Paenibacillus castaneae sp. nov., isolated from the phyllosphere of *Castanea sativa* Miller

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A bacterial strain, designated Ch-32^T, was isolated from the phyllosphere of *Castanea sativa* in Spain. Phylogenetic analysis based on 16S rRNA gene sequences placed the isolate in the genus *Paenibacillus* within the same subgroup as *Paenibacillus xinjiangensis* and *Paenibacillus glycanilyticus*, with similarities of 96.3 and 96.8 %, respectively. DNA–DNA hybridization values for strain Ch-32^T with these two species were lower than 20 %. The novel isolate was a Gram-variable, motile, sporulating rod. It produced catalase and oxidase and hydrolysed cellulose, gelatin and aesculin. Acetoin and urease production, nitrate reduction and starch hydrolysis were negative. Growth was supported by many carbohydrates and organic acids as carbon sources. MK-7 was the only menaquinone detected and anteiso-C_{15:0}, iso-C_{16:0} and C_{16:0} were the major fatty acids. The DNA G + C content was 46 mol%. Phylogenetic, DNA relatedness and phenotypic analyses showed that strain Ch-32^T should be classified as a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus castaneae* sp. nov. is proposed; the type strain is Ch-32^T (=CECT 7279^T=DSM 19417^T).

The sweet chestnut (*Castanea sativa* Miller) has been cultivated systematically throughout Europe since the Middle Ages (Conedera *et al.*, 2004) and covers large areas in the Mediterranean basin and the UK, being used for both nut and wood production (Fernández-López & Alía, 2003). The leaves of the sweet chestnut remain on the tree for some time after drying and this stage may be considered to be the first step in degradation processes in which polymer-degrading bacteria are involved. In the case of the phyllosphere of palm tree bracts, several of these bacteria have been found (Rivas *et al.*, 2007), mostly belonging to the genus *Paenibacillus*, e.g. *Paenibacillus phyllosphaerae* and *Paenibacillus cellulosilyticus* (Rivas *et al.*, 2005, 2006). In this study, a bacterial strain, Ch-32^T, was isolated from the phyllosphere of *Castanea sativa* growing in the Sierra de Francia (Salamanca, Spain) and, based on genotypic and phenotypic characterization, it should be classified as a novel species of the genus *Paenibacillus*.

Strain Ch-32^T was isolated as follows. Chestnut leaves were immersed in 50 ml pre-chilled buffer (0.1 M potassium

phosphate, pH 7.0; 0.1 % peptone) and sonicated for 10 min in an ultrasonic bath; samples (0.1 ml) were then plated on tryptic soy agar medium containing cycloheximide (0.15 mg ml⁻¹) and incubated at 30 °C. The isolate was subcultured through two rounds of single-colony purification and subsequently stored at –70 °C in 15 % glycerol. On nutrient agar, colonies of strain Ch-32^T were yellow, round, smooth and convex with approximate diameters of 1–3 mm.

Strain Ch-32^T was grown in nutrient agar for 48 h at 22 °C to check for motility by phase-contrast microscopy using the hanging drop method. Gram staining was carried out by the procedure described by Doetsch (1981) after 24 h incubation at 30 °C. The flagellation type was determined by electron microscopy after 48 h incubation of strain Ch-32^T in nutrient agar at 22 °C as described previously (Rivas *et al.*, 2005). Strain Ch-32^T was Gram-variable and motile by means of polar and subpolar flagella. Ellipsoidal subterminal spores were formed in swollen sporangia.

The 16S rRNA gene of strain Ch-32^T was analysed as described by Rivas *et al.* (2007). The sequence obtained was compared with those from GenBank using the programs BLASTN (Altschul *et al.*, 1990) and EZTAXON (Chun *et al.*, 2007). Sequences were aligned using CLUSTAL_X software (Thompson *et al.*, 1997). Distances were calculated according to Kimura's two-parameter method (Kimura, 1980). The

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *P. castaneae* Ch-32^T is EU099594.

An extended phylogenetic tree constructed using the neighbour-joining method and based on 16S rRNA gene sequences of *Paenibacillus castaneae* Ch-32^T and related species of *Paenibacillus* and *Cohnella* is available with the online version of this paper.

phylogenetic tree was inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was based on 1000 resamplings. The MEGA2.1 software package (Kumar *et al.*, 2001) was used for all analyses. The resulting neighbour-joining tree based on 16S rRNA gene sequences is shown in Fig. 1 (an extended tree, supplementary Fig. S1, is available in IJSEM Online). Similar results were obtained using the maximum-parsimony method (data not shown). The results of the phylogenetic analysis indicated that strain Ch-32^T is related to members of the genus *Paenibacillus*. Strain Ch-32^T formed a cluster with *Paenibacillus glycanilyticus* DS-1^T, *Paenibacillus xinjiangensis* B538^T, *Paenibacillus alkaliterrae* KSL-134^T, *Paenibacillus agarexedens* DSM 1327^T and *Paenibacillus agaridevorans* DSM 1355^T with gene sequence similarities of 97.7, 97.0, 96.9, 96.5 and 96.2 %, respectively.

For base composition analysis, DNA was prepared according to Chun & Goodfellow (1995). The DNA G+C content of strain Ch-32^T was 46.0 mol%, determined using the thermal denaturation method (Mandel & Marmur, 1968). DNA–DNA hybridization was performed by using the method of Ezaki *et al.* (1989) following the recommendations of Willems *et al.* (2001). DNA–DNA relatedness values for strain Ch-32^T with *P. glycanilyticus* IFO 16618^T and *P. xinjiangensis* DSM 16970^T were lower than 20 %; this suggested that the isolate was not closely related to known species according to the current species concept (Wayne *et al.*, 1987).

Chemotaxonomic analyses were carried out by the Identification Service of the DSMZ (Braunschweig,

Germany). Respiratory quinones were analysed as described by Tindall (1990) and cellular fatty acids were analysed according to the instructions of the Microbial Identification System (MIDI; Microbial ID). The menaquinone MK-7 was the only major respiratory quinone detected in strain Ch-32^T. The fatty acid profile comprised anteiso-C_{15:0} (65.17 %), C_{16:0} (9.53 %), iso-C_{16:0} (8.67 %), iso-C_{15:0} (5.42 %), anteiso-C_{17:0} (4.20 %), iso-C_{14:0} (2.10 %), C_{15:0} (1.92 %), C_{14:0} (1.55 %) and iso-C_{17:0} (1.45 %). The fatty acid profile of strain Ch-32^T differed from those of the phylogenetically related species *P. glycanilyticus* IFO 16618^T in the proportions of both anteiso-C_{15:0} and iso-C_{16:0} and it differed from that of *P. xinjiangensis* DSM 16970^T in the proportion of anteiso-C_{15:0} (Table 1).

Phenotypic characterization was performed according to standard methods described by Claus & Berkeley (1986) and Logan & Berkeley (1984) and by using the API 50CH (with CHB/E medium), API 20NE and API 20E systems (bioMérieux) according to the manufacturer's instructions. Anaerobic growth was tested in fluid tetrathionate medium (Sigma). Acetoin production, the ability to grow in 2, 5 and 7 % NaCl, nitrate reduction and phenylalanine deaminase, catalase, caseinase and oxidase activities were analysed as described by Claus & Berkeley (1986). Acid production from glucose, xylose, mannitol and L-arabinose, and gas from glucose were analysed in liquid medium as described by Claus & Berkeley (1986). Amylases and cellulases were analysed as described previously (Rivas *et al.* 2003). Growth was determined in temperatures ranging from 4 to 45 °C in YED medium (0.5 % yeast extract, 0.7 % glucose and 2 % agar). Growth at pH 5.7 and 6.8 was tested as described by

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the relatedness between *Paenibacillus castaneae* Ch-32^T and representative species of the genus *Paenibacillus*. *Lactobacillus delbrueckii* subsp. *lactis* DSM 20072^T was used as outgroup. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 2 nt substitution per 100 nt.

Table 1. Cellular fatty acid composition (%) of strain Ch-32^T and related type strains

Strains: 1, strain Ch-32^T (data from this study); 2, *P. glycanilyticus* DS-1^T (data from this study); 3, *P. xinjiangensis* B538^T (Lim *et al.*, 2006). ND, Not detected.

Fatty acid	1	2	3
Saturated straight-chain			
C _{14:0}	1.55	1.52	1.93
C _{15:0}	1.92	2.10	3.44
C _{16:0}	9.53	8.71	14.43
C _{16:0} 3-OH	ND	ND	ND
C _{17:0}	ND	0.22	ND
C _{18:0}	ND	0.40	ND
Saturated iso-branched			
C _{14:0}	2.10	3.33	2.12
C _{15:0}	5.42	6.24	9.32
C _{16:0}	8.67	23.79	9.62
C _{17:0}	1.45	1.75	3.54
Saturated anteiso-branched			
C _{15:0}	65.17	47.50	48.61
C _{17:0}	4.20	3.53	4.40
Unsaturated			
C _{16:1} ω7c	ND	ND	0.53

Claus & Berkeley (1986). Growth at pH 7–8 was tested in YED medium containing 100 mM Na₂HPO₄/NaH₂PO₄ and growth at pH 9 and 10 was tested in the same medium containing 100 mM NaHCO₃/Na₂CO₃. Results are given in the species description below. Strain Ch-32^T could be differentiated from the closely related species *P. glycanilyticus* by growth at 40 °C, gelatin and starch hydrolysis, and assimilation of fructose, *N*-acetylglucosamine, adipate and phenylacetate as carbon sources, and from *P. xinjiangensis* by its ability to grow at pH 5.7, acetoin production, gelatin hydrolysis, and assimilation of fructose, mannitol, adipate and phenylacetate as carbon sources (Table 2).

Therefore, strain Ch-32^T can be differentiated genotypically and phenotypically from previously described species and it is proposed that it should be classified as a novel species, *Paenibacillus castaneae* sp. nov.

Description of *Paenibacillus castaneae* sp. nov.

Paenibacillus castaneae (cas.ta.ne'ae. L. gen. n. *castaneae* of a chestnut tree).

Cells of the single isolate of this species (strain Ch-32^T) are Gram-variable and motile by means of polar and subpolar flagella. No chains are formed. Ellipsoidal subterminal spores are formed in swollen sporangia. Catalase- and oxidase-positive. Colonies on nutrient agar medium are yellow, round, smooth and convex with approximate

Table 2. Differential phenotypic characteristics of *Paenibacillus castaneae* sp. nov. and phylogenetically related species of the genus *Paenibacillus*

Species: 1, *P. castaneae* Ch-32^T (data from this study); 2, *P. glycanilyticus* [data from Dasman *et al.* (2002) and Yoon *et al.* (2005) for the type strain]; 3 *P. xinjiangensis* (Lim *et al.*, 2006); 4, *P. alkaliterrae* (Yoon *et al.*, 2005); 5, *P. agarixedens* [Uetanabaro *et al.* (2003) and Yoon *et al.* (2005) for the type strain]; 6, *P. agaridevorans* [Uetanabaro *et al.* (2003) and Yoon *et al.* (2005) for the type strain]. +, Positive reaction; –, negative reaction; w, weakly positive reaction; ND, not determined; v, variable reaction. Data in parentheses are for type strains (Yoon *et al.*, 2005). All species are negative for anaerobic growth and nitrate reduction.

Characteristic	1	2	3	4	5	6
Gram stain	v	+	+	+	v/–	v/–
Colony colour	Yellow	Pinkish yellow	White–cream	White–cream	White–cream	White–cream
Oxidase	+	+*†	+†	+	+	+
Growth at:						
pH 5.7	+	+	–	–	–	–
40 °C	–	+	–†	–	v (–)	–
Voges–Proskauer reaction	–	–	+†	–	–	–
Hydrolysis of:						
Gelatin	+	–	–†	–	–	–
Starch	–‡	+	–	+	+	–‡
Assimilation of:						
D-Mannose	+	+	+	+	+†	(–)
Fructose	–	+	+	+	(–)	(–)
Mannitol	+	+	–	–†	–†	ND
N-Acetylglucosamine	–	+	w†	–†	w†	ND

*Oxidase was reported as negative by Yoon *et al.* (2005).

†Data from this study.

‡Negative in flooding with Lugol's iodine solution.

diameters of 1–3 mm. The only major quinone is MK-7. The main fatty acids are anteiso-C_{15:0}, iso-C_{16:0} and C_{16:0}. Negative in tests for anaerobic growth. Grows at pH 5.7; optimal pH is 7. Grows in 2 % NaCl, but not in 5 % NaCl. Optimal growth temperature is 30 °C; unable to grow at 40 °C. Nitrate is not reduced to nitrite. Produces β -galactosidase, but not acetoin, indole, phenylalanine deaminase, urease, arginine dihydrolase, ornithine decarboxylase or lysine decarboxylase. Hydrolyses aesculin, gelatin and cellulose, but not starch or casein. In the API 20NE system, positive for assimilation of glucose, L-arabinose, mannose, mannitol, maltose and gluconate, but negative for that of N-acetylglucosamine, caprate, citrate, adipate, L-malate and phenylacetate. Acid, but not gas, is produced from glucose. In the API 50CH system, positive for acid production from L-arabinose, D-xylose, methyl β -D-xyloside, galactose, D-glucose, mannose, L-rhamnose, mannitol, maltose, melibiose, sucrose, trehalose, raffinose, gentiobiose and turanose, but negative for acid production from glycerol, erythritol, D-ribose, L-xylose, adonitol, fructose, L-sorbose, inositol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, N-acetylglucosamine, amygdalin, cellobiose, melezitose, glycogen, xylitol, L-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol and L-arabitol. Acid production from salicin and lactose is weak.

The type strain is Ch-32^T (=CECT 7279^T=DSM 19417^T), isolated from the phyllosphere of chestnut trees growing in the Sierra de Francia region, Salamanca province, Spain. The G + C content of the type strain is 46.0 mol%.

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References

- 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.
- Chun, J. & Goodfellow, M. (1995). A phylogenetic analysis of the genus *Nocardia* with 16S rRNA gene sequences. *Int J Syst Bacteriol* **45**, 240–245.
- Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y.-W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.
- Claus, D. & Berkeley, R. C. W. (1986). Genus *Bacillus* Cohn 1872, 174^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1105–1140. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Conedera, M., Krebs, P., Tinner, W., Pradella, M. & Torriani, D. (2004). The cultivation of *Castanea sativa* (Mill.) in Europe, from its origin to its diffusion on a continental scale. *Veg Hist Archaeobot* **13**, 161–179.
- Dasman, Kajiya, S., Kawasaki, H., Yagi, M., Seki, T., Fukusaki, E. & Kobayashi, A. (2002). *Paenibacillus glycanilyticus* sp. nov., a novel species that degrades heteropolysaccharide produced by the cyanobacterium *Nostoc commune*. *Int J Syst Evol Microbiol* **52**, 1669–1674.
- Doetsch, R. N. (1981). Determinative methods of light microscopy. In *Manual of Methods for General Bacteriology*, pp. 21–33. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution 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.
- Fernández-López, J. & Alía, R. (2003). *EUFORGEN Technical Guidelines for genetic conservation and use for chestnut (Castanea sativa)*. Rome, Italy: International Plant Genetic Resources Institute.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.
- Lim, J. M., Jeon, C. O., Park, D. J., Xu, L. H., Jiang, C. L. & Kim, C. J. (2006). *Paenibacillus xinjiangensis* sp. nov., isolated from Xinjiang province in China. *Int J Syst Evol Microbiol* **56**, 2579–2582.
- Logan, N. A. & Berkeley, R. C. W. (1984). Identification of *Bacillus* strains using the API system. *J Gen Microbiol* **130**, 1871–1882.
- Mandel, M. & Marmur, J. (1968). Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol* **12B**, 195–206.
- Rivas, R., Sánchez, M., Trujillo, M. E., Zurdo-Piñeiro, J. L., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2003). *Xylanimonas cellulositytica* gen. nov., sp. nov., a xylanolytic bacterium isolated from a decayed tree (*Ulmus nigra*). *Int J Syst Evol Microbiol* **53**, 99–103.
- Rivas, R., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2005). *Paenibacillus phyllosphaerae* sp. nov., a xylanolytic bacterium isolated from the phyllosphere of *Phoenix dactylifera*. *Int J Syst Evol Microbiol* **55**, 743–746.
- Rivas, R., García-Fraile, P., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2006). *Paenibacillus cellulosityticus* sp. nov., a cellulolytic and xylanolytic bacterium isolated from the bract phyllosphere of *Phoenix dactylifera*. *Int J Syst Evol Microbiol* **56**, 2777–2781.
- Rivas, R., García-Fraile, P., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2007). Characterization of xylanolytic bacteria present in the bract phyllosphere of the date palm *Phoenix dactylifera*. *Lett Appl Microbiol* **44**, 181–187.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- 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.
- Tindall, B. J. (1990). Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.
- Uetanabaro, A. P., Wahrenburg, C., Hunger, W., Pukall, R., Spröer, C., Stackebrandt, E., de Canhos, V. P., Claus, D. & Fritze, D. (2003). *Paenibacillus agarexedens* sp. nov., nom. rev., and *Paenibacillus agaridevorans* sp. nov. *Int J Syst Evol Microbiol* **53**, 1051–1057.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. &

other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Willems, A., Dognon-Bourcier, F., Goris, J., Coopman, R., de Lajudie, P., De Vos, P. & Gillis, M. (2001). DNA–DNA hybridization

study of *Bradyrhizobium* strains. *Int J Syst Evol Microbiol* **51**, 1315–1322.

Yoon, J. H., Kang, S. J., Yeo, S. H. & Oh, T. K. (2005). *Paenibacillus alkaliterrae* sp. nov., isolated from an alkaline soil in Korea. *Int J Syst Evol Microbiol* **55**, 2339–2344.