Kocuria himachalensis sp. nov., an actinobacterium isolated from the Indian Himalayas

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A reddish orange bacterium, strain K07-05^T, was isolated from soil during a study of the bacterial diversity of a cold desert of the Indian Himalayas and was studied by using a polyphasic approach. The organism had morphological and chemotaxonomic properties consistent with its classification in the genus *Kocuria*. Phylogenetic analysis of the 16S rRNA gene sequence showed that strain K07-05^T was closely related to *Kocuria rosea* DSM 20447^T and *Kocuria polaris* MTCC 3702^T (98.1 and 97.8 % sequence similarity, respectively), whereas the sequence similarity values with respect to the other *Kocuria* species with validly published names were between 96.4 and 94.2 %. However, the genomic relatedness, as shown by DNA–DNA hybridization, of strain K07-05^T and *K. polaris* MTCC 3702^T is 49.5 % and that with *K. rosea* MTCC 2522^T is 24.0 %. The DNA G + C content of the strain is 75.3 mol%. The above data in combination with the phenotypic distinctiveness of K07-05^T clearly indicate that the strain represents a novel species, for which the name *Kocuria himachalensis* sp. nov. is proposed. The type strain is K07-05^T (=MTCC 7020^T = DSM 44905^T = JCM 13326^T).

The genus *Kocuria* was taxonomically dissected from the genus *Micrococcus* to accommodate phylogenetically distinct actinobacteria (Stackebrandt *et al.*, 1995). At the time of writing, the genus *Kocuria* consists of nine species with validly published names: *Kocuria rosea* (the type species), *K. varians*, *K. kristinae* (Stackebrandt *et al.*, 1995), *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) and *K. aegyptia* (Li *et al.*, 2006).

Strain K07-05^T was isolated from a soil sample collected from Lahaul–Spiti Valley in the Indian Himalayas by the dilution-plate technique on tryptic soy agar medium (TSA; HiMedia) and maintained on glycerol stocks at –70 °C. The reference strains *K. polaris* MTCC 3702^T and *K. rosea* MTCC 2522^T were taken from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India.

Colony and cell morphologies were studied according to standard methods (Murray *et al.*, 1994). The Gram reaction was determined using the HiMedia Gram staining kit

according to the manufacturer's instructions. Physiological tests, such as growth at different temperatures, pH values and NaCl concentrations, were performed by growing the strains on basal TSA medium. Catalase activity, citrate utilization (using Simmons' citrate agar) and urea hydrolysis were determined as described by Cowan & Steel (1965). The hydrolysis of casein, gelatin, Tween 80, tyrosine, starch and indole, the methyl red test, the Voges–Proskauer test and oxidase activity were assessed as described by Smibert & Krieg (1994). Nitrate reduction was tested as described by Lanyi (1987). The utilization of various carbon compounds as sole carbon sources was tested using Biolog GP2 MicroPlates in accordance with the manufacturer's instructions, except that TSA medium was used instead of Biolog Universal Growth agar medium. The inoculated plates were incubated for 24 h and the results were read with a MicroPlate Reader using Microlog 4.2 computer software to perform automated reading. The ability to use sole nitrogen sources was studied by using the methods described by Williams *et al.* (1983), with the modification that agarose (1.5 %) was used instead of agar. Acid production from various sugars was tested on minimal medium by using the method described by Smith *et al.* (1952). For cellular fatty acid analysis, the strains were grown on TSA medium at 30 °C for 36 h; fatty acid methyl ester analysis was performed by using the Sherlock Microbial Identification System (MIDI) as described previously (Pandey *et al.*, 2002).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain K07-05^T is AY987383.

The fatty acid compositions of strain K07-05^T and closely related type strains of the genus *Kocuria* are presented in a supplementary table available in IJSEM Online.

Table 1. Phenotypic properties that differentiate strain K07-05^T from closely related species of the genus *Kocuria*

Strains: 1, K07-05^T; 2, *K. rosea* MTCC 2522^T; 3, *K. polaris* MTCC 3702^T. All data are from the present study. None of the strains produces endospores and none is acid-fast; all are positive for catalase activity, growth at 37 °C^a, 5 % NaCl^d and pH 6.5–10 and acid production from glucose, maltose, mannitol, rhamnose and fructose. All give negative results in the methyl red and Voges–Proskauer tests and are negative for urease, H₂S production, hydrolysis of gelatin and casein, growth at 15 % NaCl and 42 °C and acid production from galactose^a, lactose^a, sorbitol, sucrose^a, adonitol, dulcitol and cellobiose. In the Biolog GP2 test, all strains are positive for the utilization of dextrin, D-fructose, α -D-glucose, maltotriose, D-mannitol, D-mannose, palatinose, D-psicose, D-sorbitol, sucrose, D-tagatose, turanose, D-xylose, acetic acid and adenosine and negative for α -cyclodextrin, glycogen, inulin, mannan, Tween 80, N-acetyl-D-glucosamine, N-acetyl- β -D-mannosamine, amygdalin, arabitol, arbutin, L-fucose, D-galactose, gentiobiose, D-gluconic acid, *myo*-inositol, maltose, D-melezitose, D-melibiose, methyl α -D-galactoside, methyl β -D-galactoside, methyl α -D-glucoside, methyl β -D-glucoside, methyl α -D-mannoside, D-raffinose, L-rhamnose, sedoheptulosan, stachyose, xylitol, *p*-hydroxyphenylacetic acid, α -ketoglutaric acid, α -ketovaleric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D-malic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, succinamic acid, succinic acid, N-acetyl-L-glutamic acid, L-alaninamide, D- and L-alanine, L-glutamic acid, glycyl L-glutamic acid, L-pyrroglutamic acid, putrescine, glycerol, 2'-deoxyadenosine, inosine, uridine, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-monophosphate, D-fructose 6-phosphate, α -D-glucose 1-phosphate, D-glucose 6-phosphate and DL- α -glycerol phosphate as sole carbon sources. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3
Oxidase	–	+ ^{b*}	+
Nitrate reduction	–	+	+
Hydrolysis of:			
Starch	–	–	+
Tween 80	+	–	+
Tyrosine	–	–	+
Citrate utilization	–	–	+ ^a
Growth at/on:			
5 °C	–	–	w
10 % NaCl	–	–	+
pH 5.2	–	–	+
Utilization of substrate as sole carbon source (using Biolog GP2 Microplate)			
β -Cyclodextrin	–	+	–
Tween 40	–	+	–
L-Arabinose	+	+	–
D-Cellobiose	–	+	–
D-Galacturonic acid	–	+	–
α -D-Lactose	–	–	+
Lactulose	–	–	+
Methyl-D-glucose	–	+	–
D-Ribose	+	+	–
Salicin	+	–	+
D-Trehalose	–	–	+
β -Hydroxybutyric acid	–	–	+
γ -Hydroxybutyric acid	–	+	+
L-Malic acid	–	+	+
Propionic acid	–	+	–
Pyruvic acid	+	–	–
L-Alanyl glycine	–	+	–
L-Asparagine	–	+	–
L-Serine	–	+	–
2,3-Butanediol	–	+	–
Thymidine	–	–	+

Table 1. cont.

Characteristic	1	2	3
Utilization of substrates as sole nitrogen source			
L-Ornithine	+	–	+
Histidine	–	w	–
L-Glutamic acid	+	–	+
Acid production from:			
Arabinose	–	+	–
Glycerol	+	+	–
Xylose	–	–	+
Mannose	+	–	–
myo-Inositol	–	–	+
Melibiose	–	+	+
Salicin	–	–	+
Trehalose	+	–	w
Inulin	–	–	+

*Result not in line with data reported in the following studies: *a*, Reddy *et al.* (2003); *b*, Stackebrandt *et al.* (1995).

Freeze-dried cells for other chemotaxonomic analyses were prepared following growth of the strains in tryptic soy broth for 4 days at 30 °C. Whole-cell sugars were determined by using the methods described by Stanek & Roberts (1974). The peptidoglycan structure was determined by using a hydrolysate of purified cell walls, according to Schleifer (1985). The amino acids and peptides were separated by two-dimensional ascending TLC as described by Schleifer & Kandler (1972), with the modification that TLC on cellulose sheets (Merck 5577) was used instead of paper chromatography. Polar lipids and menaquinones were extracted and analysed by using the methods described by Minnikin *et al.* (1984) and Kroppenstedt (1982). The absence of mycolic acids was demonstrated by TLC (Minnikin & Goodfellow, 1976).

Genomic DNA extraction, amplification and sequencing of 16S rRNA genes and phylogenetic analysis were performed as described previously (Mayilraj *et al.*, 2005). DNA–DNA hybridization was performed by the membrane filter method (Tourova & Antonov, 1987). The G + C content of the genomic DNA was determined spectrophotometrically (Lambda 35; Perkin Elmer) using the thermal denaturation method (Mandel & Marmur, 1968).

Strain K07-05^T is strictly aerobic and the cells are Gram-positive cocci. Colonies are circular, reddish orange in colour and 0.4–3.0 mm in diameter on TSA medium. Detailed phenotypic properties that differentiate strain K07-05^T from closely related species of the genus *Kocuria* are summarized in Table 1. Most of the chemotaxonomic properties, including the fatty acid composition (presented in the species description and in Supplementary Table S1, available in IJSEM Online) were typical of members of the genus *Kocuria*. The cell-wall diamino acid was lysine and the interpeptide bridge of the peptidoglycan consisted of three

alanine residues (variation A3 α ; Schleifer & Kandler, 1972). The almost-complete 16S rRNA gene sequence of strain K07-05^T (1459 bases) was determined. The 16S rRNA gene sequence-based phylogenetic analysis revealed that strain K07-05^T forms a separate branch within the lineage that includes *K. rosea* and *K. polaris* (Fig. 1). The 16S rRNA gene sequence similarities for strain K07-05^T with respect to *K. rosea* DSM 20447^T and *K. polaris* MTCC 3702^T were respectively 98.1 and 97.8 %. The similarities with respect to the type strains of the remaining species of the genus were significantly lower (94.2–96.4 %). The DNA–DNA hybridization values for strain K07-05^T with *K. polaris* MTCC 3702^T and with *K. rosea* MTCC 2522^T were respectively 49.5 and 24.0 %, well below the 70 % cut-off point recommended for the delineation of bacterial species (Wayne *et al.*, 1987).

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences (1459 bases), showing the phylogenetic relationship between strain K07-05^T and other species of the genus *Kocuria*. *Kytococcus sedentarius* DSM 20547^T was used as an outgroup. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are given at nodes. Bar, 0.5 % sequence variation.

The levels of DNA–DNA relatedness between strain K07-05^T and other *Kocuria* species were not determined, since it has been shown that organisms with more than 3 % 16S rRNA gene sequence dissimilarity belong to different genomic species (Stackebrandt & Goebel, 1994). On the basis of the polyphasic data presented above, strain K07-05^T should be placed in the genus *Kocuria* within a novel species, for which we propose the name *Kocuria himachalensis* sp. nov.

Description of *Kocuria himachalensis* sp. nov.

Kocuria himachalensis (hi.ma.cha.len'sis. N.L. fem. adj. *himachalensis* pertaining to the Indian State of Himachal Pradesh, where the type strain was isolated).

Cells are Gram-positive, coccoid (1.0–1.5 µm in diameter) and form pairs, tetrads and clusters. Strictly aerobic and non-motile. Colonies are circular, reddish orange in colour and 0.4–3.0 mm in diameter on TSA medium. No growth occurs at 5 or 42 °C. Growth occurs at 20–37 °C (optimal temperature, 30 °C) and pH 6.5–10 (optimal pH, 7.5). Catalase-positive; oxidase test with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride is negative. Does not grow in the presence of ≥8 % NaCl. Negative for starch hydrolysis, gelatin hydrolysis, citrate utilization, indole production and nitrate reduction and in the methyl red and Voges–Proskauer tests. Hydrogen sulphide is not produced. Substrates utilized as sole carbon sources are given in Table 1. Capable of utilizing L-alanine, L-arginine, L-aspartic acid, L-asparagine, L-ornithine, L-glutamic acid, L-lysine and L-phenylalanine; negative for the use of histidine as a sole nitrogen source. Acid is produced aerobically from glucose, glycerol, maltose, mannose, mannitol, rhamnose and trehalose, but not from arabinose, galactose, inulin, lactose, *myo*-inositol, melibiose, salicin, sorbitol, xylose or sucrose. Peptidoglycan type is Lys–Ala₃ (variation A3α). Whole-cell sugars are glucose, galactose and ribose. Contains major amounts of anteiso-branched C_{15:0} (66.16 %) and iso-branched C_{15:0} (15.13 %) fatty acids. The major menaquinone is MK-8(H₂). The polar lipids are phosphatidylglycerol, diphosphatidylglycerol and two unknown glycolipids. The DNA G + C content of the type strain is 75.3 mol%.

The type strain, K07-05^T (= MTCC 7020^T = DSM 44905^T = JCM 13326^T), was isolated from soil 0.45 m below an ice glacier, 4200 m above sea level, at Kibber village in Spiti Valley, Himachal Pradesh, India.

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