Kocuria pelophila sp. nov., an actinobacterium isolated from the rhizosphere of a mangrove

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A novel spherical actinobacterium, designated RS-2-3T, was isolated from the rhizosphere of a mangrove growing on Rambut Island, Indonesia, and its taxonomic position was investigated using a polyphasic approach. Phylogenetic analysis based on 16S rRNA gene sequence comparison revealed that strain RS-2-3T was related to the members of the genus Kocuria. The highest 16S rRNA gene sequence similarity value was observed with Kocuria marina KMM 3906T (97.0%). The peptidoglycan type of strain RS-2-3T was found to be A3α with an interpeptide bridge comprising L-Ala4-D. The predominant menaquinone was MK-7(H2) and the major fatty acids were anteiso-C15:0 and iso-C15:0. The DNA G+C content was 71.8 mol%. These characteristics were consistent with those of members of the genus Kocuria. Meanwhile, physiological and biochemical characteristics revealed that strain RS-2-3T differed from the species of the genus Kocuria with validly published names. Therefore, strain RS-2-3T represents a novel species of the genus Kocuria, for which the name Kocuria pelophila sp. nov. is proposed. The type strain is RS-2-3T (=NBRC 110990=InaCC A704T).

The genus Kocuria was proposed by Stackebrandt et al. (1995) as a member of the family Micrococcaceae. At the time of writing, the genus Kocuria consists of 19 species with validly published names. In addition, two more species, namely ‘Kocuria assamensis’ (Kaur et al., 2011) and ‘Kocuria sediminis’ (Bala et al., 2012), have been proposed but these names have not been validly published at the time of writing. The peptidoglycan of members of this genus is of type A3α type (Schleifer & Kandler, 1972) with lysine as the diagnostic diamino acid. The predominant menaquinones are MK-7(H2), MK-8(H2) and/or MK-9(H2), the major fatty acid is anteiso-C15:0 and the polar lipids include diphostatidylglycerol and phosphatidyglycerol (Stackebrandt & Schumann, 2012). Type strains of species of the genus have been isolated from various samples, e.g. soil, fermented seafood, meat, air, seawater, marine sediment and mammalian skin. During the course of a study of the bacterial diversity in seashore environments, a novel actinobacterium, which was designated strain RS-2-3T, was isolated from the rhizosphere of a mangrove. Comparative 16S rRNA gene sequence analysis revealed that the isolate was phylogenetically related to the members of the genus Kocuria. In the present study, we clarified the taxonomic position of the isolate by using a polyphasic approach.

RS-2-3T was isolated from a mud sample that had been collected from the rhizosphere of a mangrove growing on Rambut Island (5°58′634″ S 106°41′596″ E), DKI Jakarta, Indonesia. The procedure employed for bacterial isolation was as described by Hamada et al. (2015). NBRC medium 802 [1.0% (w/v) peptone, 0.2% (w/v) yeast extract, 0.1% (w/v) MgSO4·7H2O and 1.5% (w/v) agar, when required; pH 7.0] was used as the basal medium for this study. Kocuria marina NBRC 110801T and Kocuria carni- phila NBRC 110786T were used as reference strains in this study.

Colonial appearance was examined after cultivation at 28°C for 2 days on an agar plate of NBRC medium 802. Morphological features were observed under light (BX-51; Olympus) and scanning electron (JSM-6060; JEOL) microscopes. The temperature range and optimum temperature for growth were determined by cultivating cultures at 5, 10, 15, 20, 25, 28, 37, 45 and 60°C on agar plates of NBRC medium 802 for 1–4 days (15–60°C) or 14 days (5 and 10°C). The pH and NaCl ranges for growth were

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RS-2-3T is LC042214.

One supplementary table and one supplementary figure are available with the online Supplementary Material.
determined by measuring the turbidity (OD_{610}) of 5 ml of the culture in test tubes after 1–4 days incubation at 28°C. The pH range and optimum pH for initial growth were established using liquid NBRC medium 802 adjusted to pH 4–11 (at 1.0 pH-unit intervals) with either 4 M HCl or 5 M KOH. Tolerance to NaCl was tested using liquid NBRC medium 802 adjusted to NaCl concentrations of 1, 5, 10, 15 and 20% (w/v). Cell motility, oxidase and catalase activities, anaerobic growth and Gram staining were determined using the methods described by Hamada et al. (2012). Other physiological and biochemical tests were performed using API ZYM and API Coryne systems (bioMérieux) according to the manufacturer’s instructions.

RS-2-3^T was observed to form orange–yellow, circular, convex and smooth colonies that were approximately 0.5–1.0 mm in diameter after 2 days cultivation. Cells of the strain were observed to be Gram-stain-positive, non-motile, non-endospore-forming and spherical (approximately 1.0 µm in diameter), occurring in pairs and tetrads (Fig. 1). The strain was catalase-positive and oxidase-negative. Growth occurred at 15–37°C and no growth was observed at 5, 10, 45 or 60°C. The pH range for growth was 6.0–9.0. Optimal growth was noted at 28°C and pH 7.0. The strain exhibited good growth with NaCl concentrations of 0–7% (w/v), moderate growth with 10% (w/v) NaCl and weak growth with 15% (w/v) NaCl; no growth was observed with 20% (w/v) NaCl. Growth under anaerobic conditions was not observed. The results of other physiological and biochemical analyses are summarized in the species description.

The PCR-based amplification and sequencing of the 16S rRNA gene of strain RS-2-3^T were performed as described previously (Hamada et al., 2015). Phylogenetic neighbours were identified and pairwise 16S rRNA gene sequence similarities were calculated using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). The CLUSTAL_X program (Thompson et al., 1997) was used to align the almost-complete 16S rRNA gene sequence of strain RS-2-3^T (1490 nt) with corresponding sequences of members of the genus Kocuria and some related taxa. Evolutionary distances were calculated using Kimura’s two-parameter model (Kimura, 1980). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms using the MEGA 6.0 program (Tamura et al., 2013). The resultant tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 replicates.

Phylogenetic analysis based on the 16S rRNA gene sequences showed that strain RS-2-3^T represented a member of the genus Kocuria. However, strain RS-2-3^T did not form a reliable cluster with any member of the genus Kocuria with a validly published name (Fig. 2). The highest 16S rRNA gene sequence similarity value was observed with K. marina KMM 3905^T (97.0%), followed by K. cariphila CCM 132^T (96.9%), Kocuria gwangalliensis SJ2^T (96.7%), Kocuria rhizophila DSM 11926^T (96.6%), Kocuria atrinae P30^T (96.6%), Kocuria saliscia 104^T (96.4%) and Kocuria varians DSM 20033^T (96.3%). RS-2-3^T showed less than 96.0% 16S rRNA gene sequence similarity with other members of the genus Kocuria. Biomass for chemotaxonomic studies, except for fatty acid analysis, was obtained by cultivating the strain in shake flasks at 28°C and 100 r.p.m. for 48 h. Amino acids and their isomers in cell-wall hydrolysates, isoprenoid quinones and the DNA G+C content were determined according to the methods described by Hamada et al. (2012). Polar lipids were extracted from 100 mg freeze-dried cells using the method described by Minnikin et al. (1975) and analysed by TLC using chloroform/methanol/water (65:25:4, by volume) in the first direction and chloroform/acetate acid/methanol/water (80:18:12:5, by volume) in the second. For fatty acid methyl ester analysis, RS-2-3^T and the reference strains were cultured on tryptic soy agar (Difco) for 24 h at 28°C. Cellular fatty acid methyl esters were analysed.

Fig. 1. Scanning electron micrographs of strain RS-2-3^T grown on NBRC medium 802 for 1 day at 28°C. Bars, 2 µm (a) and 1 µm (b).
The peptidoglycan sample of RS-2-3$^\top$ was found to contain alanine (Ala), glutamic acid (Glu) and lysine (Lys) in a molar ratio of 6.7 : 1.0 : 1.1. Enantiomeric analysis of the peptidoglycan amino acids revealed the presence of D-Ala, L-Ala, D-Glu and L-Lys. These data indicated that the peptidoglycan type of strain RS-2-3$^\top$ is A3α, with L-Lys as the diagnostic diamino acid and an interpeptide bridge comprising L-Ala-Lys. However, the number of Ala residues (four or five) in the interpeptide bridge may be disputable. The predominant menaquinone was identified as MK-7(H$_2$), with MK-8(H$_2$) present as a trace component (98:2). The major cellular fatty acids of RS-2-3$^\top$ were anteiso-C$_{15:0}$ (71.4 %) and iso-C$_{15:0}$ (14.2 %); anteiso-C$_{17:0}$ (4.0 %), C$_{18:0}$ω6c (3.3 %), iso-C$_{16:0}$ (2.2 %), C$_{16:0}$ (2.0 %), iso-C$_{17:0}$ (1.2 %), iso-C$_{14:0}$ (1.1 %) and anteiso-C$_{12:0}$ (0.8 %) were found as minor or trace components (Table S1, available in the online Supplementary Material). The detected polar lipids were phosphatidylglycerol, diphosphatidylglycerol and one unidentified glycolipid (Fig. S1). The DNA G+C content of strain RS-2-3$^\top$ was 71.8 mol%.

The result of the phylogenetic analysis based on the 16S rRNA gene sequences indicated that strain RS-2-3$^\top$ represented a member of the genus *Kocuria*, and its chemotaxonomic features were also consistent with those

![Fig. 2. Phylogenetic tree derived from 16S rRNA gene sequences of strain RS-2-3$^\top$ and members of the genus *Kocuria* and some related taxa, reconstructed with the neighbour-joining method. The 16S rRNA gene sequence of *Brevibacterium linens* DSM 20425$^\top$ (GenBank accession no. X77451) was used as the outgroup. Bootstrap values (>50 %) based on 1000 replicates are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.01 $K_{nuc}$ substitutions per nucleotide position.](image-url)
Table 1. Differential phenotypic characteristics of strain RS-2-3<sup>T</sup> and related species of the genus Kocuria

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum NaCl concentration (%) for growth</td>
<td>15</td>
<td>15*</td>
<td>10†</td>
</tr>
<tr>
<td>API ZYM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-β-glucosaminidase</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Esterase lipase (C8)</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>API Coryne</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aesculin hydrolysis</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Pyrrolidonyl arylamidase</td>
<td>w</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Glycogen</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>–</td>
<td>w</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Major cellular fatty acids (&gt;10%)</td>
<td>ai-C&lt;sub&gt;15:0&lt;/sub&gt;, i-C&lt;sub&gt;15:0&lt;/sub&gt;</td>
<td>ai-C&lt;sub&gt;15:0&lt;/sub&gt;, ai-C&lt;sub&gt;17:0&lt;/sub&gt;</td>
<td>ai-C&lt;sub&gt;15:0&lt;/sub&gt;</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>71.8</td>
<td>60*</td>
<td>71†</td>
</tr>
</tbody>
</table>

*Data from Kim et al. (2004).
†Data from Tvrzová et al. (2005).

of members of the genus. RS-2-3<sup>T</sup> differed from the type strains, of the most closely related species namely, K. marina and K. carniphila, in the following characteristics: the presence of esterase lipase (C8) and pyrrolidonyl arylamidase; the absence of N-acetyl-β-glucosaminidase, β-galactosidase and β-glucuronidase; hydrolysis of gelatin; absence of hydrolysis of aesculin; acid production from glycogen and xylose; and absence of acid production from lactose, maltose and sucrose (Table 1). In addition, although the profiles of cellular fatty acids of RS-2-3<sup>T</sup> were qualitatively similar to those of the reference strains, quantitative differences (especially for iso-C<sub>15:0</sub> and anteiso-C<sub>17:0</sub>) were found. On the basis of the phylogenetic and phenotypic data mentioned above, it is proposed that strain RS-2-3<sup>T</sup> represents a novel species of the genus Kocuria, with the name Kocuria pelophila sp. nov.

Description of Kocuria pelophila sp. nov.

Kocuria pelophila (pe.lo’phi.la. Gr. n. pelos mud; Gr. adj. philos -ô- on loving; N.L. fem. adj. pelophila mud-loving).

Cells are Gram-stain-positive, aerobic, non-motile, non-endospore-forming and spherical (approximately 1.0 µm in diameter), occurring in pairs and tetrads. Colonies are orange-yellow, circular, convex and smooth after 2 days cultivation on an agar plate of NBRC medium 802. Catalase-positive and oxidase-negative. The temperature range for growth is 15–37 °C (optimum 28 °C). The pH range for growth is 6.0–9.0 (optimum pH 7.0). Growth occurs at NaCl concentrations of 0–15 % (w/v). Using the API ZYM and API Coryne systems, activity is detected for acid phosphatase, esterase (C4), esterase lipase (C8), α-glucosidase, leucine arylamidase, phosphohydrolase (weak), pyrazinamidase, pyrrolidonyl arylamidase (weak) and trypsin (weak); no activity is detected for N-acetyl-β-glucosaminidase, alkaline phosphatase, chymotrypsin, cysteine arylamidase, fucosidase, α- and β-galactosidases, β-glucosidase, β-glucuronidase, lipase (C14), mannosidase and valine arylamidase. Using the API Coryne systems, acid is produced from glucose, glycogen and D-xylose; no acid production occurs from lactose, maltose, D-mannitol, D-ribose and sucrose. Gelatin and urea are hydrolysed, but aesculin is not. Nitrate is reduced. The peptidoglycan is of the A3α type with an interpeptide bridge comprising L-Ala<sub>4</sub>. The predominant menaquinone is MK-7 (H<sub>2</sub>). The major cellular fatty acids is anteiso-C<sub>15:0</sub> followed by iso-C<sub>15:0</sub>. The polar lipids are phosphatidyglycerol, diphosphatidylglycerol and one unidentified glycolipid.

The type strain, RS-2-3<sup>T</sup> (=NBRC 110990<sup>T</sup> =InaCC A704<sup>T</sup>), was isolated from a mud sample that had been collected from the rhizosphere of a mangrove growing on Rambut Island, DKI Jakarta, Indonesia. The DNA G+C content of the type strain is 71.8 mol%.

Acknowledgements

This work was partly supported by Science and Technology Research Partnership for Sustainable Development (SATREPS), which is a...
research program in collaborated with the Japan Science and Technology Agency (JST) and the Japan International Cooperation Agency (JICA).

References


