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Paenibacillus tylopili sp.nov., a Chitinolytic Bacterium Isolated from the Mycorhizosphere of *Tylopilus felleus*

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ABSTRACT. Two chitinolytic bacterial strains (designated MK2^T and V7) were isolated from the mycorhizosphere of the fungus *Tylopilus felleus*. The strains were facultatively anaerobic G^+ endospore formers. Physiological analysis and 16S rRNA gene PCR-RFLP assays revealed nearly identical profiles for both strains, demonstrating their relationship at the species level. Sequences specific for the genus *Paenibacillus* were found within the 16S rRNA gene sequence of the strain MK2^T. The 16S rRNA gene sequence showed the highest similarity to the sequences of *Paenibacillus amylolyticus*, *P. pabuli* and *P. xylanilyticus*. DNA–DNA relatedness of the strain with the type strain of *P. amylolyticus* was 4.95 %, of *P. pabuli* 38.0 %, and of *P. xylanilyticus* 46.3 %, indicating no relatedness between MK2^T and any of them at the species level. The most abundant fatty acids in strains MK2^T and V7 were *anteiso*-$C_{15:0}$, *iso*-$C_{16:0}$, *iso*-$C_{15:0}$ and *n*-$C_{16:0}$. DNA–DNA relatedness, morphological, physiological and chemotaxonomic analyses, and phylogenetic data based on 16S rRNA gene sequencing made it possible to describe both strains as the novel species of the genus *Paenibacillus*, for which the name *Paenibacillus tylopili* is proposed, the type strain being MK2^T (DSM 18927^T, LMG 23975^T).

Abbreviations

ARDRA	amplified 16S rDNA restriction analysis	TSA	tryptone soya agar
DSMZ	*Deutsche Sammlung von Mikroorganismen und Zellkulturen*	TSB	tryptone soya broth
FA(s)	fatty acid(s)	TSBA	trypticase soya broth agar

One distinctive characteristic of the genus *Paenibacillus* is the ability to excrete a wide variety of enzymes that degrade natural biopolymers (Shida *et al.* 1997*a*). Some species of this genus (*P. thiaminolyticus*, *P. macerans*, *P. alvei*, *P. koreensis*, *P. borealis, P. chitinolyticus*, *P. anaericanus*) are characterized by their ability to degrade chitin (Shida *et al.* 1997*a*).

Within the framework of a screening program to search for chitin-degrading microorganisms, strains MK2^T and V7 were isolated from the mycorhizosphere of the fungus *Tylopilus felleus* (BULL.) P. KARST. Here we describe these chitinolytic strains as members of the novel species *P. tylopili*.

MATERIALS AND METHODS

Isolation of chitin-degrading strains, morphological and physiological characterization. The chitinolytic strains were isolated in a medium supplemented with colloidal chitin (Sutrisno *et al.* 2004). The ability to utilize *N*-acetylglucosamine was also tested. The strains were cultivated at 25 °C on TSA for 1 d. The presence and morphology of endospores were checked in cells grown for two weeks on TSA. The temperature range for the growth was determined in TSB buffered with 50 mmol/L Tris-HCl buffer (pH 8.0). For determining the influence of pH, TSB was buffered with citrate–phosphate buffer (pH 6.0) and 50 mmol/L Tris-HCl buffer (pH 6.5–9.0). Growth was monitored by measuring the absorbance A_{600}. Most of the physiological tests were done according to Claus and Berkeley (1986).

Chemotaxonomic characterization. The diagnostic cell-wall diamino acid was determined according to Schleifer (1985). Cells for cellular FA analysis were harvested from 1-d cultures grown at 28 °C on TSBA. FAs were extracted and analyzed following the instructions of the *Microbial Identification System* operating manual (MIDI 1999).

ARDRA was performed with *Alu*I, *Hae*II, *Hae*III, *Rsa*I and *Taq*I (Kuisiene *et al*. 2002).

16S rRNA gene analysis. DNA extraction, amplification and cloning of the 16S rRNA gene were done according to Kuisiene *et al.* (2002). Sequences of the gene sequences were edited and sequence similarity was determined using the Seqbuilder and Megalign components of Lasergene 6 (DNAStar). The 16S rDNA sequence of strain MK2^T was aligned with the sequence of *Escherichia coli* (accession no. J01695), and nucleotides were determined in diagnostic positions (Ash *et al.* 1993).

Phylogenetic analysis. The 16S rRNA gene sequences were aligned using the Mega 3.1 program (Kumar *et al*. 2004). Phylogenetic tree was constructed using the Mega 3.1 program by the neighbor-joining method (Saitou and Nei 1987). Bootstrap analysis of the data (using 1000 resamplings) was carried out to evaluate the validity and reliability of the tree topology.

Determination of G+C content and DNA–DNA hybridization. G+C content of strain MK2^T was determined by HPLC (Mesbah *et al.* 1989), DNA–DNA hybridization (De Ley *et* al. 1970) was performed under optimum conditions (2× SSC at 67 °C) using a *Cary* 100Bio spectrophotometer.

RESULTS AND DISCUSSION

Two chitinolytic strains (MK2^T and V7) were isolated from the mycorhizosphere of *T. felleus*. The strains could utilize chitin but not *N*-acetylglucosamine. Electron-microscopic examination of both strains showed a typical G^+-cell envelope profile, although the cells were G^- in the KOH test and $G^\pm$ in the routine Gram-staining. Morphological characterization was identical for both strains, the physiological characteristics were highly similar, confirming that they belong to a single species (Table I).

Table I. Differentiating characteristics of *P. tylopili* and the most phylogenetically related species *1–5*[a] of the genus *Paenibacillus*[b,c]

Characteristic	*P. tylopili*	*1*	*2*	*3*	*4*	*5*
Growth at 50 °C	–	–	–	–	+	–
pH 5.6	–	+	+	+	+	–
Optimum growth temperature, °C	25	37	28–30	nd	37	37
pH optimum	8.0	7.0	nd	nd	7.0	7.0
Hydrolysis of casein	–	w	v	–	w	–
starch	+	+	+	–	+	+
gelatin	–	+	+	+	+	+
Degradation of chitin	+	nd	–	nd	nd	nd
Nitrate reduction	–	+	–	–	–	–
Tolerance of 5 % NaCl	v	–	v	+	–	+
Production of acid from lactose	+	–	+	+	–	nd
from raffinose	+	–	+	+	+	nd
G+C content, molar %	44.3	46.3–46.6	48.0–50.0	45.0	47.6–48.3	50.5

[a]1 – *P. amylolyticus* (Shida *et al*. 1997*b*) 2 – *P. pabuli* (Nakamura 1984; Shida *et al*. 1997*a*; Sánchez *et al*. 2005)
3 – *P. barcinonensis* (Sánchez *et al*. 2005) 4 – *P. illinoisensis* (Shida *et al*. 1997*b*)
5 – *P. xylanilyticus* (Rivas *et al*. 2005).
[b]All species are facultatively anaerobic, motile, positive for catalase activity and acid production from L-arabinose, glucose, maltose, mannitol, mannose, sucrose and D-xylose, and negative for urease production.
[c](+) – positive v – variable (–) – negative w – weakly positive nd – not determined.

The electrophoretic profiles (performed in order to find genomic diversity of strains MK2^T and V7) of *Rsa*I (ARDRA) differed in two bands. The patterns of *Alu*I, *Hae*II, *Hae*III and *Taq*I were identical, indicating the close genetic relationship between these strains (Fig. 1, results for *Hae*II *not shown*).

The length of 16S rRNA gene sequence of strain MK2^T was 1472 nucleotides. The sequence has been deposited in *GenBank* (EF206295). A number of nucleotides in potentially diagnostic positions were identified. Strain MK2^T belongs to the genetic group 3 of endospore-forming bacteria. Sequences specific for the genus *Paenibacillus*, the PAEN515F primer-binding site (Shida *et al*. 1997*a*) and BG3 (Ash *et al.* 1993) were found within the 16S rRNA gene sequence of our strain, indicating that the strain belongs to the genus *Paenibacillus*. The sequence was most similar to those of *P. amylolyticus* DSM 11730^T, *P. pabuli* DSM 3036^T and *P. xylanilyticus* DSM 17255^T (98.9, 97.8 and 96.9 %, respectively). The phylogenetic tree (Fig. 2) shows the phylogenetic position of this strain among the closely related species of the genus *Paenibacillus*.

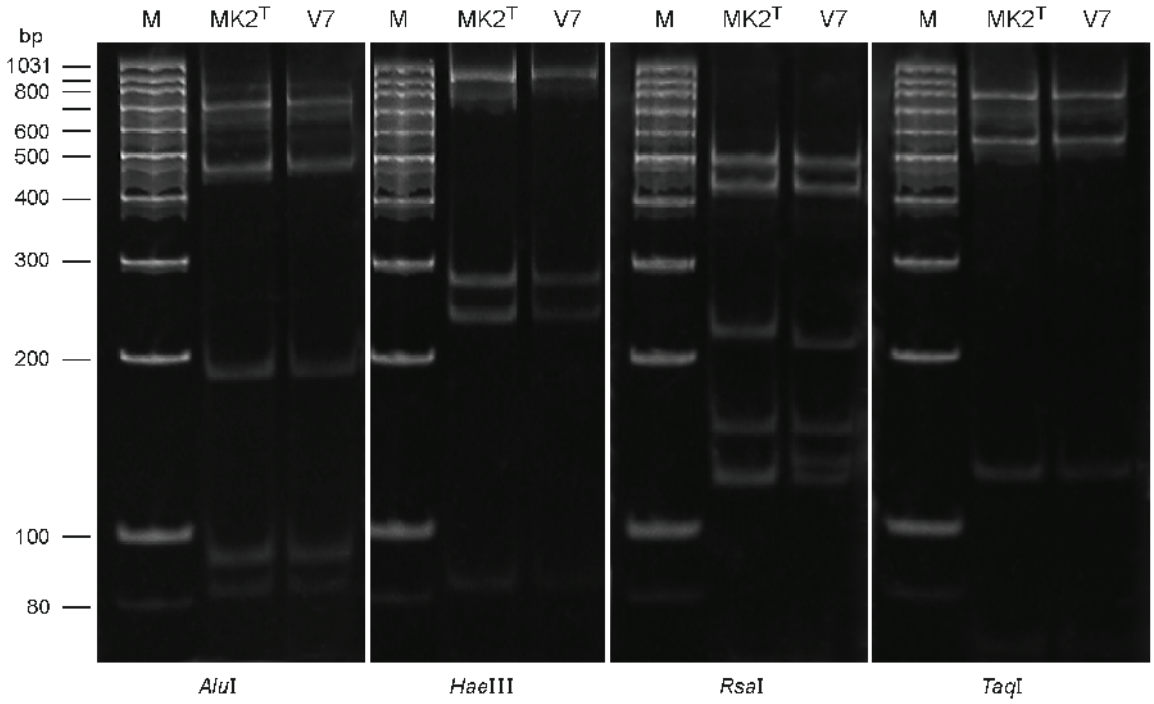

Fig. 1. ARDRA (*Alu*I, *Hae*III, *Rsa*I and *Taq*I) gel-electrophoretic profiles of strains MK2^T and V7; M – GeneRuler^{TM} 100 bp DNA ladder (*Fermentas*).

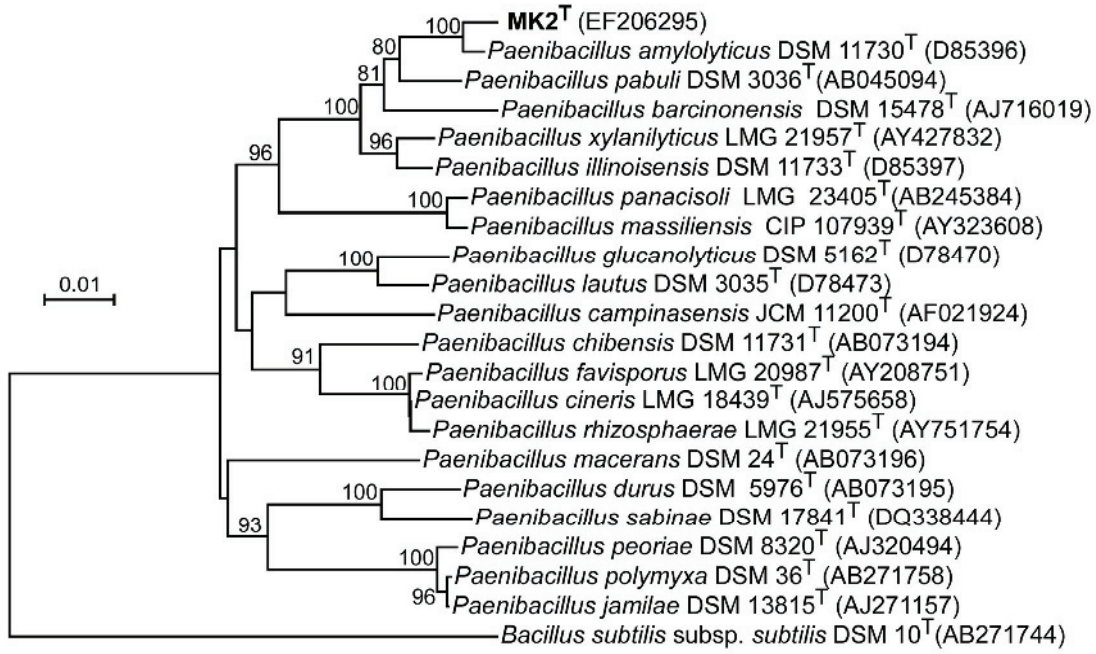

Fig. 2. Phylogenetic position of strain MK2^T among *Paenibacillus* species on the basis of 16S rRNA gene sequences; *numbers* at the *branches* – bootstrap values (%) from 1000 samplings (only the most significant values are shown). *B. subtilis* subsp. *subtilis* DSM 10^T defined as outgroup of the tree; *bar* – 0.01 nucleotide substitution per site.

DNA–DNA relatedness of the strain MK2^T with the type strain of *P. amylolyticus* was 4.95 %, with *P. pabuli* 38.0 %, and with *P. xylanilyticus* 46.3 %, indicating no relatedness between MK2^T and any of them at the species level.

anteiso-$C_{15:0}$-FA is the major cellular FA found in all members of the genus *Paenibacillus* (Ash *et al.* 1993; Shida *et al*. 1997*a*). The other major FAs of the genus are *iso*-$C_{16:0}$, *iso*-$C_{15:0}$ and *anteiso*-$C_{17:0}$ (Kämpfer 2002). The most abundant FA of strains MK2^T and V7 was also *anteiso*-$C_{15:0}$ (57.8 and 65.9 %, res-

pectively) (Table II). The other major FAs were *iso*-$C_{16:0}$, *iso*-$C_{15:0}$ and *n*-$C_{16:0}$. The value of *anteiso*-$C_{15:0}$ in MK2^T and V7 was higher than that determined for the phylogenetically related species except *P. pabuli*. Both our strains contained smaller amounts of the FA *ω*-5-*cis*-$C_{16:1}$ than the related species (Table II).

Table II. Cellular FA composition (%) of *Paenibacillus tylopili* MK2^T, *P. tylopili* V7 and the most phylogenetically related species *1–5*[a] of the genus *Paenibacillus*[b]

Fatty acid	MK2^T	V7	*1*	*2*	*3*	*4*	*5*
iso-$C_{13:0}$	0.28	–	0.24	0.14	0.15	0.18	0.14
anteiso-$C_{13:0}$	0.34	0.30	0.46	0.16	0.26	0.20	0.20
iso-$C_{14:0}$	3.87	3.60	3.47	6.74	2.75	2.81	2.35
n-$C_{14:0}$	3.58	2.75	9.61	5.40	2.32	2.39	2.32
iso-$C_{15:0}$	10.5	6.35	6.04	11.1	7.07	9.38	7.35
anteiso-$C_{15:0}$	57.8	65.9	52.4	40.4	55.3	59.3	54.0
n-$C_{15:0}$	0.82	1.23	0.78	1.38	0.77	0.28	1.27
ω-9-*cis*-$C_{16:1}$[c]	0.15	0.31	0.71	0.59	0.26	0.23	0.28
iso-$C_{16:0}$	5.41	5.25	2.39	10.0	7.51	5.78	6.55
ω-5-*cis*-$C_{16:1}$	3.15	3.65	12.7	10.3	5.25	4.14	6.64
n-$C_{16:0}$	9.00	5.76	8.81	9.44	9.28	7.81	8.89
cis-7-*iso*-$C_{17:1}$	–	0.29	0.40	0.39	0.28	0.35	0.43
iso-$C_{17:0}$	2.58	1.18	0.68	2.08	3.33	3.41	3.74
anteiso-$C_{17:0}$	2.47	3.36	1.30	1.77	5.46	3.76	5.55
n-$C_{18:0}$	–	–	–	–	–	–	0.16

[a] *1 – P. amylolyticus* DSM 11730^T *2 – P. barcinonensis* DSM 15478^T *3 – P. illinoisensis* DSM 11733^T
4 – P. pabuli DSM 3036^T *5 – P. xylanilyticus* DSM 17255^T
[b] All strains harvested after 1 d at 28 °C on TSBA. [c] Alcohol.

We therefore conclude that strains MK2^T and V7 represent the novel species of the genus *Paenibacillus*. We describe MK2^T as the type strain of this novel species, for which we propose the name *Paenibacillus tylopili* sp.nov.

Description of Paenibacillus tylopili *sp.nov.*: *Paenibacillus tylopili* [ty·lo·'pi·li NL masc. n. *Tylopilus*] – taxonomic name of a genus of fungus, from the mycorhizosphere of which the organism was isolated (Table III). G^+-cells are rod-shaped, motile, varying in length 3.0–5.9 μm and in diameter 1.2–1.6 μm. Oval central endospores are produced within the swollen sporangia. Colonies (grown for 2 d on TSA at 25 °C) are

Table III. Physiological and biochemical characteristics of *P. tylopili*

Temperature for growth		Catalase	+
optimum	25 °C	Urease	–
minimum	9 °C	Phenylalanine	not deaminated
variable growth	40 °C	Tyrosine	not decomposed
		Casein	not hydrolyzed
Range of pH for growth	6.0–9.0	Gelatin	not hydrolyzed
optimum	8.0	Starch	hydrolyzed
		Chitin	degraded
Tolerance to NaCl (%)		*N*-Acetylglucosamine	not utilized
0.5–3	good growth	Indole production	negative
5	variable	Nitrate reduction	negative
7	no growth	Voges–Proskauer	negative
		Arginine dihydrolase	negative
Growth in Sabouraud dextrose broth	negative	Lysine decarboxylase	negative
		Ornithine decarboxylase	negative
Acid production from	L-arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, raffinose, ribose, sucrose, D-xylose		
Not used as sole C source	galactitol, *myo*-inositol, rhamnose, glucitol		

1–2 mm in diameter, round, slightly raised, whitish, translucent and glossy, facultatively anaerobic. The diamino acid in the cell wall is *meso*-2,6-diaminopimelic acid. The most abundant FAs are *anteiso*-$C_{15:0}$, *iso*-$C_{16:0}$, *iso*-$C_{15:0}$ and *n*-$C_{16:0}$. The DNA G+C content is 44.3 % (mol/mol). The type strain is MK2^T, which has been deposited in the DSMZ as DSM 18927^T and in the *Belgian Coordinated Collections of Microorganisms* as LMG 23975^T.

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REFERENCES

ASH C., PRIEST F.G., COLLINS M.D.: Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus*. *Antonie van Leeuwenhoek* **64**, 253–260 (1993).

CLAUS D., BERKELEY R.C.W.: Genus *Bacillus* COHN 1872, pp. 1105–1139 in P.H.A. Sneath, N.S. Mair, M.E. Sharpe, J.G. Holt (Eds): *Bergey's Manual of Systematic Bacteriology*. Williams & Wilkins, Baltimore 1986.

DE LEY J., CATTOIR H., REYNAERTS A.: The quantitative measurement of DNA hybridization from renaturation rates. *Eur.J.Biochem.* **12**, 133–142 (1970).

KÄMPFER P.: Whole-cell fatty acid analysis in the systematics of *Bacillus* and related genera, pp. 271–299 in R. Berkeley, M. Hendrickx, N. Logan, P. De Vos (Eds): *Applications and Systematics of* Bacillus *and Relatives.* Blackwell Science, Oxford (UK) 2002.

KUISIENE N., JOMANTIENE R., VALIUNAS D., CHITAVICHIUS D.: Characterization of thermophilic proteolytic spore-forming bacteria from a geothermal site in Lithuania based on 16S rDNA RFLP and ITS-PCR analyses. *Microbiology* **71**, 712–716 (2002).

KUMAR S., TAMURA K., NEI M.: MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform.* **5**, 150–163 (2004).

MESBAH M., PREMACHANDRAN U., WHITMAN W.B.: Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. *Internat.J.Syst.Bacteriol.* **39**, 159–167 (1989).

MIDI: *Sherlock Microbial Identification System, Operating Manual*, ver. 3.0. MIDI Inc., Newark 1999.

NAKAMURA L.K.: *Bacillus amylolyticus* sp.nov., nom.rev., *Bacillus lautus* sp.nov., nom.rev., *Bacillus pabuli* sp.nov., nom.rev., and *Bacillus validus* sp.nov., nom.rev. *Internat.J.Syst.Bacteriol.* **34**, 224–226 (1984).

RIVAS R., MATEOS P.F., MARTÍNEZ-MOLINA E., VELÁZQUEZ E.: *Paenibacillus xylanilyticus* sp.nov., an airborne xylanolytic bacterium. *Internat.J.Syst.Evol.Microbiol.* **55**, 405–408 (2005).

SAITOU N., NEI M.: The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol.Biol.Evol.* **4**, 406–425 (1987).

SÁNCHEZ M.M., FRITZE D., BLANCO A., SPRÖER C., TINDALL B.J., SCHUMANN P., KROPPENSTEDT R.M., DIAZ P., PASTOR F.I.J.: *Paenibacillus barcinonensis* sp.nov., a xylanase-producing bacterium isolated from a rice field in the Ebro River delta. *Internat.J.Syst.Evol.Microbiol.* **55**, 935–939 (2005).

SCHLEIFER K.-H.: Analysis of the chemical composition and primary structure of murein. *Meth.Microbiol.* **18**, 123–156 (1985).

SHIDA O., TAKAGI H., KADOWAKI K., NAKAMURA L.K., KOMAGATA K.: Transfer of *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus curdlanolyticus*, *Bacillus glucanolyticus*, *Bacillus kobensis*, and *Bacillus thiaminolyticus* to the genus *Paenibacillus* and emended description of the genus *Paenibacillus*. *Internat.J.Syst.Bacteriol.* **47**, 289–298 (1997*a*).

SHIDA O., TAKAGI H., KADOWAKI K., NAKAMURA L.K., KOMAGATA K.: Emended description of *Paenibacillus amylolyticus* and description of *Paenibacillus illinoisensis* sp.nov. and *Paenibacillus chibensis* sp.nov. *Internat.J.Syst.Bacteriol.* **47**, 299–306 (1997*b*).

SUTRISNO A., UEDA M., ABE Y., NAKAZAWA M., MIYATAKE K.: A chitinase with high activity toward partially *N*-acetylated chitosan from a new, moderately thermophilic, chitin-degrading bacterium, *Ralstonia* sp. A-471. *Appl.Microbiol.Biotechnol.* **63**, 398–406 (2004).