Ruania albidiflava gen. nov., sp. nov., a novel member of the suborder *Micrococcineae*

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A Gram-positive, coccoid, non-spore-forming bacterium, designated strain 3-6^T, was isolated from farmland soil and subjected to a polyphasic taxonomic analysis. Comparative analysis of the 16S rRNA gene sequence revealed that the strain represented a novel member of the suborder *Micrococcineae*. Its nearest phylogenetic neighbour was the type strain of *Georgenia muralis* (94.2% 16S rRNA gene sequence similarity). Chemotaxonomic characteristics of strain 3-6^T were as follows: the major menaquinone was MK-8(H₄); the polar lipids consisted mainly of diphosphatidylglycerol, phosphatidylglycerol and one unknown glycolipid; the predominant fatty acids were anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}; mycolic acids were absent. A new murein type, L-Lys-Gly-L-Glu-L-Glu (A4₂), was found in the peptidoglycan of the cell wall. The DNA G+C content was 69.8 mol%. On the basis of morphological, chemotaxonomic and phylogenetic characteristics, it is suggested that strain 3-6^T represents a novel species of a new genus within the suborder *Micrococcineae*, for which the name *Ruania albidiflava* gen. nov., sp. nov. is proposed. The type strain of *Ruania albidiflava* is 3-6^T (=CGMCC 4.3142^T=DSM 18029^T=JCM 13910^T=PCM 2644^T).

According to the estimation performed by Hammond (1995), the number of bacteria that have been successfully isolated and identified comprises only a small portion of the total that exist in nature. In the past decade, the *Micrococcineae* (Stackebrandt *et al.*, 1997) has been one of the most studied suborders in the class *Actinobacteria*; numerous novel taxa in this suborder have been cultured and described (Martin *et al.*, 1997; Groth *et al.*, 1999a, b, 2001, 2002; von Wintzingerode *et al.*, 2001; Altenburger *et al.*, 2002; Takahashi *et al.*, 2006). In the current study, we describe a bacterium isolated from farmland soil collected in Shandong Province, China, and propose a novel species of a new genus in the suborder *Micrococcineae*.

Farmland soil samples were collected from a cotton field in Shandong Province. A 1 g soil sample was suspended in 10 ml sterile distilled water and mixed thoroughly by shaking overnight at room temperature. The suspension was

serially diluted and spread onto yeast extract–starch agar (JCM medium no. 42) plates, followed by incubation for 1 week under humid conditions at 28 °C. The organism thus isolated, designated strain 3-6^T, was picked and transferred to fresh nutrient agar for purification. The pure culture was tested for growth in various media and was maintained on nutrient agar slants at 4 °C.

For observation of colony and cell morphology, strain 3-6^T was grown on nutrient agar, R agar (Groth *et al.*, 1999b) and trypticase soy agar (TSA; BBL) for up to 5 days at 28 °C. Light and scanning electron micrographs were taken according to methods described previously (Huang *et al.*, 2004). The presence of flagella was examined via transmission electron microscopy of 48- and 72-h plate cultures. The temperature range for growth was determined by incubating inoculated slant agar cultures (R medium, pH 7.0) at 4, 10, 15, 20, 28, 37 and 45 °C. Growth was also assessed on R agar plates at initial pH values of 3.5–13.5. The lower pH values of the test medium were obtained according to the method described by Cui *et al.* (2005) and pH values above 10.5 by using a buffer system of disodium hydrogen phosphate (0.15 M)/sodium hydroxide (6 M). API 50CH and API ZYM kits (bioMérieux) were used to test acid production from carbohydrates and enzyme activities, respectively. In

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 3-6^T is DQ343153.

A scanning electron micrograph of cells of strain 3-6^T, a maximum-parsimony phylogenetic tree and tables detailing the physiological and biochemical properties and cellular fatty acid components of strain 3-6^T are available as supplementary material in IJSEM Online.

addition, susceptibility to antibiotics was assessed as described by Groth *et al.* (1999b), use of sole carbon sources for energy and growth was determined by using the methods of Yassin *et al.* (1995) and biodegradation tests were performed according to the procedures described by Gordon & Mihm (1957).

Genomic DNA extraction and PCR amplification of the 16S rRNA gene from strain 3-6^T were performed by using an established method (Chun & Goodfellow, 1995). The PCR product was purified and cloned into the vector pMD 18-T (Takara) by using the method and reagents provided by the manufacturer. The almost-complete nucleotide sequence of the 16S rRNA gene was obtained according to the process described by Gu *et al.* (2006), except that M13 sequencing primers were used. Preliminary phylogenetic analysis was performed with the BLAST search program available at NCBI. Selected sequences were loaded into the software package MEGA, version 3.1 (Kumar *et al.*, 2004), and this was followed by a series of programmed phylogenetic calculations. Neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) trees were constructed separately as described by Gu *et al.* (2006).

Cell mass of strain 3-6^T was collected from 4-day cultures in liquid R medium. Isoprenoid quinones were purified according to the methods of Collins (1985) and were analysed by HPLC (Wu *et al.*, 1989) by using an HP-1050 (Hewlett Packard) chromatograph equipped with a Zorbax ODS C18 (250 × 4.6 mm, inner diameter 5 µm) column. The mobile phase was acetonitrile/2-propanol (2:1) at a flow rate of 1 ml min⁻¹, and menaquinones were detected by absorption at 270 nm. Polar lipids were extracted and identified by two-dimensional TLC (Lechevalier *et al.*, 1977; Minnikin *et al.*, 1984). The fatty acid profile was determined according to the GC techniques described by Sasser (1990) and Kämpfer & Kroppenstedt (1996). The acid methanolysis procedure was used to detect mycolic acids (Minnikin *et al.*, 1975). To determine the peptidoglycan structure, purified cell wall was prepared according to the method of Lechevalier & Lechevalier (1970). Amino acids and peptides in the cell-wall hydrolysates were examined by TLC as described by Schleifer & Seidl (1985) and with LC-MSMS by using a micrOTOF-Q instrument (Bruker). Subsequently, GLC-MS analyses of heptafluorobutyrate derivatives of amino acids (MacKenzie & Tenaschuk, 1974) were performed to determine the enantiomeric amino acid isomers and their molar ratios. Derivatives were prepared by acid-catalysed esterification at 120 °C for 20 min with *R*-(−)-2-butanol (200 µl) and acetyl chloride (20 µl) followed by acylation with heptafluorobutyric anhydride at 150 °C for 5 min. GLC-MS analyses were performed with a Hewlett Packard 5971A system, employing an HP-1 glass capillary column (0.2 mm × 12 m), a temperature program of 100–270 °C at 5 °C min⁻¹, with helium as the carrier gas and at a split ratio of 1:50. The N-terminal amino acid of the interpeptide bridge was determined by dinitrophenylation as described by Schleifer (1985). Whole cell-wall sugars were

analysed according to the method of Lechevalier & Lechevalier (1980). The G+C content of the genomic DNA of strain 3-6^T was determined by the thermal denaturation method (Marmur & Doty, 1962) with *Escherichia coli* K-12 as a control.

After 3 days incubation, strain 3-6^T was found to grow well and formed convex and moist colonies that were whitish-yellow in colour. Cells were small cocci, 0.5–0.8 µm in diameter (see Supplementary Fig. S1 in IJSEM Online). Neither flagella nor a rod-coccus life cycle were detected when grown in the media mentioned above. Growth was observed between 20 and 37 °C, with optimum growth at 28 °C. Growth was also observed on R agar at initial pH values ranging from 5.5 to 12.5, with optimum growth at pH 6.5–10.5. Detailed physiological and biochemical characteristics of strain 3-6^T, including acid production from carbohydrates, assimilation of sole carbon sources, enzyme activities and antibiotic susceptibilities, are given in Supplementary Table S1 available in IJSEM Online.

GLC-MS analysis of cell-wall amino acids revealed that the peptidoglycan of strain 3-6^T contained L-Ala, D-Ala, Gly, L-Glu, D-Glu and L-Lys in a molar ratio of 1.0:0.7:0.7:1.7:0.9:0.7. Dinitrophenylated Glu was detected in hydrolysates of cell-wall preparations treated with 1-fluoro-2,4-dinitrobenzene. From these results and from the occurrence of the peptides L-Lys←Gly and L-Glu←D-Ala in the two-dimensional TLC peptide pattern of the partial hydrolysates of the cell walls (data not shown), it was concluded that strain 3-6^T possesses a peptidoglycan of type A4α (Schleifer & Kandler, 1972) with an L-Lys←Gly←L-Glu←L-Glu interpeptide bridge, which is a novel murein type according to the DSMZ catalogue of strains (<http://www.dsmz.de/species/murein.htm>). The whole-cell sugars consisted mainly of galactose and glucose. The major menaquinone was the partially saturated menaquinone MK-8(H₄). Phosphatidylglycerol, diphosphatidylglycerol and one unknown glycolipid were found as major polar lipids. Cellular fatty acid analysis revealed predominant amounts of the branched fatty acids anteiso-C_{15:0} (37.3%), anteiso-C_{17:0} (14.7%) and iso-C_{16:0} (14.3%) and smaller amounts of C_{18:1ω9c} (9.5%), iso-C_{15:0} (7.9%), C_{16:0} (5.9%), iso-C_{17:0} (2.6%) and C_{14:0} (1.9%). The complete fatty acid profile is given in Supplementary Table S2 in IJSEM Online. Mycolic acids were not present. The G+C content of the genomic DNA of strain 3-6^T was 69.8 mol%.

The nearest phylogenetic neighbours of strain 3-6^T, as determined by analysis of the almost-complete 16S rRNA gene sequences, were found to be members of the suborder *Micrococcineae*. Highest sequence similarity was obtained with *Georgenia muralis* 1A-C^T (94.2%). The position of strain 3-6^T relative to its phylogenetic neighbours is shown in the neighbour-joining tree (Fig. 1) and was confirmed in the maximum-parsimony tree (see Supplementary Fig. S2 in IJSEM Online). The pattern of its 16S rRNA gene sequence signature nucleotides is shown in Table 1, which

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences available from the GenBank/EMBL/DDBJ databases displaying relationships between strain 3-6^T and related taxa. Distances with the Kimura two-parameter model (Kimura, 1980) were determined and clustering with the neighbour-joining method were performed by using the software package MEGA, version 3.1 (Kumar *et al.*, 2004). Bootstrap values based on 1000 replications are listed as percentages at branch points. The taxa and sequences included in the family groups are the same as shown in Supplementary Fig. S2 (available in IJSEM Online). Bar, 0.01 substitutions per nucleotide position.

indicated that strain 3-6^T does not belong to any recognized family within the suborder *Micrococcineae*, having the fewest signature nucleotide differences with *Georgenia* (seven of 33 positions) and the *Bogoriellaceae* (eight of 33 positions) (Stackebrandt *et al.*, 1997; Stackebrandt & Schumann, 2000).

Characteristics that differentiate strain 3-6^T from other representatives of the nearest neighbours detected by 16S rRNA gene sequence analysis are given in Table 2. It is evident from the genotypic and phenotypic data presented

that strain 3-6^T represents a novel species of a new genus within the suborder *Micrococcineae*, for which the name *Ruania albidiflava* gen. nov., sp. nov. is proposed.

Description of *Ruania* gen. nov.

Ruania (Ru.an'i.a. N.L. fem. n. *Ruania* named after Ji-Sheng Ruan, a Chinese microbiologist who has made great contributions to the development of actinomycete taxonomy in China).

Table 1. Patterns of 16S rRNA gene signature nucleotides that define strain 3-6^T, the genera *Beutenbergia*, *Georgenia* and *Salana* and the family *Bogoriellaceae*

Positions are numbered according to the *E. coli* numbering scheme. The following signature nucleotides were identical in all taxa analysed: 41:401 (G–C), 45:396 (U–G), 142:221 (C–G), 248:276 (C–G), 258:268 (G–C), 293:304 (G–U), 379:384 (C–G), 407:435 (A–U), 502:543 (G–C), 586:755 (C–G), 591:648 (U–A), 630 (C), 660:745 (G–C), 668:738 (A–U), 670:736 (A–U), 1244:1293 (C–G) and 1310:1327 (G–C).

Position	Strain 3-6 ^T	<i>Georgenia</i>	<i>Bogoriellaceae</i>	<i>Beutenbergia</i>	<i>Salana</i>
66:99	A–U	A–U	A–U	G–U	G–U
140:223	C–G	G–U	G–U	G–U	G–U
144:178	U–G	U–G	U–G	C–G	C–G
589:650	U–A	U–A	C–G	U–A	U–A
602:636	G–U	C–G	C–G	C–G	C–G
610	A	A	A	A	U
612:628	U–A	G–C	C–G	G–C	C–G
616:624	G–C	G–C	G–C	G–C	G–U
615:625	A–U	A–U	G–C*	A–U	A–U
839:847	C–G	C–G	C–G	U–A	C–G
863	U	U	U	A*	U
1133:1141	A–U	G–C	A–U	A–U	A–U
1134:1140	C–G	G–C	C–G	C–G	C–G
1254:1283	G–U	G–C	G–C	G–C	G–C
1263:1272	G–U	G–U	A–U	A–U	A–U
1414:1486	U–G	C–G	C–G	U–A	U–A

*Errors in the signatures indicated in Altenburger *et al.* (2002) have been corrected here.

Table 2. Differential characteristics of strain 3-6^T and related taxa

Data for reference taxa were taken from Altenburger *et al.* (2002) (*Georgenia*), Groth *et al.* (1997) (*Bogoriella*), Groth *et al.* (1999b) (*Beutenbergia*) and von Wintzingerode *et al.* (2001) (*Salana*). All taxa have the same major menaquinone, MK-8(H₄). DNA G+C contents given are for the type strain of the type species.

Characteristic	Strain 3-6 ^T	<i>Georgenia</i>	<i>Bogoriella</i>	<i>Beutenbergia</i>	<i>Salana</i>
Cell morphology	Coccoid	Rod-coccus	Irregular rod, coccoid	Rod-coccus	Rod, coccoid, club-like
DNA G+C content (mol%)	70	70	70	71	75
Interpeptide bridge	L-Lys-Gly-L-Glu-L-Glu (A4 α)	L-Lys-L-Glu (A4 α)	L-Lys-L-Ala-L-Ala-L-Glu (A4 α)	L-Lys-L-Glu (A4 α)	L-Orn-L-Glu (A4 β)
Polar lipids*	PG, DPG, 1GL	PG, DPG, PIM, 2PL, GL	PI, PG, DPG, 1PL	PI, DPG, 3PL	PG, DPG, PLs
Major fatty acid(s)	anteiso-C _{15:0} , anteiso-C _{17:0} , iso-C _{16:0}	anteiso-C _{15:0} , iso-C _{14:0} , iso-C _{15:1}	anteiso-C _{15:0}	iso-C _{15:0} , anteiso-C _{15:0}	iso-C _{16:0} , C _{14:0} , C _{16:0}

*DPG, Diphosphatidylglycerol; nGL, *n* unknown glycolipid(s); PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; nPL, *n* unknown phospholipid(s).

Gram-positive, aerobic, mesophilic, moderately halotolerant, non-acid-fast, non-motile, non-spore-forming cocci. The rod-coccus life cycle is not detected. The peptidoglycan type is A4 α with an L-Lys-Gly-L-Glu-L-Glu interpeptide bridge. The predominant menaquinone is MK-8(H₄). The main fatty acid is anteiso-C_{15:0} (12-methyl tetradecanoic acid). The major polar lipids are phosphatidylglycerol, diphosphatidylglycerol and one unknown glycolipid. Mycolic acids are absent. The 16S rRNA gene sequence signature nucleotides are listed in Table 1. The genus belongs phylogenetically to the suborder *Micrococcineae*, order *Actinomycetales*. The type species is *Ruania albidiflava*.

Description of *Ruania albidiflava* sp. nov.

Ruania albidiflava (al.bi.di.flav'va. L. adj. *albidus* white; L. fem. adj. *flava* yellow; N.L. fem. adj. *albidiflava* whitish yellow).

Cells are 0.5–0.8 μ m in diameter. Colonies are convex and moist, with a pale yellow colour. Growth occurs at 20–37 °C with an optimum at 28 °C, and at a wide initial pH range of 5.5–12.5 with an optimum at pH 6.5–10.5. Can tolerate up to 10 % (w/v) NaCl. Catalase-positive, oxidase-negative. The methyl red test is positive, but indole and Voges-Proskauer reactions are negative. Nitrate is reduced to nitrite. H₂S is not produced. Potato starch is decomposed, but adenine, casein, aesculin, gelatin, hippurate, hypoxanthine, tyrosine, urea and xanthine are not. Acids are produced from arbutin, salicin, D-ribose, methyl β -D-xylopyranoside, D-fructose, aesculin, D-maltose, D-arabinose, L-arabinose, D-xylose, L-rhamnose, D-lyxose and L-fucose, but not from glycerol, erythritol, L-xylose, D-adonitol, D-galactose, D-glucose, D-mannose, L-sorbose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, N-acetylglucosamine,

amygdalin, D-cellobiose, D-lactose, D-melibiose, sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-tagatose, D-fucose, DL-arabitol, potassium 2-ketogluconate or potassium 5-ketogluconate. Utilizes the following substrates as sole carbon sources: acetate, D-lactose, D-melezitose, L-alanine, L-cysteine, L-leucine, L-methionine, L-proline, methyl α -D-glucoside, oxalate, D-fructose, D-glucose, D-glutamic acid, D-maltose, D-ribose, D-sorbitol, D-trehalose, glycerol, L-arginine, L-fucose and sucrose. The following substrates are not utilized: citrate, D-cellobiose, D-galactose, D-inulin, D-lactulose, D-mannitol, D-mannose, D-raffinose, D-rhamnose, D-sorbose, D-xylose, dulcitol, erythritol, glycogen, inositol, L-arabinose, L-leucinamide, L-ornithine, L-phenylalanine, L-tyrosine, L-valine, malate, malonate, nicotinamide, salicin and succinate. In the API ZYM assay, tests are positive for cystine arylamidase, α -fucosidase, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase and α -mannosidase, but negative for esterase (C4), lipase (C14), trypsin, chymotrypsin and β -glucuronidase. Cells are susceptible to ampicillin (10 μ g), chloramphenicol (30 μ g), ciprofloxacin (5 μ g), erythromycin (15 μ g), gentamicin (10 μ g), kanamycin (30 μ g), neomycin (30 μ g), polymyxin B (300 IU), rifampicin (15 μ g) and streptomycin (10 μ g). Not susceptible to nitrofurantoin (300 μ g) or oxacillin (1 μ g). Whole-cell sugars are galactose and glucose. The G+C content of the genomic DNA is 69.8 mol%. Other chemotaxonomic characteristics are as described for the genus.

The type strain, 3-6^T (=CGMCC 4.3142^T = DSM 18029^T = JCM 13910^T = PCM 2644^T), was isolated from farmland soil collected in Shandong Province, China.

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