Kineococcus marinus sp. nov., isolated from marine sediment of the coast of Jeju, Korea

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A novel marine actinomycete, designated strain KST3-3^T, which was isolated from a sediment sample of the coast of Jeju, Korea, was subjected to a polyphasic taxonomic characterization. The organism was characterized morphologically by the formation of motile, coccoid cells. A phylogenetic analysis based on 16S rRNA gene sequence comparisons showed that the organism was related to the genera *Kineosporia* and *Kineococcus*, and members of the suborder *Frankineae*, and occupied the deepest branch outside a taxon encompassing members of the genus *Kineococcus*. The organism showed relatively low levels of 16S rRNA gene sequence similarity to members of the genera *Kineococcus* (93.0–93.4 %) and *Kineosporia* (93.1–93.8 %). The morphological and chemotaxonomic characteristics, albeit with a slightly higher level of sequence similarity to members of the genus *Kineosporia*, supported its classification within the genus *Kineococcus*. On the basis of the polyphasic data presented, it was evident that the organism should be assigned to a novel species of the genus *Kineococcus*, for which the name *Kineococcus marinus* sp. nov. is proposed. The type strain is strain KST3-3^T (=KCCM 42250^T =NRRL B-24439^T).

The genus *Kineococcus* was proposed by Yokota *et al.* (1993) to accommodate an aerobic, Gram-positive, motile, coccus-shaped bacterium; *Kineococcus aurantiacus* was characterized chemotaxonomically by the presence of *meso*-diaminopimelic acid, arabinose and galactose in the cell wall, MK-9(H₂) as the predominant menaquinone, diphosphatidylglycerol and phosphatidylglycerol in the polar lipid profile, 12-methyltetradecanoic acid as a major cellular fatty acid and a DNA G + C content of 73.9 mol%. The genus currently comprises two species, *Kineococcus aurantiacus* (Yokota *et al.*, 1993) and *Kineococcus radio-tolerans* (Phillips *et al.*, 2002), which were isolated from a soil sample and a radioactive working area, respectively. Phylogenetically, it was shown that members of the genus were closely related to the genus *Kineosporia* within the *Actinobacteria* based on 16S rRNA gene sequence analyses (Phillips *et al.*, 2002).

During investigations into the genetic diversity of polysaccharide-producing marine bacteria from the coast of the island of Jeju, Republic of Korea, strain KST3-3^T was isolated from a sand beach sediment and subject to morphological, cultural, physiological and chemotaxonomic characterization in addition to phylogenetic analysis based on 16S rRNA gene sequence studies. It was evident from the polyphasic evidence that the organism could be

readily differentiated from the two recognized species of the genus *Kineococcus*.

Beach sediment samples were taken at a depth of 1 m below the surface and placed directly into sterilized 50 ml Falcon tubes. For bacterial isolation, each sediment sample (1 g) was placed into a sterile plastic tube containing 9 ml sterile distilled water, which was then mixed in a tube rotator for 30 min at moderate speed. Aliquots (100 µl) of the serial diluent of the samples were transferred onto SC-SW agar plates, supplemented with 60 % (v/v) sterilized natural seawater. The isolation medium (SC-SW) consisted of 1 % soluble starch, 0.03 % casein, 0.2 % KNO₃, 0.2 % NaCl, 0.002 % CaCO₃, 1.8 % agar, 0.005 % MgSO₄·7H₂O and 0.001 % FeSO₄·7H₂O in a 60 : 40 mixture of natural seawater and distilled water. The plates were incubated at 30 °C for 14 days, and colonies were subcultured on YE-SW medium (0.4 % yeast extract, 1.0 % malt extract, 0.4 % glucose and 1.8 % agar in a 60 : 40 mixture of natural seawater and distilled water); pure cultures were maintained as 20 % glycerol suspensions at –20 and –70 °C.

Colony pigmentation was observed visually and recorded after 5 days growth at 30 °C on YE-SW agar. Cell morphology and motility were observed by using an Olympus light microscope equipped with phase-contrast optics (magnification ×400). Cells were grown for 3 days at 30 °C on YE-SW agar and suspensions were made in sterile saline for microscopic examination. For scanning electron microscopy, bacterial samples were dehydrated through a graded series of ethanol or a mixture of ethanol and isoamyl

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acetate, then critical-point-dried with CO₂. Sputter-coated specimens were examined in a Hitachi S-2600 scanning electron microscope. Cell motility was confirmed by the presence of flagella when observed by transmission electron microscope. Cells of strain KST3-3^T were strictly aerobic, motile, non-spore-forming, Gram-positive cocci. The cells occurred singly, in pairs or in clusters (Fig. 1). On most solid growth media, the strain formed capsular polysaccharide-like substances; this was confirmed by negative staining with India ink. Colonies were circular, smooth, convex and deep orange in colour.

Chromosomal DNA for phylogenetic characterization was extracted and purified by using the Wizard genomic DNA purification kit (Promega) according to the manufacturer's instructions. The 16S rRNA gene of the chromosomal DNA was amplified by PCR with primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r

(5'-TACGGYTACCTTGTTACGACTT-3') as described by Lee *et al.* (2000). After purification, the resultant PCR product was directly sequenced using an ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 3730xl; Applied Biosystems). The sequence determined was aligned with the corresponding sequences of representatives of the genus *Kineococcus* and related genera by using the CLUSTAL X program (Thompson *et al.*, 1997) and was then manually optimized according to the secondary structure of the 16S rRNA gene of *Escherichia coli* (Brosius *et al.*, 1978). Phylogenetic analyses were performed using three tree-making algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A phylogenetic tree was reconstructed with the neighbour-joining method from evolutionary distances calculated using the method described by Jukes & Cantor (1969). Reliability of the tree topology was evaluated by using the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs in the PHYLIP package (Felsenstein, 1993), based on 1000 bootstrapped trees generated.

The almost complete 16S rRNA gene sequence of strain KST3-3^T, comprising a continuous stretch of 1406 nt, was determined and compared with those of representatives of the suborder *Frankineae* and the genera *Kineococcus* and *Kineosporia*. A total of 1268 unambiguous aligned positions present in all strains between positions 72 and 1452 (*E. coli* numbering system) were used for phylogenetic analysis. *Nocardia asteroides* was used as an outgroup taxon for tree construction. A phylogenetic tree (Fig. 2), based on 16S rRNA gene sequence comparisons, showed that strain KST3-3^T was loosely related to members of the genera *Kineosporia* and *Kineococcus*, and formed the deepest branch outside a taxon encompassing members of the genus *Kineococcus*. The phylogenetic relationship was supported by a low bootstrap value of 45 %. The organism showed 16S rRNA gene sequence similarity values of 93.0–93.4 % to members of the genus *Kineococcus*, and slightly higher values to members of the genus *Kineosporia* (93.1–93.8 %). By contrast, the 16S rRNA gene sequence similarity value between *Kineococcus aurantiacus* and *Kineococcus radiotolerans* was 98.9 %, a value considerably higher than that (93 %) reported previously (Phillips *et al.*, 2002). To date, the taxonomic status of the genera *Kineococcus* and *Kineosporia* remains uncertain at the hierarchical classification structure above the family level. It was shown from our phylogenetic analyses (Fig. 2) that members of these genera were phylogenetically related to members of the suborder *Frankineae* (Stackebrandt *et al.*, 1997).

For chemotaxonomic characterization, strain KST3-3^T was cultivated on YE-SW broth for 3 days at 30 °C and harvested by centrifugation at 3000 r.p.m. for 20 min. Cell biomass was washed twice with distilled water and freeze-dried. The isomer of diaminopimelic acid and acyl type of the cell wall and sugar composition of whole-cell hydrolysates were

Fig. 1. Electron microscopy of strain KST3-3^T grown on YE-SW agar for 3 days at 30 °C. (a) Scanning electron micrograph of cells. Bar, 5 μm. (b) Transmission electron micrograph of cells. Bar, 1 μm.

Fig. 2. Phylogenetic tree derived from 16S rRNA gene sequences showing the relationship of strain KST3-3^T with members of the genera *Kineococcus* and *Kineosporia*, and representatives of the suborder *Frankineae*. The tree was reconstructed by using the neighbour-joining method from evolutionary distances calculated using the Jukes–Cantor coefficient. *Nocardia asteroides* was used as an outgroup taxon (not shown). Asterisks indicate branches of the tree that were also recovered using both maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) tree-making algorithms. Numbers at nodes indicate percentages of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets (only values >40% are indicated). Bar, 1 substitution per 100 nt.

analysed according to the methods of Stanek & Roberts (1974), Uchida & Aida (1984) and Saddler *et al.* (1991). Polar lipids were extracted according to the small-scale method of Minnikin *et al.* (1984). Purified menaquinones were identified by HPLC (Kroppenstedt, 1985). The phospholipid composition was determined by the method of Minnikin *et al.* (1977) and analysis of mycolic acids was performed according to the method of Minnikin *et al.* (1980). Extraction of cellular fatty acids and determination of the fatty acid compositions were by GC according to the instructions of the Microbial Identification System (MIDI). The test strain was grown for 3 days at 30 °C on trypticase soy broth agar. The G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989).

The results of chemical analyses are given in the species description below, indicating that the organism has chemotaxonomic markers typical for members of the genus *Kineococcus* (Yokota *et al.*, 1993). The fatty acid profile of strain KST3-3^T was represented by the predominance of anteiso-C_{15:0} (54.4%) and C_{16:0} (7.5%). Other cellular fatty acids detected as minor components were C_{12:0} (2.9%), iso-C_{15:0} (1.3%), C_{15:0} (2.6%), iso-C_{16:1} H (2.3%), iso-C_{16:0} (2.9%), anteiso-C_{17:1}ω₉c (1.8%), anteiso-C_{17:0} (4.4%), C_{17:1}ω₈c (3.6%), C_{17:0} (1.7%), C_{18:1}ω₉c (2.1%), C_{18:0} (3.7%) and 10-methyl C_{18:0} (2.1%), in addition to a mixture of C_{16:1}ω₇c and/or iso-C_{15:0} 2-OH (2.7%). Thus, strain KST3-3^T showed a considerable difference in the relative amount of anteiso-C_{15:0} from the type strains of *Kineococcus aurantiacus* and *Kineococcus radiotolerans* (Phillips *et al.*, 2002; Yokota *et al.*, 1993), although the fatty acid profile of *Kineococcus aurantiacus* was determined under analytical conditions different from those described above. The G+C content of strain KST3-3^T was 76.6 mol%, a value slightly higher than that reported for *Kineococcus aurantiacus* (73.3 mol%; Yokota *et al.*, 1993).

Decomposition of adenine, hypoxanthine, DL-tyrosine and xanthine was examined as described by Gordon *et al.* (1974). The temperature for growth was tested at 4, 10, 20, 30, 37, 40 and 45 °C. NaCl tolerance was studied on yeast extract-malt extract agar (ISP medium 2) containing NaCl at final concentrations of 1–9% (w/v). Nitrate reduction and hydrolysis of casein, gelatin and starch were examined by using the methods of MacFaddin (1980). The production of hydrogen sulfide was detected on trypticase soy broth (Difco) by using lead acetate strips. Catalase activity was determined with a 3% (v/v) hydrogen peroxide solution. Urease activity was determined by a colour change in Bacto urea broth (Difco). The ability to use a variety of substrates as sole carbon sources was tested using GP2 microplates of the Microlog system (Biolog), containing 95 substrates. Cells were grown for 3 days at 30 °C on YE-SW agar and suspended in 2% (w/v) sea salts solution (Sigma). Aliquots of 150 µl of the suspension were transferred to each well and the plates were incubated for 48 h at 30 °C. Reduction of the tetrazolium dye was determined by measuring the absorbance at 595 nm using a microplate reader.

Results of the physiological characterization are given in the species description below. Cells were aerobic, oxidase-negative and catalase-positive and showed growth at 4–37 °C and pH 5.1–10.1. No growth was observed at or above 40 °C. Strain KST3-3^T was readily differentiated from members of the genus *Kineococcus* on the basis of an array of physiological properties (Table 1).

In conclusion, the relatively low levels of 16S rRNA gene sequence similarity between strain KST3-3^T and related genera of the class *Actinobacteria* suggest that the organism merits novel generic status. However, the phylogenetic joining of strain KST3-3^T to a clade encompassing members of the genus *Kineococcus* was strongly supported by morphological characteristics (motile, coccoid-shaped cells) and chemotaxonomic characters (e.g. principal

Table 1. Differential characteristics of strain KST3-3^T and type strains of the genus *Kineococcus*

Strains: 1, strain KST3-3^T; 2, *Kineococcus aurantiacus* IFO 15268^T; 3, *Kineococcus radiotolerans* ATCC BAA-149^T. +, Positive; –, negative; ND, not determined. Data for reference strains were taken from Yokota *et al.* (1993) and Phillips *et al.* (2002).

Characteristic	1	2	3
Utilization of:			
D-Galactose	+	–	+
D-Lactose	+	ND	–
Maltose	+	–	–
D-Mannose	+	–	+
Raffinose	+	–	–
L-Rhamnose	+	–	–
D-Ribose	+	–	–
D-Trehalose	+	–	ND
Glycerol	+	–	+
Mannitol	+	–	+
Inositol	+	–	+
Urease activity	–	+	–
Hydrolysis of:			
Aesculin	+	–	ND
Gelatin	+	–	ND
Starch	+	–	ND
Temperature range for growth (°C)	4–37	9–36	11–41
pH range for growth	5·1–10·1	6·0–9·0	5·0–9·0
Growth on 7 % NaCl	+	–	ND

diamino acid of peptidoglycan, major menaquinone, phospholipid composition and fatty acid profiles). The genera *Kineococcus* and *Kineosporia* are readily differentiated from each other on the basis of morphological and chemical features (Yokota *et al.*, 1993; Itoh *et al.*, 1989; Kudo *et al.*, 1998). It was evident from the phenotypic and genetic data presented that the isolate belonged to the genus *Kineococcus*, but represented a novel species within the genus, for which the name *Kineococcus marinus* sp. nov. is proposed.

Description of *Kineococcus marinus* sp. nov.

Kineococcus marinus (ma.ri' nus. L. masc. adj. *marinus* of the sea, the origin of the sample from which the type strain was isolated).

Aerobic, motile, non-spore-forming, oxidase-negative, catalase-positive, Gram-positive cocci. Cells occur singly, in pairs or in clusters. Forms capsular polysaccharide-like substances on most solid media. Colonies are circular, smooth, convex and deep orange in colour. Glucose fermentation, H₂S production and nitrate reduction are not observed. The temperature range for growth is 4–37 °C with an optimum at 30 °C. No growth is observed at or above 40 °C. Growth occurs at pH 5·1–10·1, with an optimum at pH 7·1. β-Galactosidase is present, but urease activity is not detected. DNA, aesculin, gelatin and starch are hydrolysed,

but casein and elastin are not. Salt is required for growth; good growth occurs at 1–4 % NaCl, moderate growth at 5–8 % NaCl and poor growth at 9 % NaCl. α-Cyclodextrin, β-cyclodextrin, dextrin, glycogen, L-arabinose, D-arabitol, arbutin, D-cellobiose, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-gluconic acid, α-D-glucose, myo-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melezitose, methyl β-D-galactoside, 3-methyl D-glucoside, methyl α-D-glucoside, methyl α-D-mannoside, palatinose, D-psicose, D-raffinose, L-rhamnose, D-ribose, salicin, sedoheptulosan, stachyose, sucrose, D-tagatose, D-trehalose, turanose, xylitol, D-xylose, acetic acid, α- and β-hydroxybutyric acids, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, α-ketovaleric acid, lactamide, L-lactic acid, D- and L-malic acid, monomethyl succinate, propionic acid, succinamic acid, L-alaninamide, D- and L-alanine, L-asparagine, glycyl L-glutamic acid, L-serine, glycerol, adenosine, 2'-deoxyadenosine, inosine, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-monophosphate, D-fructose 6-phosphate, α-D-glucose 1-phosphate and DL-α-glycerol phosphate are utilized as sole carbon and energy sources for growth. The following substrates are not utilized: inulin, mannan, Tweens 40 and 80, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, amygdalin, maltotriose, D-melibiose, methyl α-D-galactoside, methyl β-D-glucoside, D-sorbitol, γ-hydroxybutyric acid, D-lactic acid methyl ester, methyl pyruvate, succinic acid, *N*-acetyl-L-glutamic acid, L-alanyl glycine, L-glutamic acid, L-pyroglutamic acid, putrescine, 2,3-butanediol, thymidine, uridine and D-glucose 6-phosphate. The diagnostic diamino acid of the peptidoglycan is *meso*-diaminopimelic acid. Whole-cell hydrolysates contain arabinose and galactose as characteristic sugars. The glycan moiety of the murein structure is acetylated. Mycolic acids are not detected. The predominant menaquinone is MK-9(H₂). Polar lipid contains phosphatidylglycerol and phosphatidylinositol (phospholipid-type PI pattern *sensu* Lechevalier *et al.*, 1981). The major cellular fatty acid is 12-methyltetradecanoic acid (anteiso-C_{15:0}). The G + C content of the DNA is 76·6 mol%.

The type strain, KST3-3^T (=KCCM 42250^T=NRRL B-24439^T), was isolated from a sediment sample of the coast of Jeju, Republic of Korea.

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