

Kocuria indica sp. nov., isolated from a sediment sample

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An actinobacterial strain was isolated from a sediment sample from Chora Island, in Goa province, India, and subjected to a taxonomic investigation. The isolate, designated NIO-1021^T, was a Gram-stain positive, aerobic, non-motile and coccoid. Strain NIO-1021^T was identified as a member of the genus *Kocuria* by a polyphasic approach. Strain NIO-1021^T could be differentiated from other members of the genus *Kocuria* on the basis of physiology and 16S rRNA gene sequence analysis. The 16S rRNA gene sequence similarity of strain NIO-1021^T to the most closely related species, *Kocuria marina* KCTC 9943^T, was 98.6% with 19 nt differences). Furthermore, DNA–DNA hybridization analysis revealed that the novel strain had lower relatedness with the type strains of other members of the genus *Kocuria*. The strain formed a monophyletic clade with *K. marina* with 100% bootstrap values. The major phospholipids were phosphatidylglycerol, diphosphatidylglycerol and two unidentified lipids. 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 NIO-1021^T was 60.5 mol%. Chemotaxonomic and phylogenetic properties of the strain were consistent with its classification as representing a member of the genus *Kocuria*. On the basis of phenotypic, chemotypic and molecular characteristics, strain NIO-1021^T is considered to represent a novel species of the genus *Kocuria*, for which the name *Kocuria indica* sp. nov. is proposed, with strain NIO-1021^T (=NCIM 5455^T=DSM 25126^T=CCTCC AA 209050^T) as the type strain.

The genus *Kocuria* was first proposed by Stackebrandt *et al.* (1995) on the basis of a detailed phylogenetic and chemotaxonomic analysis of the genus *Micrococcus* (Stackebrandt *et al.*, 1995). This led further to the description of the genera *Dermacoccus*, *Kocuria*, *Kytococcus* and *Nesterenkonia*. The genus *Kocuria* embraced three species formerly classified as members of the genus *Micrococcus*, i.e. *Kocuria rosea* (*Micrococcus roseus*; Flüge 1886), *Kocuria varians* (*Micrococcus varians*; Migula, 1900) and *Kocuria kristinae* (*Micrococcus kristinae*; Kloos *et al.*, 1974). Based upon 16S rRNA gene sequence similarities the genus *Kocuria* represents one of the deepest lineages in the family

Micrococcaceae. The genus comprises four clades. The first contains the type species *Kocuria rosea* as well as *Kocuria aegyptia* (Li *et al.*, 2006), *Kocuria flava* (Zhou *et al.*, 2008), *Kocuria himachalensis* (Mayilraj *et al.*, 2006), *Kocuria polaris* (Reddy *et al.*, 2003) and *Kocuria turfanensis* (Zhou *et al.*, 2008). The second clade is closely related and comprises *Kocuria halotolerans* (Tang *et al.*, 2009), *Kocuria koreensis* (Park *et al.*, 2010a) and *Kocuria kristinae* (Kloos *et al.*, 1974; Stackebrandt *et al.*, 1995). The third clade includes only *Kocuria palustris* (Kovács *et al.*, 1999). The last clade appears to be the deepest lineage in the family and comprises *Kocuria atrinae* (Park *et al.*, 2010b), *Kocuria carniphila* (Tvrzová *et al.*, 2005), *Kocuria gwangalliensis* (Seo *et al.*, 2009), *Kocuria marina* (Kim *et al.*, 2004), *Kocuria rhizophila* (Kovács *et al.*, 1999), *Kocuria salsicia* (Yun *et al.*, 2011) and *Kocuria varians* (Migula, 1900, Stackebrandt *et al.*, 1995). However, the biological

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of *Kocuria indica* sp. nov. NIO-1021^T is HQ858010.

Two supplementary figures and a supplementary table are available with the online version of this paper.

significance of these clades is not currently supported by chemotaxonomic or other evidence, so their importance is not yet certain. Species of the genus *Kocuria* are coccoid, Gram-stain positive, catalase-positive, non-halophilic (except *Kocuria halotolerans*), mesophilic, non-encapsulated, non-endospore-forming and non-motile. Strains are chemo-organotrophic and metabolism is strictly respiratory. The cell-wall peptidoglycan type is A3a (L-Lys-L-Ala₃₋₄); however, mycolic acids and teichoic acids are absent. Menaquinones are hydrogenated; the predominant menaquinones are MK-7(H₂), MK-8, or MK-9(H₂), either alone or in combination. Polar lipids include diphosphatidylglycerol and phosphatidylglycerol; phosphatidylinositol is present in one species. The major fatty acid is anteiso-C_{15:0} (Stackebrandt *et al.*, 1995). The DNA G + C content ranges from 66 to 75 mol% (Reddy *et al.*, 2003). The genus currently comprises 18 recognized species with *Kocuria rosea* as the type species.

During an investigation exploring actinobacterial diversity in the marine environment of Chorao Island of Goa province, India, a coccoid actinobacterial strain was isolated and was subjected to a polyphasic investigation to determine its taxonomic position.

Strain NIO-1021^T was isolated from a marine sediment sample taken from Chorao Island. After primary isolation and purification on marine agar 2216 (Difco) at 28 °C for 2 weeks, the purified strain was subcultured on the same medium and stored as slants at 4 °C and as 20% (v/v) glycerol suspensions at -70 °C. Biomass for chemical and molecular studies was obtained by cultivation in shake flasks (with shaking at about 140 r.p.m.) using tryptic soy broth (TSB) medium at 28 °C for 1 week. Gram staining was carried out by using the standard Gram reaction and cell motility was confirmed by the development of turbidity throughout a tube containing semisolid medium (Leifson, 1960). The morphological characteristics of strain NIO-1021^T, including spore-chain morphology, spore size and surface ornamentation, were assessed by light and scanning electron microscopy (XL30; ESEM-TMP; Philips) of 8-day-old cultures on International Streptomyces Project (ISP) 4 medium (Shirling & Gottlieb, 1966). Aerial spore-mass colour, substrate mycelium pigmentation and coloration of the diffusible pigments of strain NIO-1021^T were recorded on ISP media (Shirling & Gottlieb, 1966), Czapek's agar, potato-glucose agar and nutrient agar prepared as described by Dong & Cai (2001). Growth at different temperatures (4, 10, 20, 28, 37, 45, 50 and 55 °C) was tested on ISP-4 plates by incubating the cultures for 72 h. The pH range for growth [measured at pH 4, 5, 6, 7, 8, 9 and 10, using the buffer system described by Xu *et al.* (2005)] and NaCl tolerance (0, 1, 3, 5, 7, 10, 15 and 20% w/v) were tested at 28 °C for 72 h by culturing the strains on ISP-4 agar and in broth medium. Catalase, oxidase and gelatinase activities, starch hydrolysis, nitrate reduction and urease were assessed as described by Smibert & Krieg (1994). Other physiological and biochemical tests were performed as described by Gordon *et al.* (1974).

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene from strain NIO-1021^T were performed as described by Li *et al.* (2007). The resulting 16S rRNA gene sequence was compared with available 16S rRNA gene sequences from GenBank using the BLAST program to determine an approximate phylogenetic affiliation. Multiple alignments with sequences of the most closely related actinobacteria and calculations of levels of sequence similarity were carried out using CLUSTAL X (Thompson *et al.*, 1997). 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 using the neighbour-joining method of Saitou and Nei (1987) from *K*_{nuc} values (Kimura, 1980) using MEGA version 4.0 (Tamura *et al.*, 2007). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

Morphological and physiological characterizations were performed for strain NIO-1021^T. Cells were grown on ISP-4 (pH 7.0) at 28 °C for 4 days. Cell morphology and surface ornamentation was observed by light microscopy and scanning electron microscopy (Fig. S1, available in the online Supplementary Material). Oxidase reagent (bioMérieux) was used for testing oxidase activity and catalase activity was determined by bubble formation in a 3% (v/v) H₂O₂ solution. Semisolid 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 ISP-4 medium at 4, 10, 20, 28, 37, 45, 50 and 55 °C for 72 h. Growth at various NaCl concentrations (0, 1, 3, 5, 7, 10, 15 and 20%; w/v) was determined at 28 °C in broth medium that contained all of the constituents of ISP-4 (except NaCl), supplemented with appropriate concentrations of NaCl. The pH range for growth was determined from pH 4.0 to pH 10.0 (at intervals of 1.0 pH unit) using ISP-4. The strain was characterized biochemically using the API CH50 and API ZYM systems (bioMérieux). ISP-4 plates were used to examine hydrolysis of starch and Tweens 20, 40, 60 and 80 [final concentration of 1% (v/v)].

Colonies of strain NIO-1021^T were aerobic, Gram-stain positive, non-motile cocci 1–1.5 µm in diameter. Strain NIO-1021^T grew well on ISP-2, ISP-3, ISP-4, marine agar media and nutrient agar; moderate growth was recorded on ISP-5 and ISP-7. No aerial mycelium was formed on ISP-2, ISP-4, ISP-5 or nutrient agar. After incubation on ISP-4 (pH 7.0) at 28 °C for 48 h, strain NIO-1021^T formed opaque, lemon-yellow, circular colonies with entire margins. Strain NIO-1021^T was catalase-positive, but oxidase-negative. It grew at temperatures between 15 and 37 °C (optimum, 30–37 °C), at pH 6–9 (optimum, pH 7–8) and in the presence of 0–15% (w/v) NaCl. NaCl was not required for its growth. Strain NIO-1021^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 NIO-1021^T and related species of the genus *Kocuria* is presented in Table 1 and in the species description. It is evident from Table 1 that there were phenotypic differences between strain NIO-1021^T and *Kocuria marina* KCTC 9943^T and *Kocuria salsicia* KACC 21128^T.

The nearly complete 16S rRNA gene sequence of strain NIO-1021^T (1435 bp) was determined and compared with the corresponding sequences of other bacterial strains in the GenBank database. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain NIO-1021^T should be assigned to the genus *Kocuria*. The phylogenetic tree, based on 16S rRNA gene sequence data from

strain NIO-1021^T and corresponding sequences from the type strains of species of the genus *Kocuria* as well as *Micrococcus luteus* DSM 20030^T (GenBank accession no. AJ536198), was reconstructed using the neighbour-joining algorithm (Fig. 1). The comparative analysis of 16S rRNA gene sequences and phylogenetic relationships showed that strain NIO-1021^T lies in a subclade in the tree with *Kocuria marina* KCTC 9943^T (AY211385) (supported by a bootstrap value of 100%, Fig. 1), with which it shares a 16S rRNA gene sequence similarity of 98.6%. The affiliation of strain NIO-1021^T and its closest neighbour, *Kocuria marina* KCTC 9943^T, was also supported by the neighbour joining, maximum-parsimony and maximum-likelihood algorithms with bootstrap values of 100%, 99.8% and 99.5% (PHYMLIP version 3.6), respectively (Felsenstein, 2002).

Table 1. Differential phenotypic characteristics of strain NIO-1021^T and related species of the genus *Kocuria*

Strains: 1, NIO-1021^T; 2, *Kocuria marina* KCTC 9943^T; 3, *Kocuria salsicia* KACC 21128^T. In this study, API ZYM kit results were obtained for the strains. All strains were positive for glucose assimilation, catalase, 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, cystine arylamidase, α -galactosidase, β -galactosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -glucosidase, β -glucuronidase, α -mannosidase, α -fucosidase and the assimilation of aesculin and capric acid. All strains were capable of growth at 37 °C. +, Positive; -, negative; w, weakly positive.

Characteristic	1	2	3
Nitrate reduction	+	+	+
H ₂ S production	-	-	+
Hydrolysis of:			
Starch	+	-	+
Urea	-	+	+
Growth at/ in:			
5 °C	-	+	-
5% NaCl	+	+	-
10% NaCl	+	+	-
15% NaCl	+	+	-
Assimilation of:			
Maltose	-	+	+
D-Mannitol	+	-	-
D-Mannose	+	-	+
Phenylacetic acid	-	-	+
Potassium gluconate	-	+	+
Trisodium citrate	-	w	+
Enzyme activities			
Acid phosphatase	+	-	-
Alkaline phosphatase	-	-	+
Esterase (C4)	+	w	-
Leucine arylamidase	+	w	-
Urease	-	-	+
DNA G + C content (mol%)	60.5	60.0	60.6

Genomic DNA of strain NIO-1021^T was extracted and purified with the Qiagen Genomic-tip system 100/G and then enzymically degraded into nucleosides, as described previously (Tamaoka & Komagata, 1984). The quantitative microplate DNA-DNA hybridizations were carried out under optimal conditions as described by Ezaki *et al.* (1989). One of the two DNAs for hybridization was labelled while the other was immobilized, and the reciprocal experiments were performed. The concentration of the two DNAs was strictly controlled. Six replications for hybridization were performed for each sample and the highest and lowest values in each sample were excluded. The relatedness values are expressed as the means of the remaining four values and the results of DNA-DNA hybridizations were taken from the means of the two relatedness values.

For the determination of the DNA G + C content, genomic DNA of strain NIO-1021^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). The genomic DNA G + C content of strain NIO-1021^T was 60.5 mol%, which was similar to those of related reference strains (60–72 mol%; Kim *et al.*, 2004; Stackebrandt *et al.*, 1995).

The determined DNA-DNA relatedness values between strain NIO-1021^T and *Kocuria marina* KCTC 9943^T and *Kocuria salsicia* KACC 21128^T were 28.0 and 39%, respectively [standard deviations were 2.0 and 2.0%, respectively (Table S1)], which are well below the 70% cut-off point for recognition of genomic species (Wayne *et al.*, 1987), thus indicating that strain NIO-1021^T should be considered as representing a distinct genomic species of the genus *Kocuria*.

Whole-cell sugars were analysed according to the procedures developed by Hasegawa *et al.* (1983). Polar lipids were extracted, examined using two-dimensional TLC and identified 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, v/v) and the second was developed in chloroform/

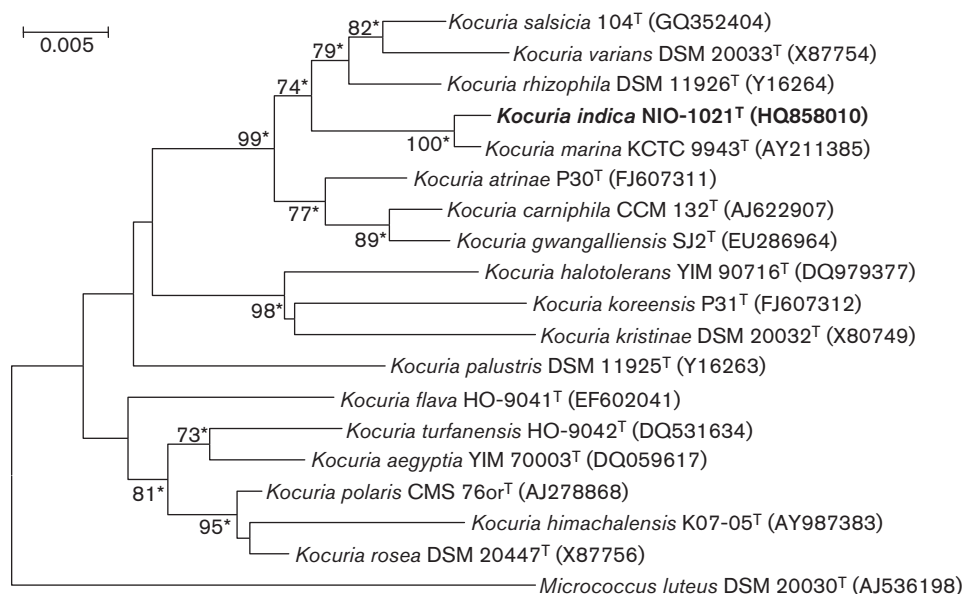


Fig. 1. Neighbour-joining tree based on the 16S rRNA gene sequences of representative species of the genus *Kocuria* and strain NIO-1021^T. Kimura's two-parameter model was used to calculate phylogenetic distances between species. The topology of the entire tree was conserved in all trees reconstructed using two different algorithms. Asterisks indicate the branches found in phylogenetic consensus trees generated with the maximum-parsimony and maximum-likelihood methods. Numbers at nodes are levels of bootstrap support from 1000-resampled datasets. Bar, 0.005 nucleotide substitutions per position.

methanol/acetic acid/water (80:12:15:4, v/v). 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 the protocol of Minnikin *et al.* (1984) and were separated by using HPLC (Kroppenstedt, 1982). For analysis of fatty acids, strain NIO-1021^T was cultured on trypticase soy agar (TSA; Difco) at 28 °C for 72 h. Preparation and analysis of fatty acid methyl esters were performed as described by Sasser (1990) with the Microbial Identification System (MIDI) using the Microbial Identification software package (Sherlock Version 6.1; MIDI database: TSBA6).

Strain NIO-1021^T contained lysine, glutamic acid and alanine, a profile that is characteristic of members of the genus *Kocuria*. Whole-cell-wall sugars were galactose, glucose and ribose. Phospholipids contained phosphatidylglycerol, diphosphatidylglycerol and two unidentified phospholipids (Fig. S2). The predominant menaquinone was MK-7(H₂). Strain NIO-1021^T exhibited a qualitatively similar cellular fatty acid profile to *Kocuria marina* KCTC 9943^T (Kim *et al.*, 2004) and *Kocuria salsicia* (Yun *et al.*, 2011) with the major fatty acids being anteiso-C_{15:0} (61.5%), iso-C_{16:0} (11.7%) and anteiso-C_{17:0} (9.4%). Minor amounts of straight-chain and unsaturated fatty acids were also detected (Table 2). Therefore, based on these characteristics determined from phenotypic,

chemotaxonomic and phylogenetic analyses described above, it is proposed that strain NIO-1021^T is representative of a novel species of the genus *Kocuria*, for which the name *Kocuria indica* sp. nov. is proposed.

Table 2. Fatty acid composition (percentages) of NIO-1021^T and the most closely related species of the genus *Kocuria*

Strains: 1, NIO-1021^T; 2, *Kocuria marina* KCTC 9943^T; 3, *Kocuria salsicia* KACC 21128^T. All data are from the present study. –, Not detected.

Fatty acid	1	2	3
iso-C _{14:0}	2.6	2.7	3.6
C _{14:0}	2.9	2.7	1.4
iso-C _{15:0}	3.1	2.6	21.2
anteiso-C _{15:0}	61.5	56.2	50.2
C _{15:1}	–	0.1	–
iso-C _{16:0}	11.7	14.6	9.6
C _{16:0}	6.3	5.3	3.0
iso-C _{17:0}	0.4	0.4	1.5
anteiso-C _{17:0}	9.4	10.9	7.1
iso-C _{18:0}	0.1	0.3	0.1
iso-C _{18:1}	0.5	0.4	–
C _{18:0}	0.5	0.3	0.1
anteiso-C _{19:0}	0.5	0.7	–

Description of *Kocuria indica* sp. nov.

Kocuria indica (in 'di.ca. L. fem. adj. *indica* pertaining to India, Indian).

Cells are Gram-stain positive, aerobic, non-motile and coccoid on ISP-4. Catalase-positive, oxidase-negative. Colony colour is lemon-yellow on ISP-2, ISP-3, ISP-4 and ISP-5, cream on ISP-6 and deep yellow on Bennett's agar. Aerial mycelia are absent. Diffusile pigment is not observed. The temperature range for growth is 10–45 °C and optimum growth is observed at 28–30 °C. Cells can grow in the presence of 0–15 % NaCl. The pH range for growth is 4.0–10 and the optimum is pH 7.0–8.0. Cellobiose, D-galactose, D-glucose, glycogen, D-fructose, inositol, cupric acid, D-mannitol, D-mannose, melibiose, melezitose, D-ribose, L-rhamnose, salicin, phenylacetic acid or trisodium citrate, trehalose and turanose can be used as sole carbon sources for energy and growth, but not glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, D-adonitol, methyl β -D-xylopyranoside, L-sorbose, dulcitol, D-sorbitol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, maltose, lactose, sucrose, inulin, raffinose, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, 2-ketogluconate or 5-ketogluconate. Positive for hydrolysis of starch. Negative for urea hydrolysis and H₂S production. Positive for nitrate reduction, acid phosphatase, esterase (C4) and leucine arylamidase; negative for indole production, arginine dehydrogenase, alkaline phosphatase lipase (C14), valine arylamidase, trypsin, α -chymotrypsin, cystine arylamidase, α -galactosidase, β -galactosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -glucosidase, β -glucuronidase, α -mannosidase, α -fucosidase and urease. The peptidoglycan contained lysine, glutamic acid and alanine and cell-wall sugars were galactose, glucose and ribose. The predominant menaquinone is MK-7(H₂). Phospholipids consisted of phosphatidylglycerol, diphosphatidylglycerol and two unidentified lipids. The cellular fatty acid profile includes the main constituents anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0} with lesser amounts of C_{16:0}, iso-C_{15:0}, C_{14:0} and iso-C_{14:0}.

The type strain is NIO-1021^T (NCIM 5455^T=DSM 25126^T=CCTCC AA 209050^T), isolated from a sediment sample from an intertidal region of mangroves at Chorao Island in Goa province, India. The DNA G+C content of the type strain is 60.5 mol%.

Acknowledgements

S.G.D thanks the Council for Scientific and Industrial Research (CSIR), New Delhi, for financial support, from grants under the 12th five-year plan.

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