Kocuria atrinae sp. nov., isolated from traditional Korean fermented seafood

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A novel actinobacterium, strain P30^T, was isolated from jeotgal, a traditional Korean fermented seafood. Cells were aerobic, Gram-positive, non-motile and coccoid. Optimal growth occurred at 30–37 °C, at pH 8–9 and in the presence of 0–2 % (w/v) NaCl. Based on 16S rRNA gene sequence analysis, strain P30^T was phylogenetically closely related to *Kocuria carniphila*, *Kocuria gwangalliensis*, *Kocuria rhizophila*, *Kocuria marina*, *Kocuria rosea* and *K. varians* with levels of similarity of 98.6, 98.2, 98.1, 97.4, 97.3 and 97.3 %, respectively, to the type strains of these species. Levels of DNA–DNA relatedness between strain P30^T and the type strains of *K. carniphila*, *K. rhizophila*, *K. marina*, *K. rosea* and *K. varians* were 37, 43, 37, 25 and 17 %, respectively. The predominant menaquinone of strain P30^T was MK-7. Major cellular fatty acids were anteiso-$C_{15:0}$, iso-$C_{15:0}$ and iso-$C_{16:0}$. The genomic DNA G+C content of strain P30^T was 70.2 mol%. Based on these data, strain P30^T is considered to represent a novel species of the genus *Kocuria*, for which the name *Kocuria atrinae* sp. nov. is proposed. The type strain is P30^T (=KCTC 19594^T=JCM 15914^T).

The genus *Kocuria* within the family *Actinobacteria* was first described by Stackebrandt *et al.* (1995), and at the time of writing comprises 16 recognized species, namely *Kocuria rosea*, *K. varians*, *K. kristinae* (Stackebrandt *et al.*, 1995), *K. erythromyxa* (Rainey *et al.*, 1997), *K. palustris*, *K. rhizophila* (Kovacs *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. gwangalliensis* (Seo *et al.*, 2009), *K. halotolerans* (Tang *et al.*, 2009) and *K. koreensis* (Park *et al.*, 2010). Strains of these species have been isolated from desert soil, saline water, seawater, air, marine sediment, the rhizoplane of narrow-leaved cattail, a cold desert soil of the Indian Himalayas and fermented seafood (Stackebrandt *et al.*, 1995; Rainey *et al.*, 1997; Kovacs *et al.*, 1999; Reddy *et al.*, 2003; Kim *et al.*, 2004; Tvrzová *et al.*, 2005; Mayilraj *et al.*, 2006; Li *et al.*, 2006; Zhou *et al.*, 2008; Seo *et al.*, 2009; Tang *et al.*, 2009; Park *et al.*, 2010). In the present study, we describe the detailed phylogenetic characterization of a novel species of the genus *Kocuria*.

Strain P30^T was isolated from a Korean traditional fermented seafood by using the standard dilution-plating method on marine agar 2216 (MA; BBL) medium at 30 °C and was transferred several times to obtain a pure culture.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P30^T is FJ607311.

Salinity tolerance and requirement were investigated in identical marine broth (MB; BBL) medium without NaCl and with NaCl at various concentrations (1, 2, 3 and 5–30 %, w/v, at intervals of 5 %). For determination of the optimum culture conditions for strain P30^T, growth was tested at 4, 10, 15, 20, 25, 30, 37 and 40 °C on MA medium and at pH 3–10 (at intervals of 1.0 pH unit) in MB medium adjusted by the addition of HCl or NaOH (prior to sterilization). Morphological and physiological studies for strain P30^T were performed with cells grown on MA medium at 30 °C for 2 days. Growth under anaerobic conditions was tested after 7 days incubation at 30 °C on MA medium in an oxygen-free $N_2/CO_2/H_2$ (8 : 1 : 1) anaerobic chamber. Gram staining reaction was performed by using a Gram Stain kit (BBL). Cell morphology of strain P30^T was observed by light microscopy (E600; Nikon). Semisolid agar was used for tests of motility (Tittsler & Sandholzer, 1936). Oxidase and catalase activities were determined by using a 3 % (v/v) H_2O_2 solution and an oxidase reagent (bioMérieux), respectively. Activity of 19 hydrolytic enzymes was determined by using an API ZYM kit (bioMérieux). Substrate utilization by strain P30^T was tested by using API 20NE (bioMérieux) and Biolog GP2 metabolic fingerprinting plates (Biolog).

Colonies grown on MA medium were pale yellow, circular, smooth and opaque. Cells of strain P30^T were aerobic, Gram-positive, non-motile and coccoid (1.0–1.5 μm in diameter). The strain was able to grow at 25–37 °C, at

pH 7–9 and in the presence of 0–4 % NaCl. Strain P30^T and all reference strains tested were catalase-positive and oxidase-negative (Stackebrandt *et al.*, 1995; Kovacs *et al.*, 1999; Kim *et al.*, 2004; Tvrzová *et al.*, 2005). The morphological, cultural, physiological and biochemical characteristics of strain P30^T and the type strains of five closely related *Kocuria* species are summarized in Table 1.

Chromosomal DNA was extracted and purified according to the method described by Sambrook *et al.* (1989). The 16S rRNA gene was amplified and purified by using a PCR Pre-Mix (Solgent) and purification kit (Solgent), respectively. 16S rRNA gene sequencing and phylogenetic analysis were performed as described by Roh *et al.* (2008). Phylogenetic relationships between strain P30^T and representatives of the genus *Kocuria* were performed by using MEGA 4 (Tamura *et al.*, 2007). Levels of 16S rRNA gene sequence similarity were determined by using the EzTaxon server (www.eztaxon.org; Chun *et al.*, 2007). Distance matrices were computed based on the method described by Kimura (1980) and were used to construct dendrograms by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) methods. Bootstrap analysis was conducted with 1000 replicates to evaluate the stability of the trees. DNA–DNA hybridization experiments were performed by using photobiotin-labelled DNA probes and microwell plates as described by Ezaki *et al.* (1989).

As shown in the phylogenetic tree based on the neighbour-joining method (Fig. 1), strain P30^T formed a monophyletic cluster with *K. carniphila* CCM 132^T. On the basis of 16S rRNA gene sequences, strain P30^T was a member of the genus *Kocuria* and was related most closely to *K. carniphila* CCM 132^T, *K. gwangalliensis* DE 706^T, *K. rhizophila* DSM 11926^T, *K. marina* KMM 3905^T, *K. rosea* DSM 20447^T and *K. varians* DSM 20033^T at levels of similarity of 98.4, 98.2, 98.1, 97.5, 97.3 and 97.3 %, respectively. Levels of DNA–DNA relatedness between strain P30^T and its five closest relatives (16S rRNA gene sequence similarity >97.0 %), namely *K. carniphila* CCM 132^T, *K. rhizophila* DSM 11926^T, *K. marina* KMM 3905^T, *K. rosea* DSM 20447^T and *K. varians* DSM 20033^T, were 37, 43, 37, 25 and 17 %, respectively. These values are clearly below the 70 % recommended cut-off for the delineation of bacterial species (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987). The low levels of DNA–DNA relatedness clearly confirmed that strain P30^T represents a novel species.

The genomic DNA G+C content of strain P30^T was determined according to the fluorimetric method by using SYBR Green I as described by Gonzalez & Saiz-Jimenez (2002). Cell biomass for analyses of cellular fatty acids and menaquinones was obtained from cultures grown on MA for 2 days at 30 °C. For quantitative analysis of cellular fatty acids, cells of P30^T and of *K. carniphila* CCM 132^T, *K. rhizophila* DSM 11926^T and *K. varians* DSM 20033^T were tested. Fatty acids were saponified, methylated and extracted by using the standard protocol provided by the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990), and were analysed by GC (Hewlett Packard 6890). TLC was used for analysis of menaquinones, as described by Hiraishi *et al.* (1996). Three replicate trials for each experiment were performed.

The G+C content of the genomic DNA of strain P30^T was 70.2 mol%, within the range of 66–75 mol% reported for

Table 1. Differential characteristics between strain P30^T and closely related species of the genus *Kocuria*

Strains: 1, P30^T; 2, *K. carniphila* CCM 132^T; 3, *K. rhizophila* DSM 11926^T; 4, *K. varians* DSM 20033^T; 5, *K. marina* KMM 3905^T; 6, *K. rosea* DSM 20447^T. Data for 1–3 are from the present study; data for 4–6 are from Stackebrandt *et al.* (1995), Kim *et al.* (2004) and Tvrzová *et al.* (2005). +, Positive; −, negative; W, weakly positive; NR, not reported.

Characteristic	1	2	3	4	5	6
Growth at/with:						
5 °C	−	−	−	−	+	−
10 % NaCl	+	−	+	−	+	−
15 % NaCl	−	−	−	−	+	−
Reduction of nitrates to nitrites	+	+	−	−	+	+
L-Arginine dihydrolase	+	−	−	−	−	−
Urease	+	−	−	+	+	−
Aesculin hydrolysis	+	−	−	−	NR	W
Gelatin hydrolysis	−	−	+	+	+	−
Starch hydrolysis	+	−	−	−	−	+
Alkaline phosphatase	−	+	+	−	−	−
β-Galactosidase	−	+	−	+	+	−
β-Glucuronidase	−	−	−	−	+	−
Utilization of:						
Dextrin	+	+	+	+	NR	−
Glycogen	−	−	+	+	NR	−
Tween 40	+	+	+	+	+	−
Tween 80	+	−	+	+	−	−
N-Acetyl-D-glucosamine	−	−	+	−	−	−
N-Acetyl-β-D-mannosamine	−	+	−	−	−	−
L-Arabinose	−	+	+	+	−	+
L-Fucose	−	+	+	+	+	+
D-Galacturonic acid	−	−	−	−	NR	−
myo-Inositol	−	+	−	−	−	−
Maltose	+	−	−	NR	+	−
D-Mannitol	−	+	−	−	−	+
Melibiose	−	+	−	+	−	−
Methyl-α-D-galactoside	−	+	−	−	NR	−
3-Methylglucose	−	−	−	W	NR	−
D-Sorbitol	+	+	−	+	−	+
Xylitol	+	−	+	+	NR	−
β-Hydroxybutyric acid	+	+	−	+	NR	−
D-Malic acid	−	−	+	+	NR	+
N-Acetyl-L-glutamic acid	+	−	−	+	NR	−
L-Glutamic acid	+	+	−	−	NR	−
Glycerol	+	+	−	−	NR	+
Adenosine	+	+	−	+	NR	−
Uridine	+	−	+	+	NR	W
D-Glucose 6-phosphate	−	−	−	+	NR	W

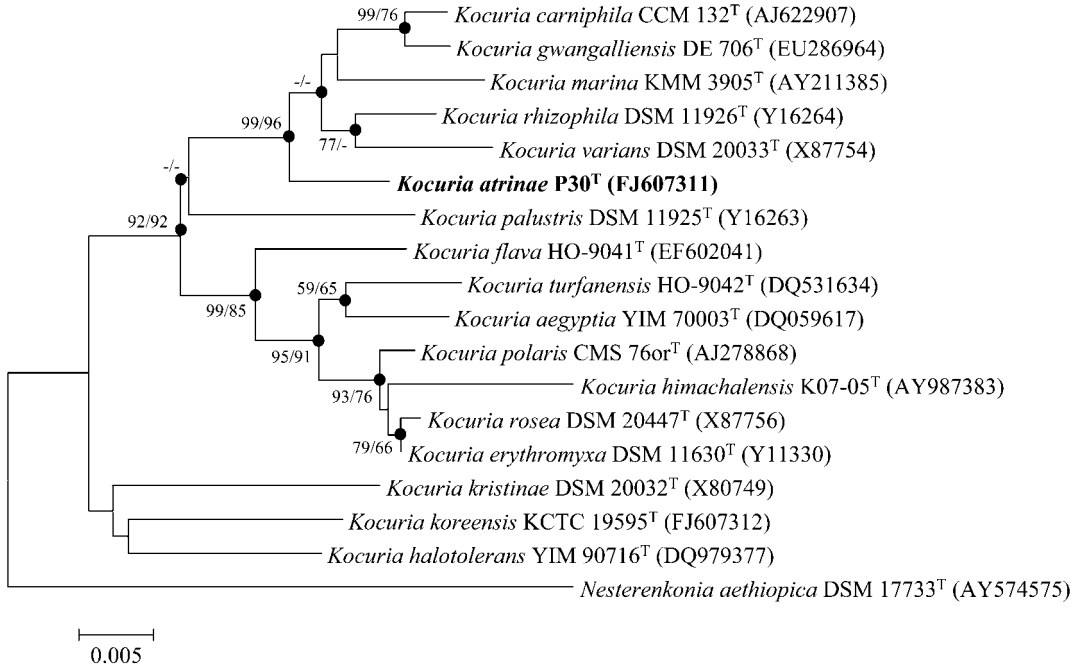

Fig. 1. Neighbour-joining phylogenetic consensus tree based on 16S rRNA gene sequences showing the relationship between strain P30^T and representative members of the genus *Kocuria*. Filled circles indicate generic branches that were also recovered with the maximum-parsimony method. *Nesterenkonia aethiopica* DSM 17733^T served as an outgroup. Numbers at nodes are bootstrap values calculated from neighbour-joining/maximum-parsimony probabilities as percentages of 1000 replications; only values >50 % are shown. GenBank accession numbers are shown in parentheses. Bar, 0.005 substitutions per nucleotide position.

members of the genus *Kocuria* (Stackebrandt *et al.*, 1995; Rainey *et al.*, 1997; Kovacs *et al.*, 1999; Reddy *et al.*, 2003; Kim *et al.*, 2004; Tvrzová *et al.*, 2005; Mayilraj *et al.*, 2006; Li *et al.*, 2006; Zhou *et al.*, 2008). The cellular fatty acid profiles of strain P30^T and the type strains of five related *Kocuria* species are shown in Table 2. Strain P30^T contained the following cellular fatty acids (>1.0 % of the total): anteiso-$C_{15:0}$ (53.7 %), iso-$C_{15:0}$ (14.1 %), iso-$C_{16:0}$ (10.3 %), anteiso-$C_{17:0}$ (7.2 %), $C_{16:0}$ (4.8 %), iso-$C_{14:0}$ (4.4 %) and $C_{14:0}$ (1.5 %). A large proportion of anteiso-$C_{15:0}$ was also reported for some *Kocuria* species (Stackebrandt *et al.*, 1995; Rainey *et al.*, 1997; Kovacs *et al.*, 1999; Reddy *et al.*, 2003; Kim *et al.*, 2004; Tvrzová *et al.*, 2005; Mayilraj *et al.*, 2006; Li *et al.*, 2006; Zhou *et al.*, 2008). The predominant menaquinone of strain P30^T was MK-7.

Taken together, these phenotypic, genotypic and phylogenetic characteristics indicate that strain P30^T represents a novel species of the genus *Kocuria*, for which the name *Kocuria atrinae* sp. nov. is proposed.

Description of *Kocuria atrinae* sp. nov.

Kocuria atrinae (a.tri′na.e. N.L. n. *Atrina* a scientific zoological genus; N.L. gen. n. *atrinae* of *Atrina*, isolated from *Atrina pectinata*).

Cells are Gram-positive, aerobic, non-motile and coccoid with a diameter of 1.0–1.5 μm. Colonies are pale yellow, circular, smooth and opaque after 2 days incubation on MA at 30 °C. Cells grow at 25–37 °C, at pH 7–9 and in the presence of 0–4 % NaCl. Optimal growth conditions on MA or in MB medium are 30–37 °C, pH 8–9 and 0–2 % NaCl. Catalase-positive and oxidase-negative. Reduces nitrate to nitrite, does not produce indole and is unable to ferment D-glucose. Positive for L-arginine dihydrolase, urease, esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and α-glucosidase. Negative for alkaline phosphatase, esterase (C4), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase. Hydrolyses aesculin but not gelatin or PNPG. According to growth on Biolog GP2 plates, assimilates dextrin, Tweens 40 and 80, D-fructose, D-gluconic acid, α-D-glucose, maltose, maltotriose, D-mannose, D-psicose, D-ribose, D-sorbitol, trehalose, turanose, xylitol, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, α-ketovaleric acid, D-lactic acid methyl ester, L-malic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, propionic acid, pyruvic acid, succinic acid, *N*-acetyl-L-glutamic acid, L-asparagine, L-glutamic acid, putrescine, glycerol, adenosine, 2′-deoxyadenosine, inosine, thymidine, uridine, thymidine 5′-monophosphate and DL-α-glycerol phosphate, but not α-cyclodextrin, β-cyclodextrin, glycogen, inulin, mannan, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosa-

Table 2. Cellular fatty acid profiles of strain P30^T and closely related species of the genus *Kocuria*

Strains: 1, P30^T (grown on MA); 2, *K. carniphila* CCM 132^T (MA); 3, *K. rhizophila* DSM 11926^T (MA); 4, *K. varians* DSM 20033^T (MA); 5, *K. marina* KMM 3905^T (MA); 6, *K. rosea* DSM 20447^T (trypticase soy broth). Data for 1–4 are from the present study; data for 5 and 6 are from Kim *et al.* (2004) and Stackebrandt *et al.* (1995), respectively. Values are percentages of the total fatty acids. tr, Trace (<1.0 %); −, not detected.

Fatty acid	1	2	3	4	5	6
$C_{12:0}$	tr	tr	−	−	−	−
iso-$C_{13:0}$	tr	−	tr	tr	−	−
anteiso-$C_{13:0}$	tr	tr	tr	tr	−	−
iso-$C_{14:0}$	4.4	2.4	1.9	tr	1.9	1.8
$C_{14:0}$	1.5	1.8	1.3	1.7	2.3	1.5
iso-$C_{15:0}$	14.1	3.1	12.4	4.3	2.5	7.6
anteiso-$C_{15:0}$	53.7	65.3	47.7	54.2	74.0	70.9
$C_{15:0}$	tr	tr	−	1.0	−	−
$C_{16:1}$	−	−	−	−	−	5.9
iso-$C_{16:0}$	10.3	4.3	9.7	6.9	7.0	1.6
$C_{16:0}$	4.8	7.0	2.3	11.6	2.9	1.6
anteiso-$C_{17:1}$	−	−	−	−	3.0	−
iso-$C_{17:0}$	tr	tr	1.6	−	−	1.3
anteiso-$C_{17:0}$	7.2	10.7	20.8	8.4	4.3	16.0
$C_{18:1}\omega 9c$	−	tr	−	−	−	tr
$C_{18:0}$	tr	1.8	−	−	−	tr

mine, amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, *myo*-inositol, α-D-lactose, lactulose, D-mannitol, melezitose, melibiose, methyl-α-D-galactoside, methyl-β-D-galactoside, 3-methylglucose, methyl-α-D-glucoside, methyl-β-D-glucoside, methyl-α-D-mannoside, palatinose, raffinose, L-rhamnose, sedoheptulosan, stachyose, sucrose, D-tagatose, D-xylose, γ-hydroxybutyric acid, lactamide, D-malic acid, L-malic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-alanylglycine, glycyl-L-glutamic acid, L-pyroglutamic acid, L-serine, 2,3-butanediol, adenosine 5′-monophosphate, uridine 5′-monophosphate, D-fructose 6-phosphate, α-D-glucose 1-phosphate or D-glucose 6-phosphate. The predominant menaquinone is MK-7. Major cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$ and iso-$C_{16:0}$. The DNA G+C content of the type strain is 70.2 mol%.

The type strain, P30^T (=KCTC 19594^T=JCM 15914^T), was isolated from a traditional fermented seafood in Korea.

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