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Kocuria salsicia sp. nov., isolated from salt-fermented seafood

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Strain 104^T was isolated from a traditional salt-fermented seafood in Korea. It was a Gram-positive, non-motile, coccus-shaped bacterium. It formed lemon–yellow, opaque colonies that were circular with entire margins. Optimal growth occurred at 30–37 °C, pH 7–8 and in the presence of 0–2 % (w/v) NaCl. Phylogenetic analysis of 16S rRNA gene sequences from strain 104^T and reference species of the genus *Kocuria* indicated that strain 104^T formed an independent line. The G+C content of the chromosomal DNA was 60.6 mol%. MK-7 was the major menaquinone and the predominant fatty acids were anteiso-$C_{15:0}$ (76.7 %), anteiso-$C_{17:0}$ (10.9 %) and iso-$C_{16:0}$ (4.5 %). Strain 104^T was most closely related to *Kocuria rhizophila* TA68^T (98.9 % 16S rRNA gene sequence similarity). The DNA–DNA hybridization value between strain 104^T and *K. rhizophila* TA68^T was 14.1 ± 3.4 %. On the basis of this polyphasic taxonomic analysis, strain 104^T appears to represent a novel species in the genus *Kocuria*. The name *Kocuria salsicia* sp. nov. is proposed. The type strain is 104^T (=KACC 21128^T=JCM 16361^T).

Phylogenetic and chemotaxonomic analyses of the genus *Micrococcus* indicated that certain species were taxonomically distinct and these findings led to the establishment of the new genus *Kocuria* in 1995. This genus contains 17 recognized species, namely, *K. kristinae*, *K. rosea*, *K. varians* (Stackebrandt *et al.*, 1995), *K. erythromyxa* (Rainey *et al.*, 1997; later reclassified as a later synonym of *K. rosea*), *K. palustris*, *K. rhizophila* (Kovács *et al.*, 1999), *K. polaris* (Reddy *et al.*, 2003), *K. marina* (Kim *et al.*, 2004), *K. carniphila* (Tvrzová *et al.*, 2005), *K. aegyptia* (Li *et al.*, 2006), *K. himachalensis* (Mayilraj *et al.*, 2006), *K. flava*, *K. turfanensis* (Zhou *et al.*, 2008), *K. halotolerans* (Tang *et al.*, 2009), *K. atrinae* (Park *et al.*, 2010a), *K. koreensis* (Park *et al.*, 2010b) and *K. gwangalliensis* (Seo *et al.*, 2009). Kocuriae have been isolated from a variety of environments, such as the narrow-leaved cattail (Kovács *et al.*, 1999), an Antarctic cyanobacterial mat sample (Reddy *et al.*, 2003), marine sediment (Kim *et al.*, 2004), saline desert soil (Li *et al.*, 2006), fermented food (Park *et al.*, 2010a, b) and seawater (Seo *et al.*, 2009). In this study, we describe the isolation of a novel strain from a salt-fermented seafood ('gajami-sikhae') from Korea. Following isolation on marine agar 2216 (MA, BBL) at 30 °C, a pure culture was obtained by repeated restreaking and the new strain was characterized and described.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 104^T is GQ352404.

A supplementary table is available with the online version of this paper.

Chromosomal DNA extraction and purification processes were performed as described by Sambrook *et al.* (1989). Two universal primers for bacterial 16S rRNA genes (8f, 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492r, 5′-AAGGA-GGTGATCCAGCCGC-3′; Baker *et al.*, 2003) and a PCR pre-Mix (Solgent), were used for colony PCR amplification of the 16S rRNA gene from strain 104^T. Following purification, the PCR products were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer's instructions. An ABI Prism 3730XL DNA Analyzer (Applied Biosystems) was used to analyse the reaction mixtures and 16S rRNA gene sequence fragments were assembled with SeqMan (DNASTAR). The CLUSTAL_X (v. 1.8) program (Thompson *et al.*, 1997) was used to perform a multiple sequence alignment between 16S rRNA gene sequences from strain 104^T and phylogenetically related taxa (obtained from the NCBI database). Phylogenetic relationships were determined using the MEGA4 program (Tamura *et al.*, 2007). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) methods. Confidence values were evaluated using a bootstrap analysis based on 1000 resamplings. The 16S rRNA gene sequence of strain 104^T indicated phylogenetic affiliation with the genus *Kocuria*, a finding supported by high bootstrap values (Fig. 1). The 16S rRNA gene sequence of strain 104^T exhibited 98.9, 98.7, 98.2, 98.1, 98.1 and 97.9 % similarity to the type strains *K. rhizophila* TA68^T, *K. varians*

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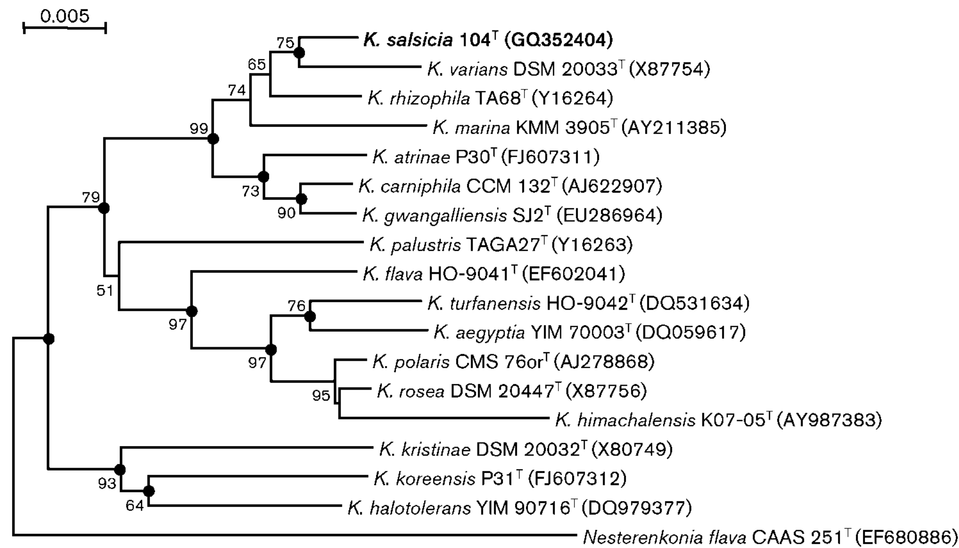

Fig. 1. Neighbour-joining phylogenetic tree of 16S rRNA gene sequences showing the relationship between strain 104^T and all recognized species of the genus *Kocuria*. Filled circles indicate branches found in phylogenetic consensus trees generated with the maximum-parsimony and neighbour-joining methods. Numbers at nodes indicate the levels of bootstrap support (>50 %) based on 1000 resamplings. Bar, 0.005 changes per nucleotide position.

DSM 20033^T, *K. marina* KMM 3905^T, *K. atrinae* P30^T, *K. carniphila* CCM 132^T and *K. gwangalliensis* SJ2^T, respectively.

DNA–DNA hybridization was performed using photobiotin-labelled DNA probes and microwell plates (Ezaki *et al.*, 1989). Separate species would be expected to exhibit DNA–DNA hybridization values of <70 % (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987). The DNA–DNA relatedness values between strain 104^T and its six closest phylogenetic neighbours were as follows (n=5): *K. rhizophila* TA68^T, 14.1 ± 3.4 %; *K. varians* DSM 20033^T, 27.1 ± 3.8 %; *K. marina* KMM 3905^T, 14.1 ± 2.7 %; *K. atrinae* P30^T, 15.2 ± 4.4 %; *K. carniphila* CCM 132^T, 12.7 ± 2.8 %; and *K. gwangalliensis* SJ2^T, 14.0 ± 3.2 %. These low DNA–DNA hybridization values indicated that strain 104^T represented a distinct genomic species.

Morphological and physiological characterizations were performed for strain 104^T and the six reference strains. Cells were grown on MA (pH 7.3) at 30 °C for 2 days. Cell morphology was observed by light microscopy. A non-staining method was used to determine the Gram reactions (Buck, 1982). Oxidase reagent (bioMérieux) was used for testing oxidase activity and catalase activity was determined by bubble formation in a 3 % (v/v) H_2O_2 solution. Semi-solid agar (motility test medium; marine agar) was used to examine motility (Tittsler & Sandholzer, 1936). In order to determine the temperature range for growth, cells were grown on MA medium at 4, 15, 25, 30, 37 and 45 °C for 48 h. Growth at various NaCl concentrations (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 and 20 %; w/v) was determined at 30 °C in broth medium that contained all of the constituents marine broth (MB), except NaCl, supplemented with appropriate concentrations of NaCl. The pH range for growth was determined from pH 2.0–13.0 (at intervals of 1.0 pH unit) using MB. The strain was characterized biochemically using the API 20NE and API ZYM systems (bioMérieux). MA plates were used to examine hydrolysis of starch and Tweens 20, 40, 60 and 80 (final concentration of 1 %; v/v). After incubation on MA (pH 7.3) at 30 °C for 48 h, strain 104^T formed opaque, lemon–yellow, circular colonies with entire margins. Cells were aerobic, Gram-positive, non-motile cocci of 1–1.5 μm in diameter. Strain 104^T was catalase-positive, but oxidase-negative. It grew between 15 and 37 °C (optimum, 30–37 °C), at pH 6–9 (optimum, pH 7–8) and in the presence of 0–4 % (w/v) NaCl (optimum, 0–2 %). Strain 104^T hydrolysed starch and urea, but not Tweens 20, 40, 60 or 80. A detailed species description is presented below. A phenotypic comparison of strain 104^T and six related species of the genus *Kocuria* is presented in Table 1.

The DNA G + C content was determined fluorometrically using SYBR Green and real-time PCR (Gonzalez & Saiz-Jimenez, 2002), with *Escherichia coli* K-12, *K. rhizophila* TA68^T and *K. marina* KMM 3905^T used for calibration. The genomic DNA G + C content of strain 104^T was 60.6 mol%, which was similar to related reference species (60–72 mol%; Kim *et al.*, 2004; Stackebrandt *et al.*, 1995). To determine the fatty acid compositions, strain 104^T and reference strains were grown on MA at 30 °C for 48 h. Cellular fatty acids were prepared and analysed according to standard protocols of the Sherlock Microbial Identification System (MIDI). The cellular fatty acid compositions of each strain are shown in Supplementary Table S1 (available in IJSEM Online). Strain 104^T exhibited a qualitatively similar cellular fatty acid profile to *K. marina* KMM 3905^T (Kim *et al.*, 2004), with the

Table 1. Differential phenotypic characteristics of strain 104^T and related species of the genus *Kocuria*

Taxa: 1, strain 104^T; 2, *K. varians* DSM 20033^T; 3, *K. rhizophila* TA68^T; 4, *K. marina* KMM 3905^T; 5, *K. carniphila* CCM 132^T; 6, *K. atrinae* P30^T; 7, *K. gwangalliensis* SJ2^T. In this study, API kit results were obtained for strains 2–7. Additional results were obtained from Stackebrandt *et al.* (1995), Kovács *et al.* (1999), Kim *et al.* (2004), Tvrzová *et al.* (2005), Park *et al.* (2010a) and Seo *et al.* (2009). All strains were positive for catalase, alkaline phosphatase, esterase lipase C8 and naphthol-AS-BI-phosphohydrolase. All strains were negative for oxidase, indole production, L-arginine dihydrolase, lipase C14, valine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase and the assimilation of L-arabinose and capric acid. +, Positive; −, negative; W, weakly positive; NR, not reported.

Characteristic	1	2	3	4	5	6	7
DNA G+C content (mol%)	60.6	66–72	69.4	60	71	70.2	65.2
Hydrolysis of:							
Starch	+	−	−	+	−	NR	−
Tween 80	−	−	+	+	−	NR	NR
Reduction of nitrate to nitrite	+	−	−	+	+	+	+
D-Glucose fermentation	−	−	−	−	−	+	−
Urease	+	+	−	+	−	−	−
β-Glucosidase (aesculin hydrolysis)	−	W	−	−	−	−	−
Protease (gelatin hydrolysis)	W	+	+	+	−	W	+
β-Galactosidase (PNPG hydrolysis)	−	−	−	+	+	−	−
Assimilation of:							
N-Acetylglucosamine	−	−	W	−	−	−	−
Adipic acid	+	W	+	+	+	−	−
D-Glucose	+	+	+	+	+	−	−
L-Malic acid	+	W	+	+	+	−	−
Maltose	+	+	+	+	+	−	W
D-Mannitol	−	−	−	−	+	−	W
D-Mannose	+	+	+	W	+	−	W
Phenylacetic acid	+	+	+	−	+	−	−
Potassium gluconate	+	+	+	+	+	−	−
Trisodium citrate	+	W	−	W	−	−	−
Enzyme activities:							
Acid phosphatase	−	−	W	−	W	W	W
Cystine arylamidase	−	−	−	−	−	−	W
Esterase (C4)	−	W	W	W	W	W	W
β-Galactosidase	−	+	−	−	+	−	−
α-Glucosidase	−	−	W	−	+	W	+
β-Glucuronidase	−	−	−	−	W	W	W
Leucine arylamidase	−	W	+	W	W	+	+

major fatty acids being anteiso-$C_{15:0}$ (76.7 %), anteiso-$C_{17:0}$ (10.9 %) and iso-$C_{16:0}$ (4.5 %). Quinone extraction and identification were performed according to the method of Komagata & Suzuki (1987). As observed for the reference species, the main isoprenoid quinone of strain 104^T was MK-7.

This taxonomic study of strain 104^T adopted a polyphasic approach and included molecular phylogenetic analyses, the determination of DNA–DNA relatedness and genomic DNA G+C content and analysis of the fatty acid profile, as well as data from physiological and biochemical tests. These findings indicate that strain 104^T represents a novel species of the genus *Kocuria* for which the name *Kocuria salsicia* sp. nov. is proposed.

Description of *Kocuria salsicia* sp. nov.

Kocuria salsicia (sal.si′ci.a. L. fem. adj. *salsicia* salted, salty).

Colonies are lemon–yellow in colour, opaque and circular with entire margins. Cells are Gram-positive, non-motile and coccus-shaped, measuring 1–1.5 μm in diameter. Cells are catalase-positive and oxidase-negative. Grows from 15 to 37 °C (optimum, 30–37 °C), at pH 6–9 (optimum, pH 7–8) and in the presence of 0–4 % (w/v) NaCl (optimum, 0–2 %). Cells are positive for alkaline phosphatase, esterase lipase C8 and naphthol-AS-BI-phosphohydrolase, but negative for esterase C4, lipase C14, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities. Reduces nitrate to nitrite. Indole is not produced. Starch, urea and gelatin (weak reaction) are hydrolysed, but not casein, Tweens 20, 40, 60 and 80 or aesculin. Negative for *p*-nitrophenyl-β-D-galactopyranoside, D-glucose fermentation and L-arginine dihydrolase

activity. Positive for assimilation of D-glucose, D-mannose, maltose, potassium gluconate, adipic acid, L-malic acid, trisodium citrate and phenylacetic acid, and negative for assimilation of L-arabinose, D-mannitol, *N*-acetylglucosamine and capric acid. Menaquinone MK-7 is the major quinone and the major cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$.

The type strain, 104^T (=KACC 21128^T=JCM 16361^T) was isolated from Korean salt-fermented seafood. The DNA G+C content of the type strain is 60.6 mol%.

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