Actinophytocola burenkhanensis sp. nov., isolated from Mongolian soil

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A Gram-positive, aerobic, non-motile actinomycete, strain MN08-A0203^T, that formed pale yellow to orange-brown colonies and non-fragmented branched substrate mycelium is described. The strain, which produced very scanty aerial mycelium-like structures and scanty formation of spherical bodies on the aerial mycelium on Bennett's agar medium, was studied in detail to determine its taxonomic position. On the basis of 16S rRNA gene sequence similarity studies, strain MN08-A0203^T grouped with the genus *Actinophytocola*, being most closely related to the type strain of *Actinophytocola oryzae* (97.8%). Chemotaxonomic data [menaquinone MK-9(H₄); iso-C_{16:0} (27%), iso-C_{15:0} (18%), C_{16:1} H (8%), C_{16:0} 9-methyl (8%) as major fatty acids; glucose, galactose, ribose, arabinose, mannose, rhamnose and xylose (trace) as whole cell sugars; diphosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine and ninhydrin-positive phosphoglycolipids as polar phospholipids] supported allocation of the strain to the genus *Actinophytocola*. Furthermore, the results of DNA–DNA hybridization of strain MN08-A0203^T with the type strain of *Actinophytocola oryzae* revealed that the two strains were genetically distinct from each other. Moreover, physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain MN08-A0203^T from closely related species. Thus, MN08-A0203^T represents a novel species of the genus *Actinophytocola*, for which the name *Actinophytocola burenkhanensis* sp. nov. is proposed, with MN08-A0203^T (=NBRC 105883^T =VTCC D9-23^T) as the type strain.

The genus *Actinophytocola* was proposed by Indananda *et al.* (2010) as a member of the family *Pseudonocardiaceae* Embley *et al.* (1988) emend. Stackebrandt *et al.* (1997). Based on 16S rRNA gene sequence analysis and 16S rRNA signature nucleotide patterns, the family currently contains 18 genera with validly published names, as described previously by Indananda *et al.* (2010). The single member of the genus *Actinophytocola* forms rod-shaped spores on aerial mycelium, but no sporangium-like structures or fragmentation of substrate mycelium are observed. The vegetative mycelia are light melon yellow and aerial mycelium is pale peach on International *Streptomyces* project (ISP) 3 medium. The cell wall contains *meso*-diaminopimelic acid, alanine,

glutamic acid and acetylated muramic acid. The whole-cell sugars are arabinose, galactose, mannose, rhamnose and ribose. Mycolic acids are absent. The major fatty acids are iso-C_{16:0}, iso-C_{15:0} and C_{16:0}. The diagnostic menaquinone is MK-9(H₄). The polar phospholipids consist of phosphatidylethanolamine and hydroxyphosphatidylethanolamine. The DNA G+C content is 71.1 mol% (Indananda *et al.*, 2010).

During the characterization of organisms from soil samples collected near Burenkhan, Khuvsgul province, Mongolia, strain MN08-A0203^T was isolated 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) at 28 °C; it had slightly pale yellow to orange-brown-coloured vegetative mycelium. Subcultivation was done on yeast extract-soluble starch agar medium (YS agar medium; 2 g yeast extract, 10 g soluble starch and 15 g agar per litre; pH 7.3) and Bennett's agar (yeast extract, 0.1 %;

Abbreviation: ISP, International *Streptomyces* project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MN08-A0203^T is AB535095.

A supplementary table and a supplementary figure are available with the online version of this paper.

beef extract, 0.1%; NZ amine, type A, 0.2%; maltose monohydrate, 1.0%; and agar, 1.5%; pH 7.3) at 28 °C for 3 weeks and the strain was kept as hyphal fragments in 20 % (v/v) glycerol at –80 °C.

The strain was then grown on ISP 2–7 (Shirling & Gottlieb, 1966), Bennett's and YS agar media and cultural characteristics were observed after incubation at 28 °C for 3–5 weeks. The colour of the substrate hyphae was determined from the reverse side as described by Shirling & Gottlieb (1966). Cell morphology was observed by light and scanning electron microscopy. Spore motility was tested by light microscopic observation of cells suspended in phosphate buffer (pH 7.0, 1 mM) for about 2 h at 28 °C. For scanning electron microscopy, agar blocks containing the organisms (cultivated on Bennett's agar medium for 28 days at 28 °C) were fixed with 1 % osmium tetroxide vapour; dried samples were sputter-coated and viewed (Tamura *et al.*, 1994). Strain MN08-A0203^T produced very scanty aerial mycelium and spore chains; formation of spherical structures above the agar was scanty (Fig. 1). Very scanty aerial hyphae were found only on Bennett's agar after 5 weeks of incubation.

Strain MN08-A0203^T grew slowly; colonies became visible after approximately 1–2 weeks of incubation and showed good growth only on Bennett's agar medium. Very scanty sporulation occurred only on Bennett's agar medium after 4–5 weeks incubation at 28 °C (Fig. 1). Pale brown soluble pigments were produced only on Bennett's agar medium after 4 weeks of incubation. Strain MN08-A0203^T and *Actinophytocola oryzae* could be differentiated by their growth intensity, substrate mycelium colour, production of aerial mycelium and production of diffusible pigmentation on Bennett's agar medium, and aerial mass colour on

various agar media (Table 1) (Indananda *et al.*, 2010). The complete cultural characteristics of strain MN08-A0203^T can be found in Table 1.

Physiological and biochemical characteristics of strain MN08-A0203^T were tested after incubation at 28 °C for 5 weeks. NaCl tolerance was examined on YS agar medium prepared with 0, 1, 2, 3, 4, 5, 6, 7 and 8 % (w/v) NaCl. Growth at different temperatures was examined on YS agar medium incubated at 4, 10, 15, 20, 25, 28, 30, 37, 40 and 45 °C. For the nitrate reduction test, ISP 8 medium (full-strength) (Gordon & Mihm, 1957) was used. Decomposition of urea was determined on Christensen urea agar containing 2 % urea (Gordon *et al.*, 1974). Degradation of casein and other compounds (final concentration 0.5 %) was determined using YS agar 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 the modified methods of Kloos *et al.* (1974) and by using commercial biochemical test kits such as the API Coryne test systems (bioMérieux) according to the manufacturer's instructions. Constitutive enzymic activities were tested using an API ZYM test kit according to the manufacturer's instructions (bioMérieux; Humble *et al.*, 1977). Detailed results are given in the species description. The isolate showed a characteristic pattern with API ZYM and API Coryne test systems. Strain MN08-A0203^T could be differentiated from the closely related species in the genus *Actinophytocola* by hydrolysis of urea and starch, calcium malate assimilation, degradation of gelatin, growth temperature, NaCl utilization in traditional agar-based tests and pH tolerance (Table 2). Based on the results of the API ZYM test kit, the strain was: positive for alkaline phosphatase, esterase lipase (C₈), leucine

Fig. 1. Scanning electron micrographs of aerial mycelium with developing branches (a) and spore chain-like formation (b) and scanty spherical body formation on the aerial mycelium (c, d) of strain MN08-A0203^T grown on Bennett's agar medium for 35 days at 28 °C. Arrows indicate spherical bodies on the aerial mycelium. Bars, 2 µm (a, c, d) and 1 µm (b).

Table 1. Cultural characteristics of the type strains of *A. burenkhanensis* sp. nov. and the closely related species *A. oryzae*

Results for the various media are presented as follows: growth/substrate mycelium colour/aerial mycelium/soluble pigmentation. –, Negative; +, positive; ++, strongly positive; ±, weakly positive. Colours are represented by numbers in parentheses and were taken from Rayner (1970). Both strains were incubated on the various agar media at 28 °C for 5 weeks.

Agar medium	<i>A. burenkhanensis</i> sp. nov. MN08-A0203 ^T	<i>A. oryzae</i> NBRC 105245 ^T
Bennett's	++/Hazel (88), light to moderate yellowish brown/±/+	+/Hazel (88), light to moderate yellowish brown/±/–
YS	±/Buff (45), pale yellow/–/–	++/Umber (9), strong brown/±/–
ISP 2	+/Buff (45), pale yellow/–/–	++/Cinnamon (62), moderate orange/–/–
ISP 3	+/Buff (45), pale yellow/–/–	+/Hazel (88), light to moderate yellowish brown/±/–
ISP 4	±/Colourless/–/–	++/Cinnamon (62), moderate orange/+ +/–
ISP 5	No growth	++/Umber (9), strong brown/±/–
ISP 6	+/Hazel (88), light to moderate yellowish brown/–/–	No growth
ISP 7	±/Colourless/–/–	+/Umber (9), strong brown/–/–
Water	±/Colourless/–/–	±/Colourless/+ +/–

arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase and *N*-acetyl- β -glucosaminidase; weakly positive for esterase (C_4) and valine arylamidase; and negative for lipase (C_{14}), cystine arylamidase, trypsin, α -chymotrypsin, β -glucuronidase, α -mannosidase and α -fucosidase.

Genomic DNA was extracted and purified as described by Marmur (1961) and Saito & Miura (1963), but with the slight modification that, after lysis, 20 % SDS and protease K were used to denature proteins and phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) was used to remove denatured proteins. 16S rRNA gene sequences were analysed as described by Tamura & Hatano (2001). 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 (Altschul *et al.*, 1990) and EzTaxon (Server version 2.1)

analyses (Chun *et al.*, 2007) were used to compare the 16S rRNA gene sequence of strain MN08-A0203^T with those of other related sequences from the NCBI nucleotide database. Sequence alignments were generated by using CLUSTAL X (Thompson *et al.*, 1997) with corresponding sequences available in DDBJ/EMBL/GenBank. Evolutionary distance matrices (Kimura two-parameter model) were calculated and phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987) method in the CLUSTAL X package, and minimum evolution (Rzhetsky & Nei, 1992), maximum-parsimony (Eck & Dayhoff, 1966) and neighbour-joining tree-making algorithms in the MEGA4 package (Tamura *et al.*, 2007). Alignment gaps and ambiguous bases were removed prior to phylogenetic analyses by using BioEdit version 7.0.5.3 (Hall, 1999). Evolutionary distances were computed using the maximum-likelihood method (Felsenstein, 1981) in the BioEdit package (version 7.0.5.3) and the NUCML program in MOLPHY (Adachi & Hasegawa, 1995; Mori *et al.*, 2003). Stability of the resultant tree

Table 2. Differential phenotypic properties of strain MN08-A0203^T and *A. oryzae* GMKU 367^T

Strains: 1, *A. burenkhanensis* sp. nov. MN08-A0203^T; 2, *A. oryzae* GMKU 367^T (=NBRC 105245^T). All data are from this study. +, Positive; –, negative; ±, weakly positive. Both strains are positive for decomposition of tyrosine and casein, but negative for decomposition of Tweens 20 and 80 and adenine, and nitrate reduction.

Characteristics	1	2
Growth temperature (°C) (optimum)	15–30 (25–30)	20–30 (25–30)
NaCl tolerance (%) (optimum)	0–3 (0–2)	0–2 (0–2)
Growth pH (optimum)	5–9 (7–9)	6–11 (9–11)
Liquefaction of gelatin	+	–
Decomposition of hypoxanthine	+	–
Assimilation of calcium malate	–	+
Decomposition of arbutin	+	–
Urea hydrolysis	±	+
Starch hydrolysis	+	±
Catalase	–	+

It is evident from Fig. 2 that strain MN08-A0203^T formed a distinct clade in the genus *Actinophytocola* 16S rRNA gene tree with the type strain of the closely related species *A. oryzae* (97.8 %); this strong association was supported by the maximum-likelihood, minimum evolution, maximum-parsimony and neighbour-joining tree-making algorithms (Fig. 2). This lineage was also independent and the branching point of the strain MN08-A0203^T lineage was strongly supported with a bootstrap value of 100 % using the neighbour-joining and maximum-likelihood methods. Therefore, strain MN08-A0203^T could be differentiated from the closely related type strain of *A. oryzae*. The DNA G + C content of strain MN08-A0203^T was 70.6 mol%.

Menaquinone analysis was 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 menaquinone was

Moreover, it has been shown that strain MN08-A0203^T and the type strain of *A. oryzae* have 97.8% 16S rRNA gene sequence similarity. To investigate the genomic relatedness between them, DNA–DNA hybridization was performed; the level of DNA–DNA relatedness was very low (7–12%), thus supporting previous results indicating that these two strains belong to two different genomic species (Supplementary Table S1) (Ezaki *et al.*, 1989). Therefore, it is concluded that strain MN08-A0203^T represents a novel species within the genus *Actinophytocola*, for which the name *Actinophytocola burenkhanensis* sp. nov. is proposed. The epithet pertains to the recovery of the strain from the soil samples near Burenkhan, Khuvsgul province in Mongolia.

Actinophytocola burenkhanensis (bu.ren.kha.nen'sis. N.L. masc. adj. *burenkhanensis* isolated from soil of Burenkhan, Khuvsgul province, Mongolia).

Table 3. Fatty acid compositions (%) of *A. burenkhanensis* MN08-A0203^T sp. nov. and *A. oryzae* NBRC 105245^T

Strains: 1, *A. burenkhanensis* sp. nov. MN08-A0203^T; 2, *A. oryzae* NBRC 105245^T. Data from the present study. —, Not detected.

Fatty acid	1	2
Saturated straight-chain		
C _{14:0}	—	1.50
C _{15:0}	—	1.59
C _{16:0}	1.30	3.89
C _{17:0}	0.80	0.41
Saturated branched		
iso-C _{14:0}	4.25	1.35
iso-C _{15:0}	18.13	8.43
anteiso-C _{15:0}	1.62	0.44
iso-C _{16:0}	26.86	39.04
iso-C _{16:0} 2-OH	5.45	15.70
iso-C _{16:0} 10-methyl	—	1.15
anteiso-C _{16:0}	1.92	—
C _{16:0} 9-methyl	8.23	—
C _{16:0} 10-methyl	—	4.22
C _{17:0} 10-methyl	1.09	—
iso-C _{17:0}	2.90	3.26
anteiso-C _{17:0}	3.49	1.38
anteiso-C _{17:0} 2-OH	0.78	0.57
iso-C _{16:1} H	8.43	9.13
C _{16:1} cis 9	5.06	0.76
anteiso-C _{17:1} C	0.55	—
C _{17:1} cis 9	4.39	—
Summed feature 4*	3.76	1.91
Unknown	0.99	5.28

*Summed feature 4 comprised iso-C_{15:0} 2-OH and/or C_{16:1} trans 9.

Aerobic, Gram-positive. Cells grow well but slowly only on Bennett's agar medium, forming branched and non-fragmented substrate hyphae. Sporulation is very weak and, when sporulation occurs on Bennett's agar, the surface of the colony changes to a yellow-brown colour. Produces irregularly shaped spore-chain-like structures with scanty spherical body formation on scanty aerial mycelium on Bennett's agar. Pale brownish soluble pigment is produced only on Bennett's agar after 5 weeks of incubation at 28 °C. No distinct or true aerial hyphae are found on other agar media tested. Colour of substrate mycelium is light yellow to moderate yellowish brown on different agar media. Good growth occurs only on Bennett's agar; moderate growth is seen on ISP 2, ISP 3 and ISP 6 agar media; grows weakly on YS, ISP 7 and water agar media; and no growth on ISP 5 agar medium is seen after 5 weeks of incubation at 28 °C. Optimal growth is at 25–30 °C; no growth is observed at 37 °C. Tolerates up to 3 % NaCl. The results of biochemical tests are as follows: nitrate is not reduced; urea (weak) and starch are hydrolysed; positive for decomposition of arbutin, hypoxanthine, casein, tyrosine and gelatin; and negative for decomposition of Tweens 20 and 80 and adenine, and assimilation of calcium. According to the API

ZYM enzyme assay: positive for alkaline phosphatase, esterase lipase (C₈), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase; weakly positive for esterase (C₄) and valine arylamidase; and negative for lipase (C₁₄), cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase and α-fucosidase. According to the API Coryne enzyme assay: positive for pyrazinamidase, alkaline phosphatase, β-galactosidase and hydrolysis of gelatin; weakly positive for pyrrolidonyl arylamidase and α-glucosidase; negative for β-glucuronidase, N-acetyl-β-glucosaminidase, β-glucosidase (aesculin) and urease; negative for catalase and nitrate reduction; positive for fermentation of glucose, ribose, mannose, maltose and lactose; and negative for fermentation of xylose, sucrose and glycogen. Peptidoglycan contains *meso*-diaminopimelic acid; mycolic acids are absent. The only menaquinone is MK-9(H₄). Characteristic phospholipids are diphosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine and ninhydrin-positive phosphoglycolipids. Major fatty acids are iso-C_{16:0}, iso-C_{15:0}, C_{16:1} H and C_{16:0} 9-methyl; the whole-cell sugars contain glucose, galactose, ribose, arabinose, mannose, rhamnose and xylose (trace).

The type strain is MN08-A0203^T (=NBRC 105883^T =VTCC D9-23^T), isolated from soil of Burenkhan, Khuvsgul province, Mongolia. The DNA G+C content of the type strain is 70.6 mol%.

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