Paenibacillus macquariensis subsp. *defensor* subsp. nov., isolated from boreal soil

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Two Gram-variable, aerobic, motile, rod-shaped, endospore-forming bacterial strains, M4-2^T and M4-1, were isolated from soil samples collected from Oblast Magadan, Russian Far East, as micro-organisms antagonistic to the psychrophilic phytopathogenic fungus *Typhula ishikariensis*. Strains M4-2^T and M4-1 were identified as members of the genus *Paenibacillus* by phenotypic and phylogenetic analyses based on 16S rRNA gene sequences. The strains contained anteiso-C₁₅:0 as the major fatty acid (63.0–64.7 %) and MK-7 as the major isoprenoid quinone. The DNA G + C contents were 42.8 and 41.7 mol%, respectively. 16S rRNA gene sequence analysis showed that strains M4-2^T and M4-1 exhibited high similarities with *Paenibacillus macquariensis* DSM 2^T (99.5 and 99.7 %, respectively) and *Paenibacillus antarcticus* LMG 22078^T (99.4 and 99.5 %, respectively). There were no clear differences in the phenotypic characteristics and chemotaxonomic and phylogenetic data between the novel isolates and *P. macquariensis* DSM 2^T. DNA–DNA hybridization experiments between strain M4-2^T and *P. macquariensis* DSM 2^T and *P. antarcticus* LMG 22078^T revealed reassociation values of 56 and 49 %, respectively. Multilocus sequence analysis confirmed the differences between the new isolates and reference strains that were observed with the DNA–DNA hybridization studies. On the basis of the results described above, it is proposed that the isolates represent a novel subspecies of *P. macquariensis*, *Paenibacillus macquariensis* subsp. *defensor* subsp. nov. The type strain is M4-2^T (=JCM 14954^T=NCIMB 14397^T).

Snow moulds are psychrophilic or psychrotolerant fungal pathogens of perennial grasses and winter cereals in the

Abbreviations: meso-DAP, meso-diaminopimelic acid; ITS, internal transcribed spacer; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains M4-2^T and M4-1 are AB360546 and AB360547, and of strains M4-1 and M4-2^T, *P. macquariensis* DSM 2^T and *P. antarcticus* LMG 22078^T, for the *rpoB* sequences are AB468054–AB468057, for the 16S rRNA gene and 16S–23S ITS sequences are AB468952–AB468955, and for the *gyrB* sequences are AB469015–AB469018, respectively.

A table of DNA–DNA reassociation values and figures showing the macromorphology of *Typhula ishikariensis* co-cultured with *Paenibacillus macquariensis* subsp. *defensor* subsp. nov. and an extended phylogenetic tree of the genus *Paenibacillus* based on 16S rRNA gene sequences are available as supplementary material with the online version of this paper.

northern hemisphere (Hoshino, 2006). Some chemical fungicides have been developed for snow mould diseases, but a surplus of fungicides in the soil can pollute underground waters (Katsura *et al.*, 1994). Studies of the biological control of snow moulds have been carried out using non-pathogenic psychrophilic fungi such as *Typhula phacorrhiza* (Hsiang *et al.*, 1999) and psychrotolerant bacteria such as *Pseudomonas fluorescens* (Oshiman, 2000). However, these candidates are not suitable for processing as biocontrol preparations because of their heat sensitivity. Kim *et al.* (1997) suggested that cold-adapted, endospore-forming bacteria could be potential biocontrol agents against snow moulds.

We have isolated many spore-forming, cold-adapted bacteria from various soil materials in the Arctic and the

frigid zone and we have selected strains that showed antagonistic activities against the psychrophilic fungal pathogen *Typhula ishikariensis*. Strains M4-2^T and M4-1, belonging to the genus *Paenibacillus*, were isolated from the frigid zone. The genus *Paenibacillus* comprises members of rRNA group 3 as defined by Ash *et al.* (1993) and this genus includes several psychrotolerant species from Antarctica, including *P. antarcticus* (Montes *et al.*, 2004), *P. cineris* and *P. cookii* (Logan *et al.*, 2004), *P. macquariensis* (basonym: *Bacillus macquariensis*; Marshall & Ohye, 1966), *P. validus* (Pepi *et al.*, 2005) and *P. wynnii* (Rodríguez-Díaz *et al.*, 2005). Another isolate from Antarctic ice that was phylogenetically related to the genus *Paenibacillus* was described by Christner *et al.* (2001). However, only a few species have been isolated in the Arctic (*P. polymyxa* in Devon Island; Jordan *et al.*, 1978) and the frigid zone (*P. borealis* in Finland; Elo *et al.*, 2001). In this study, we conducted phenotypic characterizations, chemotaxonomic analyses and phylogenetic analyses based on 16S rRNA gene sequences and DNA–DNA reassociation values. The results show that the isolates should be classified in a novel subspecies of *P. macquariensis*.

Strains M4-2^T and M4-1 were isolated from boreal soil samples collected from the Koni Peninsula, Magadan Nature Reserve, Oblast Magadan, Russian Far East. A soil sample (0.5 g) was suspended in sterilized water (5 ml) and treated at 80 °C for 2 h, and serial decimal dilutions of the suspension were mixed with a nutrient agar medium (Eiken Chemical) and plated. After incubation at 10 °C for 14 days, colonies were cultured in 5 ml nutrient broth (Eiken Chemical) at 10 °C with vigorous shaking for 7 days. The fungus *T. ishikariensis* strain Esashi was cultured on potato dextrose agar (Difco) at 10 °C for 14 days. Liquid bacterial inoculum (10 µl) was placed drop-wise on the fungus and the isolated bacterium and fungus were co-cultured at 10 °C for 30 days. Strains M4-2^T and M4-1 inhibited the mycelial growth and sclerotium production of *T. ishikariensis* (see Supplementary Fig. S1,

available in IJSEM Online). As far as is known, the inhibition of sclerotium production by a bacterium has never been demonstrated in previous studies; however, the characteristic disappeared during repeated subcultures.

For the phylogenetic analysis of strains M4-2^T and M4-1, DNA was extracted using a DNA extraction kit (Isoplant II; Nippon Gene), the 16S rRNA gene was amplified by PCR with primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3') and the purified PCR product was sequenced according to the methods of Matsuyama *et al.* (2006). The complete 16S rRNA gene sequence was compiled using GENETYX (Software Development). Multiple alignments of the sequence were performed using CLUSTAL W (Thompson *et al.*, 1994). A phylogenetic tree was constructed by the neighbour-joining method (Kimura, 1983; Saitou & Nei, 1987). Sequence similarities were calculated using GENETYX. The level of DNA–DNA relatedness was determined fluorometrically by the method of Ezaki *et al.* (1989).

Results of the comparative analysis of the 16S rRNA gene sequence of strains M4-2^T (1551 bp) and M4-1 (1548 bp) showed that these strains were phylogenetically affiliated to species of the genus *Paenibacillus*. Similarity with previously reported strains was determined and phylogenetic trees were constructed with the neighbour-joining method (Fig. 1 and Supplementary Fig. S2 in IJSEM Online). Strains M4-2^T and M4-1 were included in a subcluster within the genus *Paenibacillus*, with the closest relative being *P. macquariensis* DSM 2^T. The 16S rRNA gene sequence similarities between strains M4-2^T and M4-1 and *P. macquariensis* DSM 2^T and *P. antarcticus* LMG 22078^T were 99.5 and 99.7%, and 99.4 and 99.5%, respectively. The 16S rRNA gene sequence similarities to the other strains tested were below 96%. Stackebrandt & Goebel (1994) suggested that a sequence similarity greater than 97% indicates conspecificity.

To further verify the taxonomic position of strains M4-2^T and M4-1, DNA–DNA hybridization experiments were

Fig. 1. Phylogenetic tree based on comparative analysis of 16S rRNA gene sequences of strains M4-2^T and M4-1 and phylogenetically related species of the genus *Paenibacillus*, constructed with the neighbour-joining method. Bootstrap values (>50%) based on 1000 resamplings are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

performed. Using DNA from strain M4-2^T as the probe, DNA–DNA reassociation values of 56 and 49% were obtained with *P. macquariensis* DSM 2^T and *P. antarcticus* LMG 22078^T, respectively (Supplementary Table S1). Using DNA from *P. macquariensis* DSM 2^T as the probe with the most closely related three strains, M4-2^T, M4-1 and *P. antarcticus* LMG 22078^T, revealed similar results. These results confirm that strains M4-2^T and M4-1 have a distinct taxonomical position among the recognized species of the genus *Paenibacillus*.

Although the isolates could not be separated from *P. macquariensis* DSM 2^T on the basis of the 16S rRNA gene sequence analysis results, the DNA–DNA reassociation results showed that the isolates were distinct from *P. macquariensis* DSM 2^T. To confirm the above results, multilocus sequence analysis (MLSA) was performed. Sequences of the *rpoB*, 16S rRNA and 16S–23S ITS and *gyrB* genes were obtained for the new isolates, *P. macquariensis* DSM 2^T and *P. antarcticus* LMG 22078^T with the primer sets and amplification conditions described by Dahllöf *et al.* (2000), Xu & Côté (2003) and Yamamoto & Harayama (1995), respectively. The amplified DNA fragments were cloned into a pT7Blue-2 T-vector (Novagen), according to the manufacturer's instructions. Transformants were selected on Luria–Bertani agar plates containing ampicillin (60 µg ml⁻¹), X-Gal (30 µl 0.4% X-Gal per plate) and IPTG (30 µl 0.1 M IPTG per plate). Plasmids were extracted from positive clones and the nucleotide sequences of their inserts were determined with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and analysed using an ABI 3100 sequencer (Applied Biosystems). Neighbour-joining trees of the sequences of the *rpoB*, 16S rRNA and 16S–23S ITS and *gyrB* genes showed that the isolates could be distinguished from *P. macquariensis* DSM 2^T (Fig. 2a, b, c). Comparing strain M4-2^T with strain M4-1, *P. macquariensis* DSM 2^T and *P. antarcticus* LMG 22078^T, the *rpoB* gene sequence similarities were 99.1, 98.9 and 64.6%, the 16S rRNA gene and 16S–23S ITS sequence similarities were 100, 98.0 and 96.8% and the *gyrB* sequence similarities were 99.6, 98.0 and 92.9%, respectively. Thus, the MLSA data supports the DNA–DNA hybridization results.

The morphological, physiological and biochemical characteristics of strains M4-2^T and M4-1 and reference strains were examined according to the methods of Barrow & Feltham (1993) and Montes *et al.* (2004). DNA G+C content was determined according to the method of Tamaoka & Komagata (1984). Whole-cell fatty acid and isoprenoid quinones were analysed as described previously (Yumoto *et al.*, 1998, 2001). *meso*-Diaminopimelic acid (*meso*-DAP) in the cell wall was identified by TLC (Staneck & Roberts, 1974). Cells grown at late-logarithmic to early-stationary phase in nutrient broth (Eiken Chemical) were used for the chemotaxonomic analyses.

The cultural properties and cell morphology of strains M4-2^T and M4-1 are given in the species description. The

physiological characteristics that could be used to differentiate strains M4-2^T and M4-1 from their closest phylogenetic relatives are listed in Table 1. The DNA G+C contents of strains M4-2^T and M4-1 were 42.8 and 41.7 mol%, respectively, which are within the range observed for members of the genus *Paenibacillus* (Shida *et al.*, 1997). The fatty acid analysis of strains M4-2^T and M4-1 (Table 2) revealed that anteiso-C_{15:0} (63.0–64.7%) and iso-C_{15:0} (14.9–15.6%) were the predominant fatty acids. This fatty acid profile was almost the same as those of members of the genus *Paenibacillus* (Ash *et al.*, 1993), and anteiso-C_{15:0} is the predominant fatty acid in all members of the genus *Paenibacillus* (Ash *et al.*, 1993; Shida *et al.*, 1997). Strains M4-2^T and M4-1 contained lower percentages of iso-C_{13:0} and anteiso-C_{13:0} than *P. macquariensis* DSM 2^T and *P. antarcticus* LMG 22078^T. HPLC analysis of isoprenoid quinones isolated from strains M4-2^T and M4-1 revealed that menaquinone-7 was the major quinone. Cell walls from the new isolates contained *meso*-DAP.

All of the characteristics determined for strains M4-2^T and M4-1 are in accordance with those described for the genus *Paenibacillus*. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that the isolates are very similar to *P. macquariensis* DSM 2^T and the paucity of phenotypic and chemotaxonomic differences do not support the assignment of these new strains to a novel species. However, DNA–DNA reassociation and MLSA results indicate that the two isolates are distinct from *P. macquariensis* DSM 2^T. Therefore, we propose to designate them as a subspecies of *P. macquariensis*, according to the species concept of prokaryotes (Rosselló-Mora & Amman, 2001; Stackebrandt *et al.*, 2002).

Description of *Paenibacillus macquariensis* subsp. *defensor* subsp. nov.

Paenibacillus macquariensis subsp. *defensor* [de.fen'sor. L. n. *defensor* (nominative in apposition) defender, protector, because the isolates inhibit the production of sclerotia by the psychrophilic phytopathogenic fungus *Typhula ishikariensis*].

Cells are rod-shaped (0.5–0.9 × 1.9–3.5 µm) and motile by means of peritrichous flagella. Oval spores form centrally in swollen sporangia. Colonies grown on nutrient agar and potato dextrose agar are circular, slightly convex, bright and cream coloured. Cells are facultatively anaerobic and Gram-variable. Cells grow between 4 and 31 °C (optimum 10–15 °C). Oxidase- and catalase-positive. Nitrate reduction, production of dihydroxyacetone and Voges–Proskauer reaction are negative. Positive for β-glucosidase and β-galactosidase. Hydrolyses starch and DNA, but not casein or gelatin. The following substrates are utilized for growth and acid production: ribose, D-fructose, L-arabinose, D-xylose, methyl α-D-xyloside, D-glucose, methyl α-D-glucoside, gluconate, amygdalin, galactose, mannitol, N-acetylglucosamine, arbutin, salicin, lactose, cellobiose, maltose, melibiose, melezitose, D-mannose, sucrose, methyl

Fig. 2. Phylogenetic trees based on sequences of *rpoB* (a), 16S rRNA gene and 16S–23S ITS (b) and *gyrB* (c) from strains M4-2^T and M4-1 and phylogenetically related species of the genus *Paenibacillus*, constructed with the neighbour-joining method. Bootstrap values (>50%) based on 1000 resamplings are shown at branch nodes. Bars, 0.1 substitutions per nucleotide position.

Table 1. Phenotypic characteristics that differentiate strains M4-2^T and M4-1 from type strains of phylogenetically related *Paenibacillus* species

Strains: 1, M4-2^T; 2, M4-1; 3, *P. macquariensis* DSM 2^T; 4, *P. antarcticus* LMG 22078^T. All data are from this study. All strains were negative for casein hydrolysis, gelatin liquefaction, nitrate reduction and Voges–Proskauer test. All strains produced acid from *N*-acetylglucosamine, amygdalin, L-arabinose, cellobiose, D-fructose, galactose, β-gentiobiose, D-glucose, methyl α-D-xyloside, lactose, maltose, D-mannose, melibiose, methyl α-D-glucoside, raffinose, ribose, salicin, starch, sucrose, trehalose, turanose and D-xylose, and none of the strains produced acid from adonitol, D-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, glycerol, inositol, inulin, 2-ketogluconate, 5-ketogluconate, rhamnose, sorbitol, L-sorbose, tagatose, xylitol and L-xylose. All strains hydrolysed starch and DNA and were able to grow at 4–5 °C and at 10 °C. None of the strains grew in medium containing 5 % NaCl or at 32 °C. +, Positive; –, negative.

Characteristic	1	2	3	4
Growth at (°C):				
0–1	–	–	+	–
31	+	+	–	+
Growth with 0.001 % lysozyme	+	+	–	+
Oxidase	+	+	–	+
Acid production from:				
Arbutin	+	+	+	–
L-Fucose	–	–	+	–
Gluconate	+	+	+	–
Glycogen	+	+	+	–
Mannitol	+	+	+	–
Methyl α-D-mannoside	+	+	+	–
Melezitose	+	+	+	–
DNA G + C content (mol%)	42.8	41.7	39.0	40.7

Table 2. Cellular fatty acid compositions (%) of strains M4-2^T and M4-1 and type strains of phylogenetically related *Paenibacillus* species

Strains: 1, M4-2^T; 2, M4-1; 3, *P. macquariensis* DSM 2^T; 4, *P. antarcticus* LMG 22078^T. All data are from this study.

Fatty acid	1	2	3	4
iso-C _{13:0}	<0.2	<0.2	0.7	0.6
anteiso-C _{13:0}	<0.2	<0.2	2.1	0.8
iso-C _{14:0}	2.7	3.1	3.3	5.0
C _{14:0}	1.4	1.3	1.7	3.0
iso-C _{15:0}	15.6	14.9	12.3	12.3
anteiso-C _{15:0}	63.0	64.7	61.3	54.5
C _{15:0}	2.5	1.4	3.8	1.5
iso-C _{16:0}	5.4	6.1	4.8	4.5
iso-C _{16:1}	0.6	0.5	0.5	0.9
C _{16:0}	3.9	3.7	5.1	8.8
C _{16:1}	1.3	0.9	1.6	5.5
iso-C _{17:0}	1.4	1.3	1.2	1.0
anteiso-C _{17:0}	1.9	1.9	1.9	1.6

α-D-mannoside, trehalose, raffinose, turanose, β-gentiobiose, starch and glycogen. Acids are not produced from erythritol, L-xylose, xylitol, adonitol, D-arabinose, D-arabitol, L-arabitol, dulcitol, D-fucose, glycerol, inositol, inulin, 2-ketogluconate, 5-ketogluconate, rhamnose, sorbitol, L-sorbose or tagatose. The cell wall contains *meso*-DAP. The major isoprenoid quinone is MK-7. The major fatty acids are anteiso-C_{15:0} and iso-C_{15:0}.

The type strain, M4-2^T (=JCM 14954^T=NCIMB 14397^T), was isolated from boreal soil collected from the Koni Peninsula, Magadan Nature Reserve, Oblast Magadan, Russian Far East, Russia. The G + C content of the genomic DNA of the type strain is 42.8 mol%.

Description of *Paenibacillus macquariensis* subsp. *macquariensis* subsp. nov.

The creation of *Paenibacillus macquariensis* subsp. *defensor* automatically creates the subspecies *Paenibacillus macquariensis* subsp. *macquariensis* subsp. nov. The description is the same as that given for *Paenibacillus macquariensis* by Marshall and Ohye (1966). The type strain is ATCC 23464^T (=DSM 2^T=LMG 6935^T=NTCT 10419^T).

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