Paenibacillus contaminans sp. nov., isolated from a contaminated laboratory plate

Jui-Hsing Chou,¹ Jo-Hsin Lee,² Mei-Chun Lin,² Poh-Shing Chang,³
A. B. Arun,¹ Chiu-Chung Young¹ and Wen-Ming Chen²

Correspondence

Wen-Ming Chen
p62365@ms28.hinet.net

¹Department of Soil Environmental Science, College of Agriculture and Natural Resources, National Chung Hsing University, Taichung, Taiwan

²Laboratory of Microbiology, Department of Seafood Science, National Kaohsiung Marine University, No. 142, Hai-Chuan Rd, Nan-Tzu, Kaohsiung City 811, Taiwan

³Department of Aquaculture, National Kaohsiung Marine University, Kaohsiung, Taiwan

A bacterial strain, designated CKOBP-6^T, isolated from a contaminated laboratory plate, was characterized by using a polyphasic taxonomic approach. Chemotaxonomic and morphological properties of the strain were consistent with its classification in the genus *Paenibacillus*. Cells were Gram-variable, aerobic, sporulating, motile rods. 16S rRNA gene sequence analysis demonstrated that this novel isolate was unique, showing 94.6% sequence similarity to *Paenibacillus chitinolyticus* NBRC 15660^T and lower levels of similarity to *Paenibacillus gansuensis* KCTC 3950^T (93.8%) and *Paenibacillus terrigena* A35^T (93.8%). The novel isolate could be distinguished from the type strains of all of these species based on a range of phenotypic data. The major cellular phospholipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and one unknown phosphoglycerolipid. The predominant isoprenologue was an unsaturated menaquinone with seven isoprene units (MK-7). The major fatty acids of strain CKOBP-6^T were C_{16:0} (12.0%), anteiso-C_{15:0} (52.0%) and iso-C_{16:0} (16.4%). The G + C content of the genomic DNA was 51.2 mol%. It is evident from the genotypic and phenotypic data that strain CKOBP-6^T should be classified as a representative of a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus contaminans* sp. nov. is proposed. The type strain is CKOBP-6^T (=BCRC 17728^T=LMG 24216^T).

The genus *Paenibacillus* was created with 11 *Bacillus* spp. by Ash *et al.* (1993). Since its creation, several novel *Paenibacillus* species have been added to this diverse genus over the years. Presently, more than 70 species are recognized as members of the genus *Paenibacillus* (<http://www.bacterio.cict.fr/p/paenibacillus.html>). Species belonging to the genus *Paenibacillus* have been isolated from various ecological niches such as soil, rhizosphere, phyllosphere, water, insect, food, faeces, blood cultures and Antarctic sediments. Few *Paenibacillus* members have been isolated from human biological samples and the possibility that these bacteria may cause human infection is of particular concern (Roux & Raoult, 2004). Generally, members of this genus produce endospores, possess anteiso-C_{15:0} as the major cellular fatty acid and have

genomic DNA G + C contents in the range 39–54 mol% (Shida *et al.*, 1997).

The aim of the present study was to determine the taxonomic position of a novel *Paenibacillus*-like organism, isolated from a contaminated laboratory plate and designated strain CKOBP-6^T, by using a polyphasic taxonomic approach.

The pH range for growth was examined in tryptic soy broth (BD Difco) using appropriate biological buffers (pH 4–10) (Chung *et al.*, 1995). Growth of the strain at various temperatures (4, 10, 15, 25, 30, 37, 42 and 45 °C) was examined in tryptic soy broth adjusted to pH 7. Tolerance of strains to various NaCl levels was tested in nutrient broth (BD Difco) adjusted to different NaCl concentrations (0, 0.5% and 1.0–10.0%, w/v, at intervals of 1.0%). Growth was examined by measuring the turbidity (OD₆₀₀) of cultures grown at various pH, NaCl concentrations and temperatures. Anaerobic cultivation was performed on nutrient agar (BD Difco) and tryptic soy broth agar using the Oxoid AnaeroGen system.

Strain CKOBP-6^T showed good growth on DNase test agar (BD Difco) compared to other complex media, such as tryptic soy, nutrient and LB media. On DNase test agar,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CKOBP-6^T is EF626690.

An extended neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain CKOBP-6^T and species of the genus *Paenibacillus* is available as supplementary material with the online version of this paper.

strain CKOBP-6^T formed visible, colourless, semi-transparent, circular colonies with entire edges; colonies were approximately 0.5–1.0 mm in diameter after 72 h incubation at 30 °C. Strain CKOBP-6^T showed good growth at 10–37 °C, 0–2 % NaCl, and pH 6.5 and 8. Optimal growth was observed at 30 °C, 0.5 % NaCl and pH 7.0.

Cells were observed in the lag, exponential and stationary phases of growth under a phase-contrast microscope to ascertain cell shape and motility (hanging-drop method). Flagellum staining was performed using the Spot Test Flagella Stain (BD Difco). Gram Stain Set S (BD), the Ryu non-staining KOH method (Powers, 1995) and Bactident Aminopeptidase test strips (Merck) were used to ascertain Gram reaction. Poly- β -hydroxybutyrate granule accumulation was determined using light microscopy after staining cells with Sudan black (Smibert & Krieg, 1981).

Cells of strain CKOBP-6^T stained Gram-variable in Gram staining tests. The KOH test and L-alanine aminopeptidase using Bactident Aminopeptidase test strips were negative, as reported previously for Gram-positive bacteria (Gregersen, 1978). Vegetative cells were rod-shaped, 0.8–1.0 μ m wide and 2.0–3.0 μ m long. Young cells were peritrichously flagellated. Ellipsoidal spores were formed in swollen sporangia. No accumulation of poly- β -hydroxybutyrate granules was observed.

Extraction of genomic DNA, PCR amplification and sequencing of 16S rRNA genes were carried out as described by Chen *et al.* (2001). Sequence analysis was performed using a DNA sequencer (ABI PRISM 310; Applied Biosystems) and Fragment Assembly System program from the Wisconsin Package 8.1 (GCG, 1995) supplied by the National Health Research Institute of Taiwan. The resultant sequence was compared with

corresponding results taken from the GenBank/EMBL/DDBJ and RDP II databases for representatives of genera classified in the order *Bacillales*. Multiple sequence alignment of strain CKOBP-6^T with its closest relatives was performed using the software BIOEDIT (Hall, 1999) and MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were inferred using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) algorithms. The unrooted tree topologies were evaluated by bootstrap analyses of the neighbour-joining dataset (Felsenstein, 1993). A nearly complete 16S rRNA gene sequence (1472 nt) was obtained for strain CKOBP-6^T. A comparison of this sequence with those of representatives of genera classified in the order *Bacillales* showed that the organism fell within the evolutionary radiation occupied by the genus *Paenibacillus*. The maximum 16S rRNA gene sequence similarity between strain CKOBP-6^T and the type strains of members of the genus *Paenibacillus* was 94.6 %. It is evident from the phylogenetic trees based on the neighbour-joining algorithm in Fig. 1 and Supplementary Fig. S1 (available in IJSEM Online) that strain CKOBP-6^T clearly formed a monophyletic branch in the genus *Paenibacillus*. The organism was most closely related to *Paenibacillus chitinolyticus* NBRC 15660^T, sharing a 16S rRNA gene sequence similarity of 94.6 %. Strain CKOBP-6^T also shared comparatively low 16S rRNA gene sequence similarity with *Paenibacillus gansuensis* KCTC 3950^T (93.8 %) and *Paenibacillus terrigena* A35^T (93.8 %). Similar tree topologies were obtained in phylogenetic trees generated with maximum-parsimony and maximum-likelihood algorithms (data not shown).

DNA–DNA relatedness experiments were not carried out between strain CKOBP-6^T and its closest phylogenetic

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences available from GenBank/EMBL (accession numbers are given in parentheses) constructed after multiple alignments of data showing the position of strain CKOBP-6^T in the genus *Paenibacillus*. Distances and clustering with the neighbour-joining method were determined using the software package BIOEDIT. Numbers at the nodes are percentage bootstrap values based on 1000 resampled datasets; only values above 50 % are given. Bar, 0.01 nt substitutions per nt.

neighbours as the level of 16S rRNA gene sequence similarity between strain CKOBP-6^T and other *Paenibacillus* species was less than 95 %. It is now generally accepted that organisms displaying 3 % or greater 16S rRNA gene divergence are not members of the same species (Stackebrandt & Goebel, 1994). Further evidence for the separate species status of the unidentified isolate comes from phenotypic considerations.

The DNA G+C content of strain CKOBP-6^T was determined in duplicate as described by Mesbah *et al.* (1989). The G+C content was 52.1 ± 1.0 mol%.

Chemosystematic studies were carried out to establish whether strain CKOBP-6^T had a chemical profile that was consistent with its assignment to the genus *Paenibacillus*. Biomass for chemical studies was grown in shake flasks of tryptic soy broth for 5 days at 37 °C, checked for purity, harvested by centrifugation, washed twice in distilled water and freeze-dried. Polar lipids were extracted and analysed by using two-dimensional TLC as described by Ventosa *et al.* (1993). The major cellular phospholipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and an unknown phosphoglycolipid. Respiratory quinones of strain CKOBP-6^T were extracted, separated and identified as described by Collins (1985). An unsaturated menaquinone with seven isoprene units (MK-7) was the predominant isoprenologue. The diagnostic cell-wall amino acid of strain CKOBP-6^T was determined by using the TLC method (Staneck & Roberts, 1974). The isolate contained *meso*-diaminopimelic acid (wall chemotype III). Cellular fatty acids were analysed as methyl ester derivatives by GC according to the instructions of the Microbial Identification System (MIDI; Microbial ID). The fatty acid profile of strain CKOBP-6^T contained mainly anteiso-C_{15:0}, iso-C_{16:0} and C_{16:0}. All of these properties

are typical of representatives of the genus *Paenibacillus* (Shida *et al.*, 1997). The fatty acid profile of CKOBP-6^T was comparable, in general, to those of *P. chitinolyticus* NBRC 15660^T, *P. gansuensis* KCTC 3950^T and *P. terrigena* A35^T (Lee *et al.*, 2004; Lim *et al.*, 2006; Xie & Yokota, 2007) (Table 1).

Strain CKOBP-6^T was examined for a broad range of phenotypic and biochemical properties using the API 20E, API 20NE, API ZYM and API 50 CH (bioMérieux) systems according to the manufacturer's instructions. Catalase, oxidase, DNase and urease tests were carried out as described by Lányi (1987). Lipase (corn oil) and hydrolysis of starch and casein were determined as described by MacFaddin (2000). Hydrolysis of Tweens 20, 40, 60 and 80 was detected using standard methods (Gerhardt *et al.*, 1994). Additionally, sensitivity of strain CKOBP-6^T to different antibiotics was checked using antibiotic discs (Oxoid). Detailed results of biochemical characterization and antibiotic sensitivity are provided in Table 2 and the species description.

From the above data, it is evident that isolate CKOBP-6^T exhibits an overall fatty acid profile that is consistent with those of members of the genus *Paenibacillus* (Table 1). Phylogenetic analysis based on 16S rRNA gene sequencing further confirmed its provisional assignment and clearly demonstrated that strain CKOBP-6^T represents an unknown subline within the genus *Paenibacillus*. Biochemically, strain CKOBP-6^T could be differentiated from other phylogenetically and biochemically related species in the genus *Paenibacillus* (Table 2). On the basis

Table 1. Whole-cell fatty acid compositions (%) of strain CKOBP-6^T and related species

Strains: 1, CKOBP-6^T (*P. contaminans* sp. nov.); 2, *P. chitinolyticus* NBRC 15660^T; 3, *P. gansuensis* KCTC 3950^T; 4, *P. terrigena* A35^T. Data are from this study. —, Fatty acid contents of <0.5 % or not detected.

Fatty acid	1	2	3	4
C _{14:0}	1.8	0.5	2.3	1.2
C _{15:0}	—	—	0.5	3.5
C _{16:0}	12.0	7.2	9.1	6.3
C _{17:0}	0.5	—	—	—
iso-C _{14:0}	2.7	—	2.4	2.1
iso-C _{15:0}	6.5	7.8	8.3	4.3
iso-C _{16:0}	16.4	7.2	10.4	13.2
iso-C _{17:0}	2.5	3.9	4.7	1.7
anteiso-C _{15:0}	52.0	59.0	51.8	54.1
anteiso-C _{17:0}	5.4	9.1	7.2	7.1
C _{16:1} ω11c	—	—	0.5	—
iso-C _{17:1} ω10c	—	0.5	—	—

Table 2. Phenotypic and biochemical characteristics that differentiate strain CKOBP-6^T from representatives of closely related *Paenibacillus* species

Strains: 1, CKOBP-6^T (*P. contaminans* sp. nov.); 2, *P. chitinolyticus* NBRC 15660^T; 3, *P. gansuensis* KCTC 3950^T; 4, *P. terrigena* A35^T. All data are from this study, except DNA G+C content data for *P. chitinolyticus* NBRC 15660^T, *P. gansuensis* KCTC 3950^T and *P. terrigena* A35^T, which were from Kuroshima *et al.* (1996), Lim *et al.* (2006) and Xie & Yokota (2007), respectively. +, Positive; —, negative; w, weak response.

Characteristic	1	2	3	4
Anaerobic growth	+	—	—	+
Catalase	+	+	—	+
Oxidase	+	+	—	+
Nitrate reduction	—	+	—	—
Acid production from:				
Arabinose	w	—	+	—
Glucose	w	+	+	+
Lactose	—	+	+	+
Xylose	—	—	+	—
Hydrolysis of:				
Casein	—	w	+	+
Starch	—	—	—	+
DNA G+C content (mol%)	51.2	51.3	50.0	48.1

of phenotypic, chemotaxonomic and phylogenetic evidence, it is apparent that strain CKOBP-6^T represents a novel species within the genus *Paenibacillus*, for which the name *Paenibacillus contaminans* sp. nov. is proposed.

Description of *Paenibacillus contaminans* sp. nov.

Paenibacillus contaminans (con.ta'mi.nans. L. part. adj. *contaminans* contaminating).

Facultatively anaerobic, Gram-variable, sporulating, motile and rod-shaped, 0.8–1.0 µm in diameter and 2.0–3.0 µm in length. Grows at pH 6.5–8.0, 0–2.0 % NaCl and 10–37 °C. Positive for DNase, cytochrome oxidase and catalase. Negative for lipase (corn oil) and hydrolysis of skimmed milk, starch, and Tweens 20, 40, 60 and 80. Positive (API 20E) for β-galactosidase, citrate utilization, acetoin production, and acid production from glucose, arabinose and rhamnose, but negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase, gelatinase, H₂S production, indole production, and acid production from mannitol, inositol, sorbitol, sucrose, melibiose and amygdalin. In the API 20NE enzyme assay, positive for β-galactosidase, aesculin hydrolysis and assimilation of glucose and maltose, but negative for nitrate reduction, indole production, glucose acidification, arginine dihydrolase, urease, gelatinase, and assimilation of arabinose, mannose, mannitol, *N*-acetylglucosamine, caprate, adipate, malate, citrate and phenylacetate. In the API ZYM enzyme assay, positive for C4 esterase, leucine arylamidase, β-galactosidase and *N*-acetyl-β-glucosaminidase, but negative for alkaline phosphatase, C8 lipase, C14 lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase. The following carbon sources were utilized (positive with the API 50 CH system): glycerol, D-glucose, methyl α-D-glucopyranoside, amygdalin, arbutin, cellobiose, maltose, melibiose, sucrose, trehalose, gentiobiose and turanose. The following substrates were not utilized as carbon sources (negative with the API 50 CH system): erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, *N*-acetylglucosamine, aesculin, ferric citrate, salicin, D-lactose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. Resistant to sulfamethoxazole (23.75 µg) plus trimethoprim (1.25 µg); sensitive to ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), penicillin G (10 U), rifampicin (5 µg), streptomycin (10 µg) and tetracycline (30 µg). The major fatty acid components are anteiso-C_{15:0}, iso-C_{16:0} and C_{16:0}. The major cellular phospholipids are phosphatidyl-

glycerol, diphosphatidylglycerol and phosphatidylethanolamine. MK-7 is the predominant isoprenologue.

The type strain is CKOBP-6^T (=BCRC 17728^T=LMG 24216^T), isolated from a contaminated laboratory plate. The DNA G + C content of the type strain is 51.2 mol%.

Acknowledgements

W.-M. C. was supported by grants from the National Science Council, Taipei, Taiwan, Republic of China (NSC 95-2320-B-022-001-MY2 and 96-2313-B-022-001-MY3).

References

- Ash, C., Priest, F. G. & Collins, M. D. (1993). 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.
- Chen, W. M., Laevens, S., Lee, T. M., Coenye, T., de Vos, P., Mergeay, M. & Vandamme, P. (2001). *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. *Int J Syst Evol Microbiol* **51**, 1729–1735.
- Chung, Y. C., Kobayashi, T., Kanai, H., Akiba, T. & Kudo, T. (1995). Purification and properties of extracellular amylase from the hyperthermophilic archaeon *Thermococcus profundus* DT5432. *Appl Environ Microbiol* **61**, 1502–1506.
- Collins, M. D. (1985). Analysis of isoprenoid quinones. *Methods Microbiol* **18**, 329–366.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- GCG (1995). *Wisconsin Package Version 8.1 Program Manual*. Madison, WI: Genetics Computer Group.
- Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994). *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.
- Gregersen, T. (1978). Rapid method for distinction of Gram-negative from Gram-positive bacteria. *Eur J Appl Microbiol Biotechnol* **5**, 123–127.
- 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.
- Kluge, A. G. & Farris, F. S. (1969). Quantitative phyletics and the evolution of anurans. *Syst Zool* **18**, 1–32.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Kuroshima, K.-I., Sakane, T., Takata, R. & Yokota, A. (1996). *Bacillus ehimensis* sp. nov. and *Bacillus chitinolyticus* sp. nov., new chitinolytic members of the genus *Bacillus*. *Int J Syst Bacteriol* **46**, 76–80.
- Lányi, B. (1987). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1–67.
- Lee, J.-S., Pyun, Y.-R. & Bae, K. S. (2004). Transfer of *Bacillus ehimensis* and *Bacillus chitinolyticus* to the genus *Paenibacillus* with emended descriptions of *Paenibacillus ehimensis* comb. nov. and *Paenibacillus chitinolyticus* comb. nov. *Int J Syst Evol Microbiol* **54**, 929–933.

- Lim, J.-M., Jeon, C. O., Lee, J.-C., Xu, L.-H., Jiang, C.-L. & Kim, C.-J. (2006). *Paenibacillus gansuensis* sp. nov., isolated from desert soil of Gansu Province in China. *Int J Syst Evol Microbiol* **56**, 2131–2134.
- MacFaddin, J. F. (2000). *Biochemical Tests for the Identification of Medical Bacteria*, 3rd edn. Baltimore, MD: Williams & Wilkins.
- 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.
- Powers, E. M. (1995). Efficacy of the Ryu nonstaining KOH technique for rapidly determining gram reactions of food-borne and waterborne bacteria and yeasts. *Appl Environ Microbiol* **61**, 3756–3758.
- Roux, V. & Raoult, D. (2004). *Paenibacillus massiliensis* sp. nov., *Paenibacillus sanguinis* sp. nov. and *Paenibacillus timonensis* sp. nov., isolated from blood cultures. *Int J Syst Evol Microbiol* **54**, 1049–1054.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Shida, O., Takagi, H., Kadowaki, K., Nakamura, L. K. & Komagata, K. (1997). 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*. *Int J Syst Bacteriol* **47**, 289–298.
- Smibert, R. M. & Krieg, N. R. (1981). General characterization. In *Manual of Methods for General Microbiology*, pp. 409–443. 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.
- 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.
- Ventosa, A., Marquez, M. C., Kocur, M. & Tindall, B. J. (1993). Comparative study of “*Micrococcus* sp.” strains CCM 168 and CCM 1405 and members of the genus *Salinicoccus*. *Int J Syst Bacteriol* **43**, 245–248.
- Xie, C.-H. & Yokota, A. (2007). *Paenibacillus terrigena* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **57**, 70–72.