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ORIGINAL PAPER

Description of a novel actinobacterium *Kocuria assamensis* sp. nov., isolated from a water sample collected from the river Brahmaputra, Assam, India

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Abstract A Gram-positive, pale yellow pigmented actinobacterium, strain S9-65^T was isolated from a water sample collected from the river Brahmaputra, Assam, India and subjected to a polyphasic taxonomic study. The physiological and biochemical properties, major fatty acids (anteiso-C15:0 and anteiso-C17:0), estimated DNA G+C content (69.2 mol %) and 16S rRNA gene sequence analysis showed that strain S9-65^T belonged to the genus *Kocuria*. Strain S9-65^T exhibited highest 16S rRNA gene sequence similarity with *Kocuria palustris* (99.1%); however, the DNA–DNA relatedness value between strain S9-65^T and *K. palustris* was 20.6%. On the basis of differential phenotypic characteristics and genotypic distinctiveness, strain S9-65^T should be classified as representative of a novel species *Kocuria*, for which the name *Kocuria assamensis* is proposed. The type strain is S9-65^T (=MTCC 10622^T = DSM 23999^T).

Keywords DNA–DNA hybridization · FAME · 16S rRNA gene sequence

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The GenBank accession number for the 16S rDNA sequence of *Kocuria assamensis* strain S9-65^T is HQ018931.

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Introduction

The genus *Kocuria* was proposed by Stackebrandt et al. (1995) to accommodate phylogenetically distinct Actinobacteria formerly classified in the genus *Micrococcus*. The type species of the genus is *Kocuria rosea*. Members of the genus have been isolated from different sources such as air, fermented sea food, mammalian skin, soil, the rhizoplane, freshwater or seawater, marine sediment and desert soil (Kloos et al. 1974; Stackebrandt et al. 1995; Kovacs et al. 1999; Reddy et al. 2003; Kim et al. 2004; Tvrzová et al. 2005; Li et al. 2006; Mayilraj et al. 2006a, b; Zhou et al. 2008; Tang et al. 2009; Seo et al. 2009). At present the genus *Kocuria* consists of 17 species with validly published names (http://www.bacterio.cict.fr/k/kocuria.html). Here, we describe the taxonomic status of an actinobacterium,

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strain S9-65^T, isolated from a surface water sample collected from the river Brahmaputra, Assam, India (93° 08′–98° 36′E and 26° 30′–20° 45′N) by using a polyphasic approach.

Materials and methods

Strains, cultivation and phenotypic characterization

The strain S9-65^T was isolated by the dilution plate technique on tryptic soy agar medium (TSA; HiMedia, India) and incubated for 4 days at 30°C. To study its phenotypic characteristics, the isolate was routinely cultivated on TSA medium at 30°C and maintained as glycerol stocks at −70°C. The reference type strain *Kocuria palustris* strain TAGA 27^T MTCC 10490^T (=DSM 11925^T) was obtained from Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. Colony morphology, cell morphology, motility and Gram's reaction of the strain were determined by using standard methods (Barrow and Feltham 1993; Murray et al. 1994; Smibert and Krieg 1994). Phenotypic characterization was performed using TSA as basal medium and strains were incubated at their optimum growth temperatures. Physiological tests such as growth at different temperatures (between 10 and 45°C), pH (using biological buffers; Na_2HPO_4/NaH_2PO_4, $Na_2CO_3/NaHCO_3$ for pH below 8 and Na_2HPO_4/NaOH for pH above 8), NaCl concentrations and acid production from various carbohydrates and other biochemical tests were performed as described (Smibert and Krieg 1994). The API ZYM and API 20NE micro test strips were used as per the instructions of the manufacturer (bioMérieux). Sensitivity of the strain to antibiotics was tested by using antibiotic susceptibility discs (HiMedia, India) after incubation of 48 h.

Chemotaxonomic characterization

Freeze-dried cells for chemotaxonomic analysis (except for the fatty acids study) were prepared by harvesting the bacterial cells in the late exponential phase following their growth in Tryptic Soy Broth (TSB; HiMedia, India) at 30°C for 2 days. Isoprenoid quinones were extracted and purified as described by Saha et al. (2005). The purified quinones were separated by reversed phase HPLC (SCL-10AVP, Shimadzu) using the solvent system of acetonitrile and isopropanol in a ratio of 65:35 with a flow rate of 1 ml/min and monitored at a wavelength of 269 nm. For cellular fatty acid analysis, the strains were grown on tryptic soy agar medium at 30°C for 36 h and the fatty acid methyl ester analysis was performed by using Sherlock Microbial Identification System (MIDI, USA) as described previously (Sasser 1990; Pandey et al. 2002). Extraction of polar lipids was done based on the modified protocol of Bligh and Dyer (1959). Two-dimensional TLC was run for identification of polar lipids according to procedures described by Komagata and Suzuki (1987). Lipid spots were detected using the following spray reagents: molybdatophosphoric acid (5% w/v) in absolute ethanol, molybdenum blue spray reagent (1.3% Sigma), ninhydrin (0.2% w/v) in acetone and anisaldehyde reagent (Sigma) for detection of total lipids, phospholipids, aminolipids and glycolipids respectively. 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 and Kandler (1972), with the modification that TLC on cellulose sheets (Merck 5577) was used instead of paper chromatography. The G+C content of genomic DNA was determined spectrophotometrically (Lambda 35, Perkin Elmer, Waltham, MA, USA) using thermal denaturation method (Mandel and Marmur 1968).

Determination of 16S rRNA gene sequence, phylogenetic analysis and genomic relatedness

For 16S rRNA gene sequencing the genomic DNA extraction and amplification was performed as described previously (Mayilraj et al. 2006a, b). Identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (http://www.eztaxon.org/; Chun et al. 2007). The 16S rRNA gene sequence of S9-65^T and representative closely related species were retrieved from the Eztaxon server and aligned using the MEGA version 4.0 (Tamura et al. 2007). Phylogenetic trees were constructed using the neighbour-joining as well as maximum parsimony algorithms. Bootstrap analysis

was performed to assess the confidence limits of the branching. The G+C content of genomic DNA was determined spectrophotometrically (Lambda 35, Perkin Elmer, Waltham, MA, USA) using thermal denaturation method (Mandel and Marmur 1968). DNA–DNA hybridization was performed each time with freshly isolated genomic DNA and was repeated three times by the membrane filter method (Tourova and Antonov 1987).

Results and discussion

Phenotypic characteristics

Growth of the strain on TSA produced a yellow pigment after incubation on TSA for 2 days. The detailed differential phenotypic properties are shown in Table 1 and also mentioned in species description. Phenotypic data presented in the table indicated that strain S9-65^T differed from the closely related species at least by 29 characters which includes acid production from carbohydrates like adonitol, raffinose, rhamnose, cellobiose, arbinose and xylose, casein hydrolysis, nitrate reduction, hydrogen sulphide production were negative for strain S9-65^T and positive for the closely related strain *K. palustris* TAGA 27^T. There were major differences in oxidation of different carbon sources using biolog. Strain S9-65^T was sensitive to antibiotics (μg disc^{-1}) such as nitrofurantion (300), norfloxacin (10), polymyxin B (300), cephalothin (30) and oxacillin (5) in comparison to the closely related strain *K. palustris* which showed resistance to all of the above mentioned antibiotics.

Both strain S9-65^T and *K. palustris* TAGA 27^T were positive for growth at pH 8.0–10.0; NaCl concentration up to 7%; temperature between 20–37°C. Both strains were positive for citrate utilization, gelatin liquefaction; for enzyme activities of (using API ZYM) alkaline phosphatase, esterase (C4), esterase lipase (C8), α-chymotrypsin, acid phosphatase, napthol-AS-B1-phosphohydrolase, β-galactosidase and negative for lipase (C14), β-glucoronidase and α-fucosidase. Both the strains are positive (using API 20 NE) for hydrolysis of esculin and gelatin, ONPG, nitrate reduction, urease, D-glucose fermentation, assimilation of glucose, citrate, D-mannitol, D-mannose and negative for indole production, MR-VP, growth at pH 5 and 12, 15% NaCl; starch hydrolysis; arginine dihydrolase, assimilation of capric acid, adipic acid and phenylacetic acid. Both the strains were sensitive to antibiotics (μg disc^{-1}) novobiocin (30), streptomycin (10), ampicillin (10), methicillin (5), penicillin G (10 U), neomycin (30), triple sulphas (300), sulfonamide (300), kanamycin (30), trimethoprim (5), colistin (10), rifampicin (2), lincomycin (2), cepoxitin (30), gentamycin (10) and bacitracin (8) and resistance to optachin and oxytetracycline (30). Both strains were positive for acid production from sucrose, trehalose, salicin, dextrose, maltose, sorbitol, mannitol, mannose, fructose and inositol; negative for melibiose, dulcitol, lactose and galactose.

Table 1 Characters that differentiate strain S9-65^T along with the closest species *K. palustris* (MTCC 10490^T): 1, strain S9-65^T and 2, *K. palustris*

Characteristics	1	2
Growth at pH 11	+	−
Growth at 12°C	−	+
Casein hydrolysis	−	+
Enzyme assayed for (API ZYM)		
Leucine arylamidase	−	+
Valine arylamidase	−	+
Cystine	−	+
Trypsin	−	+
α- galactosidase	−	+
α-glucosidase	−	+
β-glucosidase	−	+
N-acetyl-β-glucosaminidase	−	+
α-mannosidase	−	+
Assimilation of (API 20 NE)		
Arabinose	−	+
N-acetyl-glucosamine	−	+
Potassium gluconate	−	+
Malic acid	−	+
Hydrogen sulphide production	−	+
Nitrate reduction	−	+
Acid production from		
Adonitol	−	+
Raffinose	−	+
Rhamnose	−	+
Cellobiose	−	+
Arabinose	−	+
Xylose	−	+

+ positive; − negative. All the data from present study

Chemotaxonomic characterization

The chemotaxonomic properties of strain S9-65^T (presented in the species description) were typical of members of the genus *Kocuria*. The major fatty acids (anteiso-C15:0 and anteiso-C17:0) detected in the novel strain (presented in the species description) are consistently found in members of the genus *Kocuria*. The fatty acid compositions of the reference strain *K. palustris* assayed were qualitatively similar, but quantitatively varied from those of the novel strain (Table 2). The major polar lipids were phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and four unknown phospholipids (PL) (Supplementary Figure 1). The major menaquinones detected for the strain S9-65^T were MK-9(H_2), 77.4%, and MK-8(H_2), 8.4.; MK-7(H_2), 5.1%, and MK-6(H_2), 5.3% occur in minor amounts. The diagnostic diamino acid in cell wall hydrolyzates was lysine with peptidoglycan type Lys-Ala_3 (type A3α). The DNA G+C content of strain S9-65^T was estimated to be 69.2 mol%, a value within the range of 66–75 mol% reported for 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. 2006a, b; Li et al. 2006; Zhou et al. 2008).

Table 2 Cellular fatty acid composition of strain S9-65^T along with the closest species *K. palustris* (MTCC 10490^T): 1 strain S9-65^T and 2 *K. palustris*

Fatty acid	1	2
Iso $C_{14:0}$	3.11	3.06
Iso $C_{15:0}$	1.83	ND
Iso $C_{16:0}$	2.44	2.76
$C_{14:0}$	2.03	3.06
$C_{16:0}$	1.4	2.77
ANTEISO-$C_{15:0}$	77.97	68.06
ANTEISO-$C_{17:0}$	8.37	16.92
$C_{18:1}$ 2OH	1.40	3.38
Summed feature 4*	1.46	ND

Data from present study. *ND* not detected

Summed feature 4* consists of C17: 1 iso I/anteiso B, which could not be separated by MIDI

C17:1 iso I/ anteiso B: iso I/ anteiso B are monounsaturated iso/anteiso branched C17 fatty acids where the exact location of the double bond is not known with "I" / "B" indicating the probability of several isomers like A, B, C, etc

Phylogenetic analysis and genomic relatedness

The almost complete sequence (1425 bp) of the 16S rRNA gene of strain S9-65^T was determined (GenBank accession no. HQ018931) and compared with those of other closely related taxa retrieved from the EzTaxon database. Sequence analysis revealed that strain S9-65^T shared highest 16S rRNA gene sequence identity with *K. palustris* TAGA 27^T (99.1%) and the remaining species in the genus *Kocuria* were in the range between 95.6 and 96.5% 16S rRNA gene sequence similarity. Based on the 16S rRNA gene sequence identity, the strain could be assigned to the genus *Kocuria*. The neighbour-joining phylogenetic tree (Fig. 1) as well as maximum parsimony algorithms (data not shown) demonstrated that strain S9-65^T formed a separate lineage along with the closely related species *K. palustris*. However, the DNA–DNA relatedness value between strain S9-65^T and the closely related taxon *K. palustris* TAGA 27^T (MTCC 10490^T) was 20.6% (±0.6%), which was well below the 70% threshold value recommended for the delineation of bacterial species (Stackebrandt and Goebel 1994). DNA–DNA relatedness values between strain S9-65^T and the remaining type strains of the genus *Kocuria* were not determined, since organisms with more than 3% 16S rRNA gene sequence dissimilarity are considered to belong to different genomic species (Wayne et al. 1987).

Conclusion

Based on the phenotypic and genotypic results, strain S9-65^T has to be regarded as a new species of *Kocuria*. Table 1 shows the main features that distinguish strain S9-65^T from the closely related taxon *K. palustris* TAGA 27^T (MTCC 10490^T). Therefore, from the polyphasic evidence gathered in this study it is concluded that strain S9-65^T represents a novel species of the genus *Kocuria*, for which the name *Kocuria assamensis* sp. nov. is proposed.

Description of *Kocuria assamensis* sp. nov.

Kocuria assamensis (as.sam.en'sis. N.L. masc. adj. *assamensis*, pertaining to Assam, a north-eastern state in India, where the type strain was isolated).

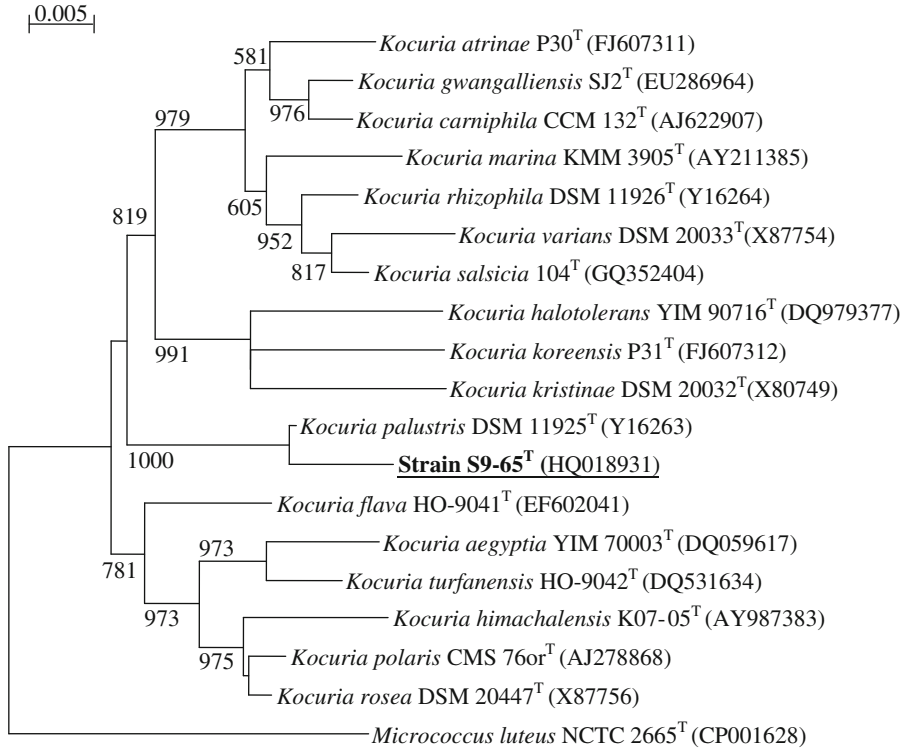

Fig. 1 Phylogenetic neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between *Kocuria assamensis* S9-65^T and related members of the genus *Kocuria*. *Micrococcus luteus* NCTC 2665^T (CP001628) was used as an out-group. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are given at *nodes*. *Bar* 0.5% sequence variation. GenBank accession numbers are given in parentheses

Cells are Gram-positive, non-spore forming, aerobic, motile, catalase-positive, oxidase-positive, short rods (0.8–1.0 × 2.0–2.5 μm). The colonies are pale yellow pigmented, circular, opaque and convex with smooth margin on TSA. Growth occurs at 25–37°C, optimal temperature is 30°C, at a pH of 6.5–11.0, optimal pH is 7.0 and at 2–7% (w/v) NaCl. The strain does not grow on Simmon's citrate and MacConkey agar and do not reduce nitrate to nitrite. Detailed phenotypic characteristics are given in Table 1. The predominant fatty acids are anteiso C15:0 and anteiso-C17:0. The major polar lipids are phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and four unknown phospholipids (PL), as shown in Supplementary Figure 1. The major menaquinones detected for the strain S9-65^T were MK-9(H_2), 77.4%, and MK-8(H_2), 8.4%; MK-7(H_2), 5.09%, and MK-6(H_2), 5.33%, occur in minor amounts. The diagnostic diamino acid in cell wall hydrolyzates was lysine with peptidoglycan type Lys-Ala_3 (type A3α). The DNA G+C content is 69.2 mol%. The type strain S9-65^T (MTCC 10622^T = DSM 23999^T) was isolated from a water sample collected from the river Brahmaputra, Assam, India.

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