acropetal budding of the aerial hyphae and the formation of blastospores are observed. Good growth occurs on Bennett's, YS, ISP 2, ISP 4, ISP 5, ISP 6 and ISP 7 agar media at 28 °C. Forms white aerial mycelium and pale yellow to moderate yellowish

brown substrate mycelium on the media tested. No diffusible pigment is produced. Grows at 15–30 °C (optimum 25–30 °C), at pH 5.0–11.0 (optimum pH 6.0–6.8) and at 0–3.0 % (w/v) NaCl (optimum 0–2.0 %). Positive for urease and decomposition of hypoxanthine, tyrosine, calcium malate and arbutin, but negative for catalase, nitrate reduction, gelatin liquefaction, hydrolysis of starch and Tweens 20 and 80 and decomposition of casein and adenine. According to the API ZYM enzyme assay, positive for alkaline phosphatase, esterase (C₄), esterase lipase (C₈), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, α -glucosidase, β -glucosidase and α -mannosidase, weakly positive for lipase (C₁₄), trypsin, α -galactosidase, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, β -galactosidase and β -glucuronidase and negative for *N*-acetyl- β -glucosaminidase and α -fucosidase. According to the API Coryne enzyme assay, positive for pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, β -galactosidase, α -glucosidase, *N*-acetyl- β -glucosaminidase, β -glucosidase and urease and hydrolysis of gelatin and negative for catalase, nitrate reduction and β -glucuronidase. The cell wall contains *meso*-diaminopimelic acid. The whole-cell sugar pattern consists of galactose, glucose, arabinose, ribose, mannose and rhamnose (trace) (type IV cell wall). Mycolic acids are absent. MK-8(H₄) is the predominant menaquinone. The major fatty acid methyl esters are iso-C_{16:0} 2-OH, iso-C_{16:1} H, iso-C_{16:0} and 10-methyl C_{16:0}. The DNA G + C content of the type strain is 73.0 mol%.

The type strain, MN08-A0297^T (=NBRC 105886^T =VTCC D9-26^T), was isolated from soil of Khuvs gul Lake, Khuvs gul province, Mongolia.

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References

- Adachi, J. & Hasegawa, M. (1995). Improved dating of the human/chimpanzee separation in the mitochondrial DNA tree: heterogeneity among amino acid sites. *J Mol Evol* **40**, 622–628.
- Akimov, V. N., Evtushenko, L. I. & Dobritsa, S. V. (1989). *Pseudoamycolata halophobica* gen. nov., sp. nov. *Int J Syst Bacteriol* **39**, 457–461.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Ara, I., Yamamura, H., Tsetseg, B., Daram, D. & Ando, K. (2010a). *Luteipulveratus mongoliensis* gen. nov., sp. nov., a new actinobacterial taxon in the family *Dermacoccaceae*. *Int J Syst Evol Microbiol* **60**, 574–579.
- Ara, I., Yamamura, H., Tsetseg, B., Daram, D. & Ando, K. (2010b). *Actinoplanes toevensis* sp. nov. and *Actinoplanes tereljensis* sp. nov., isolated from Mongolian soil. *Int J Syst Evol Microbiol* **60**, 919–927.
- Chen, H.-H., Qin, S., Li, J., Zhang, Y.-Q., Xu, L.-H., Jiang, C.-L., Kim, C.-J. & Li, W.-J. (2009). *Pseudonocardia endophytica* sp. nov., isolated from the pharmaceutical plant *Lobelia clavata*. *Int J Syst Evol Microbiol* **59**, 559–563.
- Duangmal, K., Thamchaipenet, A., Matsumoto, A. & Takahashi, Y. (2009). *Pseudonocardia acaciae* sp. nov., isolated from roots of *Acacia auriculiformis* A. Cunn. ex Benth. *Int J Syst Evol Microbiol* **59**, 1487–1491.
- Eck, R. V. & Dayhoff, M. O. (1966). *Atlas of Protein Sequences and Structure*. Silver Springs, MD: National Biomedical Research Foundation.
- Evtushenko, L. I., Akimov, V. N., Dobritsa, S. V. & Tapytkova, S. D. (1989). A new species of actinomycete, *Amycolata alni*. *Int J Syst Bacteriol* **39**, 72–77.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Gordon, R. E. & Mihm, J. M. (1957). A comparative study of some strains received as nocardiae. *J Bacteriol* **73**, 15–27.
- Gordon, R. E., Barnett, D. A., Handerhan, J. E. & Pang, C. H.-N. (1974). *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. *Int J Syst Bacteriol* **24**, 54–63.
- Gu, Q., Luo, H., Zheng, W., Liu, Z. & Huang, Y. (2006). *Pseudonocardia oroxyli* sp. nov., a novel actinomycete isolated from surface-sterilized *Oroxylum indicum* root. *Int J Syst Evol Microbiol* **56**, 2193–2197.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Hasegawa, M., Kishino, H. & Yano, T. (1985). Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* **22**, 160–174.
- Hayakawa, M. & Nonomura, H. (1987). Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. *J Ferment Technol* **65**, 501–509.
- Hayakawa, M., Otoguro, M., Takeuchi, T., Yamazaki, T. & Iimura, Y. (2000). Application of a method incorporating differential centrifugation for selective isolation of motile actinomycetes in soil and plant litter. *Antonie van Leeuwenhoek* **78**, 171–185.
- Henssen, A. (1957). Beiträge zur Morphologie und Systematik der thermophilen Actinomyceten. *Arch Mikrobiol* **26**, 373–414 (in German).
- Henssen, A. & Schäfer, D. (1971). Emended description of the genus *Pseudonocardia* Henssen and description of a new species *Pseudonocardia spinosa* Schäfer. *Int J Syst Bacteriol* **21**, 29–34.
- Huang, Y., Wang, L., Lu, Z., Hong, L., Liu, Z., Tan, G. Y. A. & Goodfellow, M. (2002). Proposal to combine the genera *Actinobispora* and *Pseudonocardia* in an emended genus *Pseudonocardia*, and description of *Pseudonocardia zijingensis* sp. nov. *Int J Syst Evol Microbiol* **52**, 977–982.
- Humble, M. W., King, A. & Phillips, I. (1977). API ZYM: a simple rapid system for the detection of bacterial enzymes. *J Clin Pathol* **30**, 275–277.

- Jiang, C., Xu, L., Yang, Y.-R., Guo, G.-Y., Ma, J. & Liu, Y. (1991). *Actinobispora*, a new genus of the order Actinomycetales. *Int J Syst Bacteriol* **41**, 526–528.
- Kämpfer, P. & Kroppenstedt, R. M. (2004). *Pseudonocardia benzenivorans* sp. nov. *Int J Syst Evol Microbiol* **54**, 749–751.
- Kämpfer, P., Kohlweyer, U., Thieme, B. & Andreesen, J. R. (2006). *Pseudonocardia tetrahydrofuranoxydans* sp. nov. *Int J Syst Evol Microbiol* **56**, 1535–1538.
- Kloos, W. E., Tornabene, T. G. & Schleifer, K. H. (1974). Isolation and characterization of micrococci from human skin, including two new species: *Micrococcus lylae* and *Micrococcus kristinae*. *Int J Syst Bacteriol* **24**, 79–101.
- Kothe, H. W., Vobis, G., Kroppenstedt, R. M. & Henssen, A. (1989). A taxonomic study of mycolateless, wall chemotype IV actinomycetes. *Syst Appl Microbiol* **12**, 61–69.
- Lechevalier, M. P. & Lechevalier, H. A. (1980). The chemotaxonomy of actinomycetes. In *Actinomycete Taxonomy* (Special Publication 6), pp. 227–291. Arlington, VA: Society for Industrial Microbiology.
- Lechevalier, M. P., Prauser, H., Labeda, D. P. & Ruan, J. S. (1986). Two new genera of nocardioform actinomycetes: *Amycolata* gen. nov. and *Amycolatopsis* gen. nov. *Int J Syst Bacteriol* **36**, 29–37.
- Lee, S. D., Kim, E. S. & Hah, Y. C. (2000). Phylogenetic analysis of the genera *Pseudonocardia* and *Actinobispora* based on 16S ribosomal DNA sequences. *FEMS Microbiol Lett* **182**, 125–129.
- Lee, S. D., Kim, E. S., Min, K.-L., Lee, W. Y., Kang, S.-O. & Hah, Y. C. (2001). *Pseudonocardia kongjuensis* sp. nov., isolated from a gold mine cave. *Int J Syst Evol Microbiol* **51**, 1505–1510.
- Lee, S. D., Kim, E. S., Kang, S.-O. & Hah, Y. C. (2002). *Pseudonocardia spinosipora* sp. nov., isolated from Korean soil. *Int J Syst Evol Microbiol* **52**, 1603–1608.
- Lee, S.-B., Strand, S. E., Stensel, H. D. & Herwig, R. P. (2004). *Pseudonocardia chloroethenivorans* sp. nov., a chloroethene-degrading actinomycete. *Int J Syst Evol Microbiol* **54**, 131–139.
- Liu, Z.-P., Wu, J.-F., Liu, Z. H. & Liu, S.-J. (2006). *Pseudonocardia ammonioxydans* sp. nov., isolated from coastal sediment. *Int J Syst Evol Microbiol* **56**, 555–558.
- Mahendra, S. & Alvarez-Cohen, L. (2005). *Pseudonocardia dioxanivorans* sp. nov., a novel actinomycete that grows on 1,4-dioxane. *Int J Syst Evol Microbiol* **55**, 593–598.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- McVeigh, H. P., Munro, J. & Embley, T. M. (1994). The phylogenetic position of *Pseudoamycolata halophobica* (Akimov et al. 1989) and a proposal to reclassify it as *Pseudonocardia halophobica*. *Int J Syst Bacteriol* **44**, 300–302.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A. & Parlett, J. H. (1984). An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233–241.
- Mori, K., Kim, H., Kakegawa, T. & Hanada, S. (2003). A novel lineage of sulfate-reducing microorganisms: *Thermodesulfobiaceae* fam. nov., *Thermodesulfobium narugense*, gen. nov., sp. nov., a new thermophilic isolate from a hot spring. *Extremophiles* **7**, 283–290.
- Nozawa, Y., Sakai, N., Arai, K., Kawasaki, Y. & Harada, K. (2007). Reliable and sensitive analysis of amino acids in the peptidoglycan of actinomycetes using the advanced Marfey's method. *J Microbiol Methods* **70**, 306–311.
- Park, S. W., Park, S. T., Lee, J. E. & Kim, Y. M. (2008). *Pseudonocardia carboxydivorans* sp. nov., a carbon monoxide-oxidizing actinomycete, and an emended description of the genus *Pseudonocardia*. *Int J Syst Evol Microbiol* **58**, 2475–2478.
- Qin, S., Su, Y. Y., Zhang, Y. Q., Wang, H. B., Jiang, C. L., Xu, L. H. & Li, W. J. (2008). *Pseudonocardia ailaonensis* sp. nov., isolated from soil in China. *Int J Syst Evol Microbiol* **58**, 2086–2089.
- Qin, S., Zhu, W. Y., Jiang, J. H., Klenk, H. P., Li, J., Zhao, G. Z., Xu, L. H. & Li, W. J. (2010). *Pseudonocardia tropica* sp. nov., an endophytic actinomycete isolated from the stem of *Maytenus austroyunnanensis*. *Int J Syst Evol Microbiol* **60**, 2524–2528.
- Rayner, R. W. (1970). *A Mycological Colour Chart*. Kew, UK: Commonwealth Mycological Institute and British Mycological Society.
- Reichert, K., Lipski, A., Pradella, S., Stackebrandt, E. & Altendorf, K. (1998). *Pseudonocardia asaccharolytica* sp. nov. and *Pseudonocardia sulfidoxydans* sp. nov., two new dimethyl disulfide-degrading actinomycetes and emended description of the genus *Pseudonocardia*. *Int J Syst Bacteriol* **48**, 441–449.
- Rzhetsky, A. & Nei, M. (1992). A simple method for estimating and testing minimum evolution trees. *Mol Biol Evol* **9**, 945–967.
- Saito, H. & Miura, K. (1963). Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim Biophys Acta* **72**, 619–629.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sakiyama, Y., Thao, N. K. N., Vinh, H. V., Giang, N. M., Miyadoh, S., Hop, D. V. & Ando, K. (2010). *Pseudonocardia babensis* sp. nov., isolated from plant litter. *Int J Syst Evol Microbiol* **60**, 2336–2340.
- Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Schäfer, J., Busse, H.-J. & Kämpfer, P. (2009). *Pseudonocardia parietis* sp. nov., from the indoor environment. *Int J Syst Evol Microbiol* **59**, 2449–2452.
- Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Staneck, J. L. & Roberts, G. D. (1974). Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.
- Takeuchi, M., Nishii, T. & Yokota, A. (1992). Taxonomic significance of arabinose in the family Pseudonocardiaceae. *Actinomycetologica* **6**, 79–90.
- Tamura, T. & Hatano, K. (2001). Phylogenetic analysis of the genus *Actinoplanes* and transfer of *Actinoplanes minutisporangius* Ruan et al. 1986 and '*Actinoplanes aurantiacus*' to *Cryptosporangium minutisporangium* comb. nov. and *Cryptosporangium aurantiacum* sp. nov. *Int J Syst Evol Microbiol* **51**, 2119–2125.
- Tamura, T., Nakagaito, Y., Nishii, T., Hasegawa, T., Stackebrandt, E. & Yokota, A. (1994). A new genus of the order Actinomycetales, *Couchioplanes* gen. nov., with descriptions of *Couchioplanes caeruleus* (Horan & Brodsky 1986) comb. nov. and *Couchioplanes caeruleus* subsp. *azureus* subsp. nov. *Int J Syst Bacteriol* **44**, 193–203.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible

strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Warwick, S., Bowen, T., McVeigh, H. P. & Embley, T. M. (1994). A phylogenetic analysis of the family *Pseudonocardiaceae* and the

genera *Actinokineospora* and *Saccharothrix* with 16S rRNA sequences and a proposal to combine the genera *Amycolata* and *Pseudonocardia* in an emended genus *Pseudonocardia*. *Int J Syst Bacteriol* **44**, 293–299.