Kocuria halotolerans sp. nov., an actinobacterium isolated from a saline soil in China

Shu-Kun Tang,^{1†} Yun Wang,^{2†} Kai Lou,² Pei-Hong Mao,³ Li-Hua Xu,¹ Cheng-Lin Jiang,¹ Chang-Jin Kim⁴ and Wen-Jun Li^{1,5}

Correspondence

Wen-Jun Li
wjli@ynu.edu.cn
or
liact@hotmail.com

¹The Key Laboratory for Microbial Resources of the Ministry of Education and Laboratory for Conservation and Utilization of Bio-Resources, Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, PR China

²Xinjiang Institute of Microbiology, Xinjiang Academy of Agricultural Science, Urumqi, Xinjiang 830091, PR China

³Institute of Ion Beam Biotechnology, College of Physics Science and Technology, Xinjiang University, Urumqi, Xinjiang 830008, PR China

⁴Functional Metabolite Research Center KRIBB, 52 Eoeun-dong, Yuseong gu, Daejeon 305-806, Republic of Korea

⁵Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, PR China

A Gram-positive actinobacterium, designated strain YIM 90716^T, was isolated from a saline soil sample collected from Ganjiahu Suosuo Forest National Nature Reserve in Xinjiang Province, north-west China. The new isolate contained lysine, glutamic acid and alanine with peptidoglycan type Lys–Ala₃ (variation A3 α). The major phospholipids were phosphatidylglycerol and diphosphatidylglycerol. The predominant menaquinone was MK-7(H₂). The major fatty acids were anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}. The DNA G + C content of strain YIM 90716^T was 68.0 mol%. Chemotaxonomic properties supported the affiliation of strain YIM 90716^T to the genus *Kocuria*. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the organism was related most closely to *Kocuria kristinae* DSM 20032^T (96.8% similarity) and showed lower levels of 16S rRNA gene similarity (<96.5%) with the type strains of other species of the genus *Kocuria*. The results of fatty acid analysis and physiological and biochemical tests allowed the genotypic and phenotypic differentiation of strain YIM 90716^T from its closest relatives. On the basis of data from the present polyphasic study, strain YIM 90716^T is considered to represent a novel species of the genus *Kocuria*, for which the name *Kocuria halotolerans* sp. nov. is proposed. The type strain is YIM 90716^T (=DSM 18442^T=KCTC 19172^T=CCTCC AB 206069^T).

The genus *Kocuria* was proposed by Stackebrandt *et al.* (1995) to accommodate phylogenetically distinct actinobacteria formerly classified in the genus *Micrococcus*. Members of the genus *Kocuria* are Gram-positive, aerobic, catalase-positive, non-halophilic, non-encapsulated, non-endospore-forming, coccoid actinobacteria. *Kocuria* species

have peptidoglycan type L-Lys–Ala₃, variation A3 α . Galactosamine and glucosamine are the major amino sugars in the cell-wall polysaccharide. The predominant menaquinones are MK-7(H₂) and MK-8(H₂). The major phospholipids are diphosphatidylglycerol and phosphatidylglycerol. The major fatty acid is anteiso-C_{15:0}. The G + C contents of the genomic DNA range from 66 to 75 mol%. The type species of the genus is *Kocuria rosea*. At the time of writing, the genus *Kocuria* comprised 13 recognized species. Members of the genus have been isolated from mammalian skin, soil, the rhizosphere, freshwater, marine sediment and desert soil (Kloos *et al.*, 1974; Stackebrandt *et al.*, 1995; Kovács *et al.*, 1999; Reddy *et al.*, 2003; Kim *et al.*, 2004; Tvřzová *et al.*, 2005; Li *et al.*, 2006; Mayilraj *et al.*, 2006; Zhou *et al.*, 2008).

†These authors contributed equally to this work.

Abbreviation: OPA, *o*-phthalaldehyde.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 90716^T is DQ979377.

A chromatogram of amino acids in purified cell-wall and whole-cell hydrolysates of strain YIM 90716^T and a table detailing the cellular fatty acid compositions of strain YIM 90716^T and *Kocuria kristinae* DSM 20032^T are available as supplementary material with the online version of this paper.

A novel actinobacterial strain, YIM 90716^T, was isolated from a saline soil sample collected from Ganjiahu Suosuo Forest National Nature Reserve in Xinjiang Province, north-west China, after 3 weeks incubation at 37 °C on modified ISP 5 medium [per litre distilled water (final pH 7.5): 1 g L-asparagine, 10 g glycerol, 5 g yeast extract, 1 g K₂HPO₄, 5 g KNO₃, 100 g NaCl and 15 g agar (NaCl was sterilized separately before being added to the medium)]. The strain was maintained on modified ISP 5 agar slants containing 5 % NaCl (w/v) at 4 °C and as glycerol suspensions (20 %, v/v) at –20 °C. Biomass for chemical and molecular studies was obtained by cultivation in shaken flasks (about 150 r.p.m.) by using modified ISP 5 medium [5 % (w/v) NaCl, pH 7.5] at 37 °C for 1 week.

Cell morphology was determined with cultures grown for 6, 12, 24 and 48 h on modified ISP 5 agar medium supplemented with 5 % (w/v) NaCl at 37 °C. Gram staining was carried out by using the standard Gram reaction and was confirmed by using the KOH lysis test method (Cerny, 1978). Cell motility was determined based on the presence of turbidity throughout the tube after incubation in a semisolid medium (Leifson, 1960). Morphological characteristics of the strain were observed by using light microscopy (model BH 2; Olympus) and transmission electron microscopy with a model H-800 transmission electron microscope (Hitachi). Growth temperature was tested at 4, 10, 20, 28, 37, 45 and 55 °C on modified ISP 5 medium containing 5 % (w/v) NaCl. For NaCl tolerance experiments, modified ISP 5 medium was used as the basal medium with salt added at concentrations of 0–30 % (w/v). The pH growth range was investigated between pH 4.0 and 10.0 at intervals of 1 pH unit, by using the buffer system: pH 4.0–5.0, 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0, 0.1 M KH₂PO₄/0.1 M NaOH; pH 9.0–10.0, 0.1 M NaHCO₃/0.1 M Na₂CO₃. Catalase activity was determined based on the production of bubbles after the addition of a drop of 3 % H₂O₂. Oxidase activity was determined based on oxidation of tetramethyl-*p*-phenylenediamine. Hydrolysis of aesculin, casein, gelatin, starch, and Tweens 40 and 80 was determined as described by Cowan & Steel (1965). Utilization of different compounds as sole carbon and energy sources or as sole nitrogen sources was tested as described by Carrasco *et al.* (2006). Enzyme activity and acid production from carbohydrates were determined by using the API ZYM, API Coryne and API 50 CH systems (bioMérieux) according to the manufacturer's instructions. The morphological, cultural and physiological properties of strain YIM 90716^T are given in Table 1 and in the species description.

A purified cell-wall preparation was obtained and hydrolysed as described by Schleifer & Kandler (1972). Amino acids in cell-wall hydrolysates were analysed by precolumn derivatization with *o*-phthalaldehyde (OPA) by HPLC (Agilent 1100; Agilent Technologies). Ten amino acid standards (10 µl, 0.2 mM) and 10 µl hydrolysed purified cell wall of strain YIM 90716^T were dissolved in 0.1 M

Table 1. Differential characteristics between strain YIM 90716^T and its nearest phylogenetic neighbour, *Kocuria kristinae*

+, Positive; –, negative; w, weak; v, variable.

Characteristic	Strain YIM 90716 ^T	<i>K. kristinae</i> DSM 20032 ^{T*}
Isolation source	Saline soil	Human skin
Colony colour	Pale yellow	Pale cream to pale orange
Oxidase	–	+
Urease	–	v
Alkaline phosphatase	+	–
β-Galactosidase	–	v
Nitrate reduction	+	–
Acid production from:		
Glycerol	w	+
Sucrose	w	+
Utilization of:		
L-Arabinose	+	–
Galactose	+	–
Ribose	+	–
Growth with:		
0 % NaCl	+	+
10 % NaCl	+	+
15 % NaCl	–	w
Major fatty acids (>10 %)	anteiso-C _{15:0} , iso-C _{16:0} , anteiso-C _{17:0}	anteiso-C _{15:0} , iso-C _{16:0}
DNA G + C content (mol%)	68.0	67.0

*Data from Stackebrandt *et al.* (1995).

(30 µl) borax buffer, and 10 µl OPA was added and allowed to react for 50 s at room temperature. Analysis of precolumn derivatives of amino acids with OPA was carried out on the Agilent 1100 HPLC system equipped with an Agilent four-unit pump, a 7125 injector, G1314A UV detector and a column oven (ABI). The analytical column used was a ZORBAX Eclipse-AAA (4.6 × 150 mm, 3.5 µm; Agilent). The wavelength for UV detection was 338 nm. Elution was carried out at a flow rate of 1.0 ml min^{–1} at 40 °C. The mobile phase A contained CH₃COONa at 0.05 mol l^{–1} and 0.3 % tetrahydrofuran, and phase B was acetonitrile/methanol (1 : 1, v/v), by using gradient elution of 0–50–50 % buffer B by a linear increase from 0 to 25 to 30 min.

Whole-cell sugars were analysed according to the procedures developed by Hasegawa *et al.* (1983). Polar lipids were extracted, examined by using two-dimensional TLC and identified by using standard procedures (Minnikin *et al.*, 1984). Polar lipids were separated by using two-dimensional TLC (silica-gel plate 60; Merck). The first direction was developed in chloroform/methanol/water

(65:25:4, by vol.) and the second was developed in chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Total lipid material and specific functional groups were detected by using Dittmer and Lester reagent (phosphate), ninhydrin (free amino groups), Dragendorff reagent (quaternary nitrogen) and anisaldehyde-sulfuric acid (glycolipids). Menaquinones were isolated according to Minnikin *et al.* (1984) and were separated by using HPLC (Kroppenstedt, 1982). For analysis of fatty acids, strain YIM 90716^T was cultured on trypticase soy agar (TSA; Difco) containing 5 % NaCl at 37 °C for 72 h. Preparation and analysis of fatty acid methyl esters were performed as described by Sasser (1990) by using the Microbial Identification System (MIDI Inc.).

Strain YIM 90716^T contained lysine, glutamic acid and alanine (HPLC; see Supplementary Fig. S1 in IJSEM Online), a profile that is characteristic of the genus *Kocuria* [peptidoglycan type Lys-Ala₃ (variation A3 α); DSMZ, 2001]. Whole cell-wall sugars were galactose and glucose. Phospholipids contained phosphatidylglycerol and diphosphatidylglycerol. The predominant menaquinone was MK-7(H₂). Strain YIM 90716^T had a cellular fatty acid profile that contained large amounts of branched fatty acids, and the major components (>10 % of the total) were anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}; minor amounts of straight-chain and unsaturated fatty acids were also detected. Details of the phospholipids, menaquinones and the cellular fatty acid profile are given in the species description (see also Supplementary Table S1 in IJSEM Online for detailed fatty acid data).

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were performed as described by Li *et al.* (2007). The sequence obtained for strain YIM 90716^T (1479 bp) was compared with 16S rRNA gene sequences of reference taxa retrieved from the GenBank and EMBL databases via BLAST searches. Multiple alignments and sequence evolutionary distance calculations were carried out by using CLUSTAL X (Thompson *et al.*, 1997) software. Gaps at the 5' and 3' ends of the alignment were omitted from further analysis. Phylogenetic analyses were performed by using three tree-making algorithms, namely the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A phylogenetic tree was constructed based on the neighbour-joining method of Saitou & Nei (1987) from K_{nuc} values (Kimura, 1980) by using MEGA version 4.0 (Tamura *et al.*, 2007). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method based on 1000 replicates (Felsenstein, 1985).

The results of the BLAST searches showed that strain YIM 90716^T had highest 16S rRNA gene sequence similarity to members of the genus *Kocuria*. A phylogenetic tree was constructed with sequences of representative strains of the genus *Kocuria*, and *Micrococcus antarcticus* T2^T was used as the outgroup. In the phylogenetic tree based on the neighbour-joining algorithm, strain YIM 90716^T clustered

with *K. kristinae* DSM 20032^T (Fig. 1). Levels of 16S rRNA gene sequence similarity between strain YIM 90716^T and the type strains of recognized species of the genus *Kocuria* ranged from 96.8 % (*K. kristinae* DSM 20032^T) to 95.4 %.

For the determination of the DNA G + C content, genomic DNA of strain YIM 90716^T was prepared according to the method of Marmur (1961). The G + C content of the DNA was determined by using reversed-phase HPLC (Mesbah *et al.*, 1989) and a value of 68 mol% was derived.

The chemotaxonomic properties of strain YIM 90716^T [L-Lys-Ala₃, variation A3 α as peptidoglycan type; galactose and glucose as major amino sugars; anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0} as major fatty acids; diphosphatidylglycerol and phosphatidylglycerol as major phospholipids; MK-7(H₂) and MK-8(H₂) as predominant menaquinones] indicated affiliation to the genus *Kocuria* (Stackebrandt *et al.*, 1995). 16S rRNA gene sequence analysis revealed that strain YIM 90716^T had closest phylogenetic affiliation to the genus *Kocuria*, which was confirmed by a high bootstrap resampling value. Therefore, both chemotaxonomic and phylogenetic results clearly indicated that strain YIM 90716^T belongs to the genus *Kocuria*. Although strain YIM 90716^T was similar to *K. kristinae* in containing anteiso-C_{15:0} as the major fatty acid and shared the same branch with *K. kristinae* DSM 20032^T in the neighbour-joining phylogenetic tree, it could be differentiated based on several morphological, physiological and chemotaxonomic properties (Table 1). Colonies of strain YIM 90716^T were pale yellow and could not grow in the presence of 15 % NaCl, whereas colonies of *K. kristinae* are pale cream to pale orange and can grow in the presence of 15 % NaCl. In addition, strain YIM 90716^T and *K. kristinae* showed differences with regard to the presence of oxidase and phosphatase, nitrate reduction and in acid

Fig. 1. Neighbour-joining phylogenetic dendrogram obtained by distance-matrix analysis of 16S rRNA gene sequences, showing the position of strain YIM 90716^T and its phylogenetic neighbours. Numbers at branch nodes are bootstrap values (based on 1000 resamplings; only values >50 % are shown). The sequence of *Micrococcus antarcticus* T2^T was used as an outgroup. Bar, 1 % sequence divergence.

production from carbohydrates (Table 1). Furthermore, strain YIM 90716^T and *K. kristinae* showed differences in their fatty acid compositions (Supplementary Table S1). For instance, strain YIM 90716^T contains anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0} as major fatty acids (>10%), as well as minor amounts of unsaturated fatty acids; *K. kristinae* contains anteiso-C_{15:0} and iso-C_{16:0} as major fatty acids (>10%) but no unsaturated fatty acids. On the basis of its phenotypic and genotypic distinctiveness, strain YIM 90716^T should be placed in the genus *Kocuria* as the type strain of a novel species, for which the name *Kocuria halotolerans* sp. nov. is proposed.

Description of *Kocuria halotolerans* sp. nov.

Kocuria halotolerans (ha.lo.to'le.rans. Gr. n. *hals*, *halos* salt; L. pres. part. *tolerans* tolerating, enduring; N.L. part. adj. *halotolerans*, salt-tolerating).

Cells are Gram-positive, coccoid (0.6–1 µm in diameter), non-motile and non-endospore-forming. Colonies are pale yellow, circular, opaque, slightly convex, smooth and approximately 1.0 mm in diameter after 24 h at 37 °C on modified ISP 5 medium containing 5% NaCl. No water-soluble exopigment is formed. Grows at 10–37 °C, at pH 6.0–9.0 and in the presence of 0–10% (w/v) NaCl, but no growth is observed at 4 or 45 °C, above pH 10 or in the presence of 15% NaCl; optimal growth occurs with 5% NaCl, at pH 7.0–8.0 and at 28–37 °C. Catalase-positive but oxidase-negative. Hydrolyses aesculin but not gelatin, starch or Tweens 20, 40 or 80. Hydrogen sulfide is not produced. Nitrate reduction is positive. In the API 50 CH test system, acid is produced from *N*-acetylglucosamine, D-adonitol, aesculin, amygdalin, L-arabinose, D-arabitol, arbutin, D-fructose, D-galactose, D-glucose, inositol, maltose, D-mannitol, D-mannose, melezitose, D-ribose, salicin, D-sorbitol, D-tagatose and turanose; acid production from glycerol, sucrose and potassium gluconate is weak. No acid is produced from D-arabinose, L-arabitol, cellobiose, dulcitol, erythritol, DL-fucose, gentiobiose, methyl α-D-glucopyranoside, glycogen, inulin, D-lactose, D-lyxose, melibiose, methyl α-D-mannopyranoside, potassium 2-ketogluconate, potassium 5-ketogluconate, raffinose, L-rhamnose, starch, L-sorbose, trehalose, xylitol, methyl β-D-xylopyranoside or DL-xylose. In the API Coryne system, positive for nitrate reduction, pyrazinamidase, alkaline phosphatase, α-glucosidase, β-glucosidase, and fermentation of D-glucose, D-ribose, maltose and D-mannitol; variable for fermentation of sucrose. Negative for pyrrolidonyl arylamidase, β-glucuronidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, urease, gelatin hydrolysis, and acid production from D-lactose, D-xylose and glycogen. In the API ZYM system, positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-glucosidase and β-glucosidase. Negative for esterase (C4), esterase lipase (C8), lipase (C14), α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-

fucosidase. The peptidoglycan type is Lys-Ala₃ (variation A3α). Whole cell-wall sugars are galactose and glucose. Major phospholipids are phosphatidylglycerol and diphosphatidylglycerol. Menaquinones are MK-7(H₂) (86.3%), MK-8(H₂) (11.8%) and MK-6(H₂) (1.8%). Major cellular fatty acids are anteiso-C_{15:0} (47.7%), iso-C_{16:0} (15.7%) and anteiso-C_{17:0} (22.1%); minor amounts of C_{16:0} (6.2%), iso-C_{14:0} (1.4%), C_{14:0} (1.3%), iso-C_{15:0} (1.1%), C_{18:2ω6,9c}/anteiso-C_{18:0} (1.4%), C_{18:1ω9c} (1.9%) and C_{18:0} (1.3%) are also detected. The DNA G+C content of the type strain is 68.0 mol%.

The type strain, YIM 90716^T (=DSM 18442^T=KCTC 19172^T=CCTCC AB 206069^T), was isolated from a saline soil sample collected from Ganjiahu Suosuo Forest National Nature Reserve, Xinjiang Province, north-west China.

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