Microbacterium kribbense sp. nov., isolated from soil

Syed G. Dastager, Jae-Chan Lee, Yoon-Jung Ju, Dong-Jin Park and Chang-Jin Kim

Correspondence
Chang-Jin Kim
changjin@kribb.re.kr

Functional Metabolomics Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, Republic of Korea

A Gram-positive, short, rod-shaped, cream-coloured actinobacterium, designated MSL-04^T, was isolated from a soil sample collected from an agricultural field on Bigeum Island (Republic of Korea) and was subjected to polyphasic characterization to unravel its taxonomic position. The optimum growth temperature and pH were 28–30 °C and pH 7.2–7.4. Chemotaxonomic characteristics such as B2β-type peptidoglycan with glycolyl residues, MK-11 and MK-12 as the major menaquinones and a fatty acid profile containing iso- and anteiso-branched fatty acids placed this organism within the genus *Microbacterium*. Phylogenetic analysis based on 16S rRNA gene sequences confirmed the position of strain MSL-04^T in the genus *Microbacterium* and showed that it belonged to the same clade as *Microbacterium ulmi* but was distinct from its nearest neighbours. The 16S rRNA gene sequence of strain MSL-04^T showed the highest levels of similarity with respect to *Microbacterium ulmi* LMG 20991^T (97.5% similarity) and *Microbacterium arborescens* IFO 3750^T (97%). The G + C content of the DNA was 71.0 mol%. The DNA–DNA relatedness between MSL-04^T and its closest neighbours (<70%) showed that the strain represents a distinct genomic species. On the basis of the phenotypic and genotypic results, strain MSL-04^T represents a novel species in the genus *Microbacterium*, for which the name *Microbacterium kribbense* sp. nov. is proposed. The type strain is MSL-04^T (=KCTC 19269^T =DSM 19265^T).

The genus *Microbacterium*, the type genus of the family *Microbacteriaceae*, was proposed by Orla-Jensen (1919) for Gram-positive, non-spore-forming, rod-shaped bacteria isolated during studies on lactic acid-producing bacteria. More recently, the description of the genus *Microbacterium* was emended to unite the genera *Microbacterium* and *Aureobacterium* (Takeuchi & Hatano, 1998). Members of the genus are widespread and have been isolated from a broad range of environmental habitats, e.g. plants, soil, water, air, steep liquor, milk products and humans. At the time of writing, the genus *Microbacterium* comprises more than 50 recognized species. Here, we describe the phenotypic and genotypic properties of a novel *Microbacterium* species.

Strain MSL-04^T was isolated from a soil sample from Bigeum Island, Republic of Korea, using R2A agar medium (Difco). Routine cultivation was performed using R2A broth at 28 °C. Bacterial growth was monitored for up to 7 days after inoculation by measuring the turbidity at 600 nm. Cell morphology was observed by using light microscopy after 24 h cell growth. Sporulation was

observed by means of phase-contrast microscopy after the cells had reached stationary phase. Gram staining was carried out on exponentially growing cells, using Hucker's modified method (Cowan & Steel, 1965). The presence of flagella was examined using Leifson's method (Cowan & Steel, 1965). Growth at different temperatures, salt concentrations (w/v) and pH values was investigated as described by Tang *et al.* (2003). The pH was adjusted by using autoclaved Na₂CO₃. The following buffers were used to control the pH in R2A medium: 0.1 M KH₂PO₄/0.1 M NaOH (pH 6.0, 7.0 and 8.0); 0.1 M NaHCO₃/0.1 M Na₂CO₃ (pH 9.0 and 10.0); 0.05 M Na₂HPO₄/0.1 M NaOH (pH 11.0); and 0.2 M KCl/0.2 M NaOH (pH 12.0). The temperature range for growth of the organism was examined at 10–50 °C (in increments of 5 °C). Salt tolerance was tested from 0 to 7% NaCl (w/v). Anaerobic growth was tested using incubation at 28 °C in 10 ml rubber-stoppered, screw-capped tubes containing R2A medium (9 ml) covered with liquid paraffin.

Biochemical and physiological tests were performed using the methods described by Shirling & Gottlieb (1966). Oxidase activity was investigated by testing for oxidation of 1% benzidine (Deibel & Ewans, 1960) and tetramethyl-*p*-phenylenediamine (Tarrand & Groschel, 1982). Catalase

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MSL-04^T is EF466125.

activity was determined by checking bubble formation in a 3 % (v/v) hydrogen peroxide solution. Acid production from carbohydrates was determined as described by Leifson (1963). Substrate utilization and enzyme activities in strain MSL-04^T were determined using API ZYM test kits (bioMérieux) according to the manufacturer's instructions.

Biomass for biochemical and chemotaxonomic characterization was prepared by culturing strain MSL-04^T in trypticase soy broth at 28 °C for 5 days and then harvesting cells by centrifugation. The peptidoglycan structure was determined by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Qualitative analyses of amino acids and peptides in peptidoglycan hydrolysates were carried out as described by Schleifer (1985) and Schleifer & Kandler (1972) with the modification that TLC on cellulose plates was used instead of paper chromatography. Quantitative analysis of amino acids in the total cell hydrolysate was performed using GC and GC-MS as described by MacKenzie (1987) and Groth *et al.* (1996).

The methods used for sugar analysis of whole-cell hydrolysates, determination of the menaquinone pattern and analysis of fatty acids were those of Li *et al.* (2004a, b) and Chen *et al.* (2004). Polar lipid profiles were determined by means of two-dimensional TLC (Minnikin *et al.*, 1977). The genomic DNA of the bacterium was extracted and purified by using the method of Marmur (1961). The DNA G + C content of strain MSL-04^T was measured using the thermal denaturation method of Marmur & Doty (1962).

The 16S rRNA gene sequence of the isolate was amplified by using a PCR as described by Cui *et al.* (2001). A phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein, 1993) and MEGA, version 2.1 (Kumar *et al.*, 2001), following multiple alignment of data using CLUSTAL_X (Thompson *et al.*, 1997). Evolutionary distances (with distance options according to Kimura's two-parameter model; Kimura, 1980, 1983) and clustering were calculated with the neighbour-joining (Saitou & Nei, 1987) (Fig. 1) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap analysis (based on 1000 resamplings)

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain MSL-04^T among species of the genus *Microbacterium*. Asterisks indicate branches that were also recovered using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) algorithms. Numbers at branching points refer to bootstrap values (based on 1000 resamplings). *Curtobacterium luteum* DSM 20542^T was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

was used to evaluate the tree topology of the neighbour-joining data (Felsenstein, 1985).

Strain MSL-04^T was found to comprise Gram-positive, aerobic, non-motile, non-spore-forming, short rods. Colonies were lemon-yellowish, circular, convex with entire margins, shiny, moist and approximately 0.4–0.6 × 0.5–0.8 mm in size after 48 h growth at 28 °C in R2A medium. Growth occurred over a wide range of pH (7.0–11.0). The physiological and biochemical characteristics, metabolic properties and substrate-utilization results obtained for strain MSL-04^T are presented in Table 1 and in the species description below.

Strain MSL-04^T contained ornithine, alanine, glycine, homoserine and hydroxyglutamic acid in the total hydrolysate of the peptidoglycan. From these data, we concluded that the peptidoglycan type was B2β (Schleifer & Kandler, 1972). Its cell-wall hydrolysate contained ribose, galactose,

glucose and trace amounts of xylose. The predominant menaquinones were MK-11 and MK-12. The main polar lipids were diphosphatidylglycerol and phosphatidylglycerol. The major cellular fatty acids of strain MSL-04^T are C_{18:1ω7c} (35.7%), anteiso-C_{15:0} (23.5%), iso-C_{16:0} (23.1%), anteiso-C_{17:0} (9.7%), iso-C_{15:0} (3.3%) and iso-C_{17:0} (2.3%). To ascertain the phylogenetic position of strain MSL-04^T, the almost-complete 16S rRNA gene sequence (1428 nt) was determined. A phylogenetic tree demonstrated that the isolate is a member of the genus *Microbacterium*, and that it forms an independent phyletic line within a monophyletic subclade. The 16S rRNA gene sequence similarity between the isolate and *Microbacterium* type strains ranged from 93.7 to 97.5%. *Microbacterium ulmi* LMG 20991^T showed the highest sequence similarity (97.5%) with respect to strain MSL-04^T. DNA–DNA hybridization was carried out using the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke,

Table 1. Physiological and biochemical characteristics that serve to differentiate strain MSL-04^T from its closest phylogenetic neighbours

Strains: 1, strain MSL-04^T; 2, *M. ulmi* LMG 20991^T (data from Rivas *et al.*, 2004); 3, *M. arborescens* NBRC 3750^T (Imai *et al.*, 1984); 4, *M. imperiale* NBRC 12610^T (Collins *et al.*, 1983). +, Positive; –, negative; v, variable reaction; w, weak reaction; ND, no data available. For the data on chemotaxonomy, components are listed in order of decreasing abundance.

Characteristic	1	2	3	4
Colony colour	Lemon yellow	White	Dirty orange	Red–orange
Motility	–	–	+	+
Catalase	–	w	+	+
Hydrogen sulfide	+	–	+	v
Urease	–	–	+	–
Growth on sole carbon sources				
<i>N</i> -Acetylglucosamine	–	–	+	w
L-Arabinose	+	–	+	+
Citrate	–	–	w	–
Malate	+	–	+	+
Mannose	+	+	–	–
Phenyl acetate	+	+	–	–
Propionate	–	+	–	–
Hydrolysis of:				
Gelatin	–	–	+	–
Starch	+	–	–	+
Acid production from:				
Glycerol	+	–	+	–
Glycogen	+	–	–	–
Melezitose	+	–	+	–
Chemotaxonomy				
Menaquinones	11, 12	12, 13, 11, 14, 10	11, 12	11, 12
Cell-wall diamino acid	Lys	Orn	Lys	Lys
Cell-wall sugars*	Rib, Gal, Glc, Xyl	Gal, Fuc, Xyl, Rha	6dTal, Man, Gal	Rha, Man, Gal
Polar lipids†	PG, DPG	PG, DPG, L	ND	DPG, PG, DMG
DNA G + C content (mol%)	71.0	69.0	71.0	71.0–75.4‡

*6dTal, 6-Deoxytalose; Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose.

†DMG, Dimannosyldiacylglycerol; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; L, unknown polar lipid.

‡Range of values for a number of strains (Collins *et al.*, 1983).

1992), under optimal hybridization conditions, between strain MSL-04^T, *M. ulmi* LMG 20991^T and *Microbacterium arborescens* NBRC 3750^T. The DNA–DNA relatedness between strain MSL-04^T and *M. ulmi* LMG 20991^T and *M. arborescens* NBRC 3750^T was 45 and 51.2 %, respectively. These values are significantly lower than the 70 % threshold recommended for the delineation of genomic species (Wayne *et al.*, 1987). Therefore, the phylogenetic and DNA–DNA hybridization results from this work demonstrate that the isolate is phylogenetically closely related to members of the genus *Microbacterium* and is not related to any previously described *Microbacterium* species.

Strain MSL-04^T shares some chemotaxonomic features with members of the genus *Microbacterium*, i.e. the major menaquinones are MK-11 and MK-12, iso- and anteiso-branched cellular fatty acids predominate and the DNA G+C content is 71.0 mol%. However, it shows considerable phenotypic and genomic differences from other recognized species of the genus *Microbacterium*.

On the basis of the results obtained using this polyphasic approach, strain MSL-04^T represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium kribbense* sp. nov. is proposed.

Description of *Microbacterium kribbense* sp. nov.

Microbacterium kribbense (krib.ben'se. N.L. neut. adj. *kribbense* arbitrary adjective formed from the acronym of the Korea Research Institute of Bioscience and Biotechnology, KRIBB, where the taxonomic studies on the type strain were performed).

Gram-positive, non-motile, catalase-negative and aerobic. Colonies are pale to lemon yellow in colour. Cells are irregular rods, 0.4–0.6 × 0.5–0.8 µm in size. Growth occurs at pH 7–11 and 20–37 °C; optimal growth occurs at pH 7.2–7.4 and 28 °C. NaCl is tolerated at concentrations up to 3 %. Arabinose, galactose, glucose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, sucrose and trehalose are assimilated, but ribose, fructose and xylose are not. Nitrate is reduced to nitrite. Aesculin, starch and Tween 80 are hydrolysed, but cellulose, gelatin and urea are not. Esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase are detected with the API ZYM enzyme assay; alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-galactosidase, α-mannosidase and α-fucosidase are negative. The diagnostic diamino acid isomer of the peptidoglycan is L-lysine. The acyl type of the peptidoglycan is N-glycolyl. The predominant menaquinones are MK-11 and MK-12. The main polar lipids are diphosphatidylglycerol and phosphatidylglycerol. The major cellular fatty acids are C_{18:1ω7c}, anteiso-C_{15:0}, iso-C_{16:0}, anteiso-C_{17:0}, iso-C_{15:0} and iso-C_{17:0}. Whole-cell hydrolysate contains ribose, galactose, glucose and xylose. The DNA G+C content of the type strain is 71.0 mol%.

The type strain, MSL-04^T (=KCTC 19269^T =DSM 19265^T), was isolated from a soil sample collected from Bigeum Island, Korea.

Acknowledgements

This work was supported by the 21C Frontier Microbial Genomics and Application Centre Program, Korean Ministry of Science and Technology (MOST), and the Korea Foundation for International Cooperation of Science and Technology (KICOS) through a grant provided by MOST in the Global Partnership Program (no. M60602000001-06E0200-00100) of the Republic of Korea.

References

- Chen, H.-H., Li, W.-J., Tang, S.-K., Kroppenstedt, R. M., Stackebrandt, E., Xu, L.-H. & Jiang, C.-L. (2004). *Corynebacterium halotolerans* sp. nov., isolated from saline soil in the west of China. *Int J Syst Evol Microbiol* **54**, 779–782.
- Collins, M. D., Jones, D. & Kroppenstedt, R. M. (1983). Reclassification of *Brevibacterium imperiale* (Steinhaus) and “*Corynebacterium laevaniformans*” (Dias and Bhat) in a redefined genus *Microbacterium* (Orla-Jensen), as *Microbacterium imperiale* comb. nov. and *Microbacterium laevaniformans* nom. rev.; comb. nov. *Syst Appl Microbiol* **4**, 65–78.
- Cowan, S. T. & Steel, K. J. (1965). *Manual for the Identification of Medical Bacteria*. Cambridge: Cambridge University Press.
- Cui, X.-L., Mao, P.-H., Zeng, M., Li, W.-J., Zhang, L.-P., Xu, L.-H. & Jiang, C.-L. (2001). *Streptimonospora salina* gen. nov., sp. nov., a new member of the family Nocardioptaceae. *Int J Syst Evol Microbiol* **51**, 357–363.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Deibel, R. H. & Ewans, J. B. (1960). Modified benzidine test for the detection of cytochrome-containing respiratory systems in microorganisms. *J Bacteriol* **79**, 356–360.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- Fitch, W. M. & Margoliash, E. (1967). Construction of phylogenetic trees: a method based on mutation distances as estimated from cytochrome *c* sequences is of general applicability. *Science* **155**, 279–284.
- Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. A. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234–239.
- HuB, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Imai, K., Takeuchi, M. & Banno, I. (1984). Reclassification of “*Flavobacterium arborescens*” (Frankland and Frankland) Bergey *et al.* in the genus *Microbacterium* (Orla-Jensen) Collins *et al.*, as *Microbacterium arborescens* comb. nov., nom. rev. *Curr Microbiol* **11**, 281–284.

- Jahnke, K. D. (1992).** BASIC computer program for evaluation of spectroscopic DNA renaturation data from Gilford System 2600 spectrophotometer on a PC/XT/AT type personal computer. *J Microbiol Methods* **15**, 61–73.
- 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.
- Kimura, M. (1983).** *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press.
- Kluge, A. G. & Farris, F. S. (1969).** Quantitative phyletics and the evolution of anurans. *Syst Zool* **18**, 1–32.
- Kumar, S., Tamura, K., Jakobsen, I.-B. & Nei, M. (2001).** MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.
- Leifson, E. (1963).** Determination of carbohydrate metabolism of marine bacteria. *J Bacteriol* **85**, 1183–1184.
- Li, W.-J., Chen, H.-H., Xu, P., Zhang, Y.-Q., Schumann, P., Tang, S.-K., Xu, L.-H. & Jiang, C.-L. (2004a).** *Yania halotolerans* gen. nov., sp. nov., a novel member of the suborder *Micrococcineae* from saline soil in China. *Int J Syst Evol Microbiol* **54**, 525–531.
- Li, W.-J., Chen, H.-H., Zhang, Y.-Q., Schumann, P., Stackebrandt, E., Xu, L.-H. & Jiang, C.-L. (2004b).** *Nesterenkonia halotolerans* sp. nov. and *Nesterenkonia xinjiangensis* sp. nov., actinobacteria from saline soils in the west of China. *Int J Syst Evol Microbiol* **54**, 837–841.
- MacKenzie, S. L. (1987).** Gas chromatographic analysis of amino acids as the *N*-heptafluorobutyryl isobutyl esters. *J Assoc Off Anal Chem* **70**, 151–160.
- Marmur, J. (1961).** A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Marmur, J. & Doty, P. (1962).** Determination of base composition of deoxyribonucleic acid from its denaturation temperature. *J Mol Biol* **5**, 109–118.
- Minnikin, D. E., Patel, P. V., Alshamaony, L. & Goodfellow, M. (1977).** Polar lipid composition in the classification of *Nocardia* and related bacteria. *Int J Syst Bacteriol* **27**, 104–117.
- Orla-Jensen, S. (1919).** *The Lactic Acid Bacteria*. Copenhagen: Høst & Sons.
- Rivas, R., Trujillo, M. E., Sánchez, M., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2004).** *Microbacterium ulmi* sp. nov., a xylanolytic, phosphate-solubilizing bacterium isolated from sawdust of *Ulmus nigra*. *Int J Syst Evol Microbiol* **54**, 513–517.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schleifer, K. H. (1985).** Analysis of the chemical composition and primary structure of murein. *Methods Microbiol* **18**, 123–156.
- Schleifer, K. H. & Kandler, O. (1972).** Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Shirling, E. B. & Gottlieb, D. (1966).** Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.
- Takeuchi, M. & Hatano, K. (1998).** Union of the genera *Microbacterium* Orla-Jensen and *Aureobacterium* Collins *et al.* in a redefined genus *Microbacterium*. *Int J Syst Bacteriol* **48**, 739–747.
- Tang, S.-K., Li, W.-J., Dong, W., Zhang, Y.-G., Xu, L.-H. & Jiang, C.-L. (2003).** Studies of the biological characteristics of some halophilic and halotolerant actinomycetes isolated from saline and alkaline soils. *Actinomycetologica* **17**, 6–10.
- Tarrand, J. J. & Groschel, D. H. M. (1982).** Rapid, modified oxidase test for oxidase-variable bacterial isolates. *J Clin Microbiol* **16**, 772–774.
- 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.
- 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.