Kineococcus gynurae sp. nov., isolated from a Thai medicinal plant

Kannika Duangmal,¹ Arinthip Thamchaipenet,² Ismet Ara,³
Atsuko Matsumoto³ and Yoko Takahashi³

Correspondence

Kannika Duangmal
fscikkd@ku.ac.th
or
kannikaduangmal@hotmail.com

¹Department of Microbiology, Faculty of Science, Kasetsart University, Chatuchak, Bangkok 10900, Thailand

²Department of Genetics, Faculty of Science, Kasetsart University, Chatuchak, Bangkok 10900, Thailand

³Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

A novel, Gram-positive, motile, coccus-shaped, orange-pigmented organism, designated strain KKD096^T, was isolated from the roots of a Thai medicinal plant, *Gynura pseudochina* DC. var. *hispida* Thwaites. Growth of strain KKD096^T occurred at temperatures of 14–34 °C, at pH 5.0–9.0 and at NaCl concentrations up to 7 % (w/v). Whole-cell hydrolysates contained arabinose and galactose as the characteristic sugars. The diagnostic diamino acid of the peptidoglycan was meso-diaminopimelic acid. The glycan moiety of the murein contained acetyl residues. The predominant menaquinone was MK-9(H₂); mycolic acids were not detected. The genomic DNA G + C content was 73.3 mol%. The major cellular fatty acid was anteiso-C_{15:0} (81.42 % of the total). Strain KKD096^T was assigned to the genus *Kineococcus* on the basis of 16S rRNA gene sequence analysis; it was most closely related to *Kineococcus radiotolerans* DSM 14245^T (97.1 % similarity). DNA–DNA hybridization revealed 39.4 % relatedness between these two taxa. On the basis of the genotypic and phenotypic data presented, strain KKD096^T is considered to represent a novel species of the genus *Kineococcus*, for which the name *Kineococcus gynurae* sp. nov. is proposed. The type strain is KKD096^T (=TISTR 1856^T=NRRL B-24568^T=BCC 26245^T=NBRC 103943^T).

The genus *Kineococcus* was proposed by Yokota *et al.* (1993) to accommodate an aerobic, Gram-positive, motile coccus with meso-diaminopimelic acid in the whole-cell hydrolysate. At the time of writing, the genus comprises three recognized species, namely *Kineococcus aurantiacus* (Yokota *et al.*, 1993), *Kineococcus marinus* (Lee, 2006) and *Kineococcus radiotolerans* (Phillips *et al.*, 2002), which were isolated from soil in India, sediment of the coast of Korea and a radioactive environment in the USA, respectively. All members of the genus are aerobic, Gram-positive, motile, coccus-shaped bacteria.

Strain KKD096^T was isolated from the roots of a Thai medicinal plant, *Gynura pseudochina* (L.) DC. var. *hispida* Thwaites, by using starch casein agar (Küster & Williams, 1964) supplemented with antibacterial and antifungal agents (nalidixic acid and ketokonazole). The plates were incubated at room temperature for 14 days. One colony

was isolated and purified on glucose yeast extract (GYE) agar [1.0 % glucose (w/v), 1 % yeast extract (w/v) and 1.5 % agar (w/v)]. The pure culture was maintained as a 20 % glycerol suspension at –20 °C or as lyophilized cells for long-term preservation. *Kineococcus aurantiacus* IFO 15268^T and *Kineococcus radiotolerans* DSM 14245^T were used for comparative purposes.

The morphological characteristics of strain KKD096^T were observed by scanning electron microscopy (JEOL-JSM 5600 LV) and transmission electron microscopy (TEM; JEM-1200EXII, JEOL).

Samples used for scanning electron microscopy and TEM were cultured for 4 days on GYE agar. Motility and production of flagella were observed by scraping cells from GYE agar plates and adding the cells to soil extract solution (5 g garden soil in 25 ml distilled water, mixed well and filtered through a 0.22 µm Millipore membrane). The cells were left at room temperature for 5–10 min before TEM observation. Negative staining of cells for TEM was performed with 1 % uranyl acetate. The ability of the novel strain to use a variety of substrates as sole carbon

Abbreviation: TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KKD096^T is EF667339.

sources was tested by using the API 50CH system (bioMérieux; 49 substrates). Enzyme activity profiles were tested by using the API ZYM system (bioMérieux; 19 enzyme activities tested). Urease activity was determined based on a colour change in urea broth (Gordon *et al.*, 1974). The temperature range for growth was determined on GYE agar by using a temperature gradient incubator (Tokyo Kagaku Sangyo). Hydrolysis of casein and gelatin was evaluated by using the media of Gordon *et al.* (1974). Reduction of nitrate and production of melanoid pigment were determined according to the method of the International *Streptomyces* Project (ISP; Shirling & Gottlieb, 1966). NaCl tolerance was studied on ISP medium 2 [containing 0.4 % yeast extract (w/v), 1 % malt extract (w/v), 0.4 % glucose (w/v) and 2 % agar (w/v)] containing NaCl at a final concentration of 1–9 % (w/v). pH tolerance was studied at pH 4–10. Catalase and oxidase activities were determined with a 3 % (v/v) hydrogen peroxide solution and 1 % tetramethyl *p*-phenylenediamine solution, respectively.

Cells used for chemotaxonomic analysis were obtained after incubation at 27 °C for 3 days in GYE broth. Isomers of diaminopimelic acid in the whole-organism hydrolysates were determined by TLC according to the method of Hasegawa *et al.* (1983). The acyl type of the cell wall was analysed according to the method of Uchida & Aida (1984). Whole-cell sugars were analysed in accordance with Becker *et al.* (1965). Menaquinones were extracted and purified by using the method of Collins *et al.* (1977), and isoprene units were analysed by HPLC with a JASCO 802-SC chromatograph equipped with a Shiseido CAPCELL PAK C18 column (Tamaoka *et al.*, 1983). Mycolic acids were detected by TLC according to the method of Tomiyasu (1982). The G + C content of the DNA, isolated following the method of Marmur (1961), was determined by HPLC according to Tamaoka & Komagata (1984). Analysis of the fatty acids in cells grown on ISP 2 medium at 28 °C for 72 h was performed according to the procedures for the Sherlock Microbial Identification System (Microbial ID).

PCR amplification of 16S rRNA gene samples was carried out by using standard methods (Duangmal *et al.*, 2005). The PCR products were purified and sequenced by using an ABI PRISM Big Dye Terminator v3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems) and universal primers. The nucleotide sequences were obtained automatically by using an Applied Biosystems DNA sequencer (ABI 377) and software provided by the manufacturer. The resultant almost-complete 16S rRNA gene sequence of strain KKD096^T (1351 bp) was compared via a BLAST search and aligned with the corresponding sequences of representatives of the genus *Kineococcus* and of related genera, retrieved from the GenBank database, by using the CLUSTAL_X (Thompson *et al.*, 1997) and PHYDIT programs (<http://plaza.snu.ac.kr/~jchun/phydit/>). A phylogenetic tree was constructed by using the TREECON program (Van de Peer & De Wachter, 1994) with the Jukes & Cantor

(1969) algorithm for distance estimation and the neighbour-joining method (Saitou & Nei, 1987) for tree construction. The resultant unrooted tree topologies were evaluated by carrying out a bootstrap analysis (Felsenstein, 1985) of the neighbour-joining data based on 1000 resampled datasets, by using the TREECON software. Levels of DNA–DNA relatedness were determined fluorometrically according to the method of Ezaki *et al.* (1989).

Cells of strain KKD096^T were Gram-positive, non-spore-forming, motile, strictly aerobic cocci. Cells occurred singly, in pairs and in clusters (Fig. 1). Colonies were circular, convex and deep orange in colour on GYE agar and ISP 2 medium after 4 days at 27 °C. The results of chemical analysis indicated that strain KKD096^T had chemotaxonomic markers typical of members of the genus *Kineococcus* (Yokota *et al.*, 1993). Strain KKD096^T contained *meso*-diaminopimelic acid in the peptidoglycan and MK-9(H₂) as the predominant menaquinone. No mycolic acids were detected. The predominant cellular fatty acid was anteiso-C_{15:0} (81.42 %). The G + C content of the DNA was 73.3 mol%. Phylogenetic analysis of the almost-complete 16S rRNA gene sequence of strain KKD096^T indicated that it formed a distinct cluster with members of the genus *Kineococcus* (except *Kineococcus marinus*) within the radiation encompassing members of the family *Kineosporiaceae* (Fig. 2). The highest levels of 16S rRNA gene sequence similarity were with *Kineococcus radiotolerans* DSM 14245^T and *Kineococcus aurantiacus* IFO 15268^T (97.1 and 97 %, respectively).

Fig. 1. Scanning electron micrograph (a) and transmission electron micrograph (b) of cells of strain KKD096^T grown for 4 days at 27 °C on GYE agar. Bars, 1 μm.

Levels of DNA–DNA hybridization were determined from duplicate experiments with its two closest phylogenetic relatives based on 16S rRNA sequencing, namely *Kineococcus aurantiacus* IFO 15268^T and *Kineococcus radiotolerans* DSM 14245^T (25 and 39.4 % DNA–DNA relatedness, respectively). These results clearly indicated that strain KKD096^T did not belong to *K. radiotolerans* or *K. aurantiacus* as these values were well below the recommended threshold value of 70 % for the definition of bacterial species (Wayne *et al.*, 1987).

Description of *Kineococcus gynurae* sp. nov.

Cells are Gram-positive, aerobic, motile, non-spore-forming, catalase-positive, oxidase-negative cocci. Colonies are circular, rough and deep orange in colour. Negative for

Table 1. Differential characteristics between strain KKD096^T and the type strains of recognized species of the genus *Kineococcus*

Characteristic	1	2	3	4
Urease	+	+	—	—
Utilization of:				
L-Arabinose	—	+	+	+
D-Galactose	+	—	+	+
D-Mannose	+	—	+	+
D-Xylose	—	+	+	+
Glycerol	—	—	+	+
Inositol	—	—	+	+
L-Rhamnose	+	—	+	—
Mannitol	+	—	+	+
Maltose	+	—	+	—
Raffinose	—	—	+	—
Ribose	—	—	+	—
Temperature range for growth (°C)	14–34	9–37	4–37	11–41
pH range for growth	5.0–9.0	6.0–9.0	5.1–10.1	5.0–9.0

rose, D-sorbitol, L-sorbose, trehalose, turanose and xylitol are utilized as sole carbon and energy sources for growth. The following substrates are not utilized: D-adonitol, amygdalin, D-arabinose, L-arabinose, arbutin, cellobiose, dulcitol, erythritol, D-fucose, L-fucose, glycogen, glycerol, inositol, inulin, methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, N-acetylglucosamine, potassium 2-ketogluconate, potassium 5-ketogluconate, potassium gluconate, raffinose, D-ribose, salicin, starch, D-tagatose, D-xylose and L-xylose. Acid phosphatase, alkaline phosphatase, esterase (C4), α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, leucine aminopeptidase, trypsin and phosphoamidase are detected with the API ZYM enzyme assay, but not chymotrypsin, cystine aminopeptidase, α -fucosidase, β -glucuronidase, lipase (C8), lipase (C14), α -mannosidase, N-acetyl- β -glucosaminidase or valine aminopeptidase. The diagnostic diamino acid of the peptidoglycan is *meso*-diaminopimelic acid. Whole-cell sugars contain arabinose and galactose. The glycan moiety of the murein is acetylated. The predominant menaquinone is MK-9(H₂). Mycolic acids are not detected. The predominant fatty acids are anteiso-C_{15:0} (81.42 %) and iso-C_{14:0} (6.34 %). Other cellular fatty acids detected as minor components are C_{14:0} 2-OH (3.42 %), C_{14:0} (2.47 %), C_{17:0} 2-OH (1.46 %) and C_{17:0} 3-OH (1.3 %).

The type strain, KKD096^T (=TISTR 1856^T=NRRL B-24568^T=BCC 26245^T=NBRC 103943^T), was isolated from the roots of *Gynura pseudochina* (L.) DC. var. *hispid*a Thwaites, collected from Bangkok, Thailand. The DNA G+C content of the type strain is 73.3 mol%.

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References

- Becker, B., Lechevalier, M. P. & Lechevalier, H. A. (1965). Chemical composition of cell-wall preparations from strains of various form-genera of aerobic actinomycetes. *Appl Microbiol* **13**, 236–243.
- Collins, M. D., Pirouz, T., Goodfellow, M. & Minnikin, D. E. (1977). Distribution of menaquinones in actinomycetes and corynebacteria. *J Gen Microbiol* **100**, 221–230.
- Duangmal, K., Ward, A. C. & Goodfellow, M. (2005). Selective isolation of members of the *Streptomyces violaceoruber* clade from soil. *FEMS Microbiol Lett* **245**, 321–327.
- 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.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Gordon, R. E., Barnett, D. A., Handerhan, J. E. & Pang, C. H.-N. (1974). *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. *Int J Syst Bacteriol* **24**, 54–63.
- Hasegawa, T., Takizawa, M. & Tanida, S. (1983). A rapid analysis for chemical grouping of aerobic actinomycetes. *J Gen Appl Microbiol* **29**, 319–322.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Küster, E. & Williams, S. T. (1964). Media for the isolation of streptomycetes: starch casein medium. *Nature* **202**, 928–929.
- Lee, S. D. (2006). *Kineococcus marinus* sp. nov., isolated from marine sediment of the coast of Jeju, Korea. *Int J Syst Evol Microbiol* **56**, 1279–1283.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Phillips, R. W., Wiegel, J., Berry, C. J., Filermans, C., Peacock, A. D., White, D. C. & Shmids, L. J. (2002). *Kineococcus radiotolerans* sp. nov., a radiation-resistant, Gram-positive bacterium. *Int J Syst Evol Microbiol* **52**, 933–938.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Tamaoka, J., Katayama-Fujimura, Y. & Kuraishi, H. (1983). Analysis of bacterial menaquinone mixtures by high performance liquid chromatography. *J Appl Bacteriol* **54**, 31–36.
- 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.
- Tomiyasu, I. (1982). Mycolic acid composition and thermally adaptive changes in *Nocardia asteroides*. *J Bacteriol* **151**, 828–837.
- Uchida, K. & Aida, K. (1984). An improved method for the glycolate test for simple identification of the acyl type of bacterial cell walls. *J Gen Appl Microbiol* **30**, 131–134.
- Van de Peer, Y. & De Wachter, R. (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* **10**, 569–570.
- 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 the reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Yokota, A., Tamura, T., Nishii, T. & Hasegawa, T. (1993). *Kineococcus aurantiacus* gen. nov., sp. nov., a new aerobic, gram-positive, motile coccus with *meso*-diaminopimelic acid and arabinogalactan in the cell wall. *Int J Syst Bacteriol* **43**, 52–57.