

## *Georgenia ruanii* sp. nov., a novel actinobacterium isolated from forest soil in Yunnan (China), and emended description of the genus *Georgenia*

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A Gram-positive, motile, short-rod-shaped strain, designated YIM 004<sup>T</sup>, was isolated from a forest-soil sample collected from Lijiang, Yunnan Province, China, and was investigated using a polyphasic taxonomic approach. The isolate contained chemotaxonomic markers that corresponded to those of its phylogenetic neighbour, *Georgenia muralis*, i.e. it possessed peptidoglycan type A4 $\alpha$  with lysine as the diagnostic cell-wall diamino acid, the predominant menaquinone was MK-8(H<sub>4</sub>) and the major fatty acid was ai-C<sub>15:0</sub>. The G+C content of the genomic DNA was 72.9 mol%. Strain YIM 004<sup>T</sup> exhibited a 16S rRNA gene sequence similarity of 97.3% and a DNA–DNA relatedness value of 18% with respect to *G. muralis* DSM 14418<sup>T</sup>. On the basis of the phenotypic and genotypic differences between the isolate and *G. muralis*, strain YIM 004<sup>T</sup> represents a novel species of the genus *Georgenia*, for which the name *Georgenia ruanii* sp. nov. is proposed. The type strain is YIM 004<sup>T</sup> (= CCTCC AB 204065<sup>T</sup> = DSM 17458<sup>T</sup> = KCTC 19029<sup>T</sup>). In addition, an emended description of the genus *Georgenia* is presented.

The genus *Georgenia* was proposed by Altenburger *et al.* (2002) with a single species, *Georgenia muralis*, the type strain of which was isolated from a medieval wall painting in the church of St Georgen in Styria, Austria. The cells were Gram-positive, oxidase- and catalase-positive, non-sporulating and non-motile and exhibited a rod–coccus cycle.

In the course of a screening programme for new antibiotics, some mesophilic bacteria that contained both type I and type II polyketide biosynthesis pathway genes were screened and identified by means of polyphasic taxonomy (Xu *et al.*, 2003, 2005a, b). The present investigation was designed to clarify the taxonomic position of another novel strain containing these genes. Strain YIM 004<sup>T</sup> was isolated from a forest-soil sample collected from Lijiang, Yunnan Province, China, after 2 weeks incubation at 28 °C on ISP 2 agar

(Shirling & Gottlieb, 1966). Comparative 16S rRNA gene sequence analysis revealed the isolate to be a member of the suborder *Micrococccineae*. On the basis of phenotypic and genotypic evidence, the novel isolate represents a novel species of the genus *Georgenia*.

All physiological and biochemical tests were performed at 28 °C. The colony morphology was determined after 3 days incubation at 28 °C on ISP 2 and TSA [trypticase soy broth (BBL), 3%, w/v; Bacto agar (Difco), 1.5%, w/v] medium. Colour determination was achieved using colour chips from the ISCC–NBS colour charts (standard sample no. 2106) (Kelly, 1964). Gram staining was carried out using the standard Gram reaction and was examined by light microscopy (BH-2 microscope; Olympus). Cell motility was investigated on Luria–Bertani swarming agar (0.3%, w/v). Cellular morphology was studied by using a JEM-1010 electron microscope (JEOL) with cells from exponentially growing cultures. Oxidase activity was determined using a 1% solution of tetramethyl-*p*-phenylenediamine (Kovács, 1956). Catalase activity was determined by assessing the production of bubbles after the addition of a drop of 3% H<sub>2</sub>O<sub>2</sub>. The temperature range and optimum for growth were

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 004<sup>T</sup> is DQ203185.

A transmission electron micrograph of cells of strain YIM 004<sup>T</sup> is available as a supplementary figure with the online version of this paper.

tested at 4–55 °C on ISP 2 medium. The pH range and optimum for growth, tolerance of sodium chloride and phenol and susceptibility to antibiotics were examined as described by Xu *et al.* (2005a). Carbon utilization and acid production were tested using Micro-biochemical tubes (Hangzhou Tianhe Micro-organism Reagent). Some other physiological properties were tested by using the API ID 32E test kit (bioMérieux).

The cells of strain YIM 004<sup>T</sup> were short rod-shaped (0.5–0.8 × 1.4–2.0 µm) and motile (amphitrichous, i.e. the cell has a single flagellum at each end, as shown in Supplementary Fig. S1 available in IJSEM Online). The cells were Gram-positive and chemo-organotrophic, with a respiratory-type metabolism. Endospores and poly-β-hydroxyalkanoate were not formed. The strain could not grow in the presence of sodium chloride at concentrations above 5%. The temperature range for growth was 10–37 °C and the pH range for growth was 6.5–10.0. Optimal growth occurred at 28–30 °C and at pH 7.0. The results of the other physiological and biochemical analyses are summarized in the species description.

Analysis of sugars from whole-cell hydrolysates was performed using procedures described by Staneck & Roberts (1974). Purified peptidoglycan preparations were obtained after disruption of the cells by shaking with glass beads and subsequent trypsin digestion according to the method of Schleifer (1985). The amino acids and peptides in the cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates using the solvent systems of Schleifer & Kandler (1972). The N-terminal amino acid of the interpeptide bridge was determined by dinitrophenylation as described by Schleifer (1985). The molar ratios of the amino acids were determined by GC and GC-MS of the *N*-heptafluorobutyryl amino acid isobutyl esters (MacKenzie, 1987). Analysis of the enantiomers of the peptidoglycan amino acids was performed by GC of the *N*-pentafluoropropionyl amino acid isopropyl esters (Frank *et al.*, 1980) on an L-Chirasil-Val column (Macherey-Nagel) as described by Groth *et al.* (1999).

Polar lipids were extracted from cultures grown in TSB, examined by two-dimensional TLC and identified using published procedures (Minnikin *et al.*, 1979; Collins & Jones, 1980). Menaquinones were isolated using the method of Collins *et al.* (1977) and were analysed by HPLC (Groth *et al.*, 1997). Analysis of the whole-cell fatty acid pattern was performed using previously described methods involving the MIDI system (Microbial ID) (Kroppenstedt, 1985; Meier *et al.*, 1993). The G+C content of the DNA was determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989).

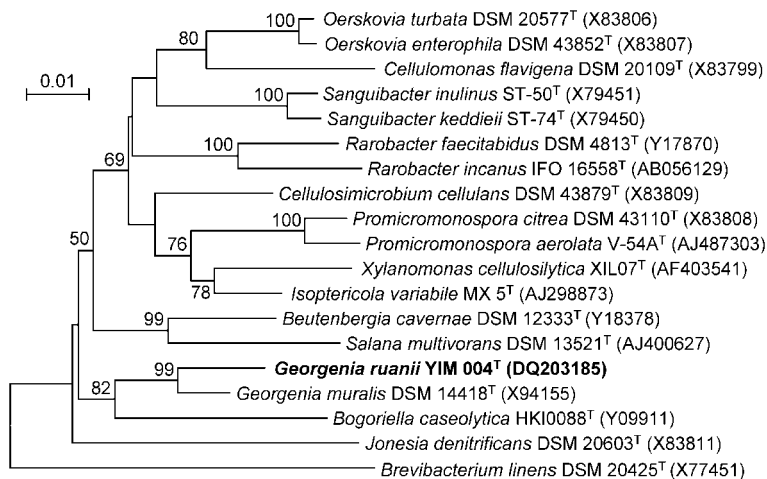
The molar ratio of the amino acids in the peptidoglycan hydrolysate (4 M HCl, 16 h at 100 °C) was approximately 3.1 : 2.4 : 1.0 for alanine/glutamic acid/lysine. Glutamic acid and smaller amounts of alanine were detected as N-terminal amino acids because of the incomplete cross-linkage of the

interpeptide bridge. Enantiomeric analysis of the peptidoglycan amino acids revealed almost equimolar amounts of L- and D-glutamic acid and an L-alanine/D-alanine ratio of approximately 2 : 1. The peptides D-Ala←L-Lys←L-Ala and L-Lys←L-Ala were detected in the partial hydrolysate (4 M HCl, 45 min at 100 °C) of the peptidoglycan of strain YIM 004<sup>T</sup>. From these data, it was concluded that strain YIM 004<sup>T</sup> possesses peptidoglycan of type A4α, with lysine as the diagnostic cell-wall diamino acid and an interpeptide bridge comprising L-Lys←L-Ala←L-Glu. The cell-wall sugars consisted of galactose and rhamnose. The polar lipids were phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and an unknown phospholipid. The menaquinone pattern was composed of MK-8(H<sub>4</sub>), MK-7(H<sub>4</sub>), MK-9(H<sub>4</sub>) (90 : 2 : 1). The major fatty acids were ai-C<sub>15:0</sub> and i-C<sub>15:0</sub>; the detailed cellular fatty acid profile is reported in the species description. The G+C content of genomic DNA from strain YIM 004<sup>T</sup> was 72.9 mol%.

Genomic DNA for PCR amplification was prepared from cells lysed by microwaves: a small amount of biomass was transferred from solid medium to a new Eppendorf tube. After the cells had been washed with 1 ml PBS (pH 8.0) and 1 ml washing buffer [50 mM Tris/HCl, pH 7.7; 25 mM EDTA; 0.1 % SDS; 0.1 % polyvinylpyrrolidone (PVP)], 50 µl lysis buffer (50 mM Tris/HCl, pH 8.0; 25 mM EDTA; 3 % SDS; 1.2 % PVP) was added to resuspend the cells, which were then heated at 700 W in a microwave oven for 45 s. Next, 400 µl warm extraction buffer [10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.5 M sodium acetate, 1.2 % PVP (pre-warmed at 65 °C)] was added immediately and mixed with the cell suspension. An equal volume of phenol/chloroform was used for extraction. The DNA was precipitated with 2-propanol. The DNA was washed with 70 % ethanol and dissolved using 20 µl TE solution.

PCR amplification and 16S rRNA gene sequencing were carried out as described previously (Cui *et al.*, 2001). After a preliminary BLAST analysis (Altschul *et al.*, 1997), the sequence of strain YIM 004<sup>T</sup> was aligned with those of related members of the class *Actinobacteria*, which had been retrieved from the GenBank database using the BLAST2 algorithm (Tatusova & Madden, 1999). Sequence similarities were determined by using CLUSTAL X (Thompson *et al.*, 1997). A phylogenetic tree was constructed, using the neighbour-joining method of Saitou & Nei (1987), from *K<sub>nuc</sub>* values (Kimura, 1980, 1983) with MEGA, version 2.1 (Kumar *et al.*, 2001). The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985), with 1000 replicates.

The almost-complete 16S rRNA gene sequence (1516 nt) was determined for strain YIM 004<sup>T</sup>. Phylogenetic analysis of strain YIM 004<sup>T</sup> revealed that the isolate was most closely related to *G. muralis* DSM 14418<sup>T</sup>, the 16S rRNA gene sequence similarity value being 97.3 %, and the two strains formed a distinct subclade within the suborder *Micrococcineae* (Fig. 1).



**Fig. 1.** Phylogenetic dendrogram, obtained by distance matrix analysis of 16S rRNA gene sequences, showing the position of strain YIM 004<sup>T</sup> and its phylogenetic neighbours. Numbers at branch nodes are bootstrap values (from 1000 resamplings); only values of 50% or above are shown. The sequence of *Brevibacterium linens* DSM 20425<sup>T</sup> was used as the root. Bar, 1% sequence divergence.

To determine the genomic DNA relatedness (%) between strain YIM 004<sup>T</sup> and *G. muralis* DSM 14418<sup>T</sup>, DNA–DNA hybridization was carried out by applying the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983) and using a UV-Vis spectrophotometer (model UV1601; Shimadzu). Genomic DNAs from strains YIM 004<sup>T</sup> and *G. muralis* DSM 14418<sup>T</sup> were prepared according to the method of Marmur (1961); purified genomic DNAs were cut to generate DNA fragments between 400 and 800 bp in size. Sheared genomic DNA (about 100 µg) from each sample was added to 2 × SSC (1 × SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) and then denatured by raising the temperature to 100 °C and cooled to the melting temperature in the spectrophotometer. The samples were kept at the melting temperature for 3 min and the absorbance at 260 nm was recorded at 30 s intervals for a total of 30 min. The initial reassociation kinetics were determined by using linear regression analysis. The percentage DNA homology for these two micro-organisms was calculated using the equation described by De Ley *et al.* (1970). All statistical analyses were performed using the EXCEL software (Microsoft). The DNA–DNA relatedness value obtained was 18% (mean from two experiments), significantly lower than the 70% value considered to be the threshold for the delineation of genomic species (Wayne *et al.*, 1987).

The results of the 16S rRNA gene sequence analysis clearly demonstrated that strain YIM 004<sup>T</sup> is closely related to *G. muralis* DSM 14418<sup>T</sup>. Both organisms contain MK-8(H<sub>4</sub>) as the predominant menaquinone and have the same peptidoglycan type (A4α) with lysine as the diagnostic cell-wall diamino acid. These properties support the inclusion of strain YIM 004<sup>T</sup> within the genus *Georgania*. However, strain YIM 004<sup>T</sup> contains an interpeptide bridge composed of L-Lys←L-Ala←L-Glu, while the peptidoglycan of *G. muralis* has peptide subunits that are cross-linked via a single L-glutamic acid residue. In addition, strain YIM 004<sup>T</sup> differs from *G. muralis* DSM 14418<sup>T</sup> by the presence of phosphatidylinositol, by possessing larger amounts of

iso-C<sub>15:0</sub> and by some other phenotypic characteristics, as shown in Table 1.

The DNA–DNA relatedness data provide conclusive evidence that the novel isolate (YIM 004<sup>T</sup>) and *G. muralis* DSM 14418<sup>T</sup> are members of different genomic species (Wayne *et al.*, 1987). Strain YIM 004<sup>T</sup> therefore represents a novel species of the genus *Georgania*, for which the name *Georgania ruanii* sp. nov. is proposed. As a consequence of newly reported characteristics not included in the original description of strain DSM 14418<sup>T</sup>, the description of the genus *Georgania* Altenburger *et al.* 2002 needs to be emended.

#### Emended description of *Georgania* Altenburger *et al.* 2002

The description is based on that given by Altenburger *et al.* (2002). Cells are cocci or short rods that may exhibit a rod–coccus life cycle. Gram-positive, non-sporulating and motile or non-motile. Aerobic, but growth under anaerobic conditions can also occur. Oxidase- and catalase-positive. The peptidoglycan type is A4α (based on lysine as diagnostic diamino acid). The major menaquinone is MK-8(H<sub>4</sub>). The polar lipids are diphosphatidylglycerol and phosphatidylglycerol; phosphatidylinositol mannoside or phosphatidylinositol can occur. There is a predominance of iso- and anteiso-branched fatty acids, with ai-C<sub>15:0</sub> as a major component. The G+C content of the genomic DNA is 70–73 mol%. Phylogenetically, the genus is a member of the suborder *Micrococcineae*, order *Actinomycetales*. The type species is *Georgania muralis*.

#### Description of *Georgania ruanii* sp. nov.

*Georgania ruanii* [ru.a'ni.i. N.L. gen. n. *ruanii* of Ruan, named in honour of Ji-Sheng Ruan (born in 1926), a Chinese microbiologist who devotes himself to the study of actinomycete taxonomy].

Displays the following properties in addition to those described above for the genus. Colonies are 1.1–1.2 mm in diameter, circular, entire, slightly convex, opaque and pale

**Table 1.** Characteristics used to differentiate between strain YIM 004<sup>T</sup> and its closest phylogenetic neighbour, *G. muralis* DSM 14418<sup>T</sup>

The following characteristics apply to both strains: A4 $\alpha$ -type peptidoglycan with lysine as the diagnostic cell-wall diamino acid; MK-8(H<sub>4</sub>) as the predominant menaquinone; the assimilation of fructose, glucose, arabinose, galactose, sucrose, maltose, cellobiose, melibiose and salicin; the hydrolysis of starch and aesculin; the presence of lysine decarboxylase,  $\beta$ -glucosidase, *N*-acetylglucosaminidase and  $\alpha$ -glucosidase activities; and the absence of H<sub>2</sub>S and indole production, urease, arginine dihydrolase,  $\alpha$ -galactosidase and  $\beta$ -glucuronidase. +, Positive; –, negative; w, weakly positive.

Characteristic	YIM 004 <sup>T</sup>	<i>G. muralis</i> DSM 14418 <sup>T</sup>
Cell morphology	Short rods	Rod–coccus cycle
Motility	+	–
pH tolerance	6.5–10.0	6.0–9.0
Maximum NaCl concentration for growth (% w/v)	5	7
Maximum temperature for growth (°C)	37	45
Utilization of D-mannose	+	w
Nitrate reduction	–	+
$\beta$ -Galactosidase	+	–
<i>N</i> -Acetyl- $\beta$ -glucosaminidase	+	–
Cystine arylamidase	–	+
Hydrolysis of Tween 80	w	–
Polar lipids*	DPG, PG, PI, 1 PL	DPG, PG, PIM, 2 PLs, GL
Peptidoglycan interpeptide bridge	L-Lys←L-Ala←L-Glu	L-Lys←L-Glu
Major fatty acids (> 5%)	ai-C <sub>15:0</sub> (78.3%); i-C <sub>15:0</sub> (14.9%)	ai-C <sub>15:0</sub> (66.6%); i-C <sub>14:0</sub> (6.0%)
DNA G + C content (mol%)	72.9	70.0

\*DPG, Diphosphatidylglycerol; GL, unknown glycolipid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; PL, unknown phospholipid.

white–yellow to straw-coloured on ISP 2 agar. Cells are short-rod-shaped, 0.5–0.8 × 1.4–2.0  $\mu$ m in size, and motile (by means of a single flagellum at each end of the cell). Chemo-organotrophic, with respiratory-type metabolism. Endospores and poly- $\beta$ -hydroxyalkanoate are not formed. Does not grow in the presence of sodium chloride at concentrations above 5%. Temperature range for growth is 10–37 °C, with optimum growth at 28–30 °C. pH range for growth is 6.5–10.0, with optimum growth at pH 7.0. Tests for the hydrolysis of gelatin, for melanin production, H<sub>2</sub>S production and indole production, for resistance to KCN, for milk coagulation and for peptonization are negative. Nitrate is not reduced to nitrite. Dextrin, rhamnose, fructose, glucose, arabinose, sorbose, lactose, galactose, sucrose, dulcitol, inositol, maltose, mannose, cellobiose, turanose, mannitol, melibiose, melezitose, raffinose, ribose, salicin, xylitol, adonitol, arabitol, galacturonate and sorbitol are utilized as sole carbon and energy sources, and acid is produced from fructose, galactose, mannose, mannitol, xylose, ribose, lactose, glucose and sucrose. Starch and aesculin are hydrolysed and acetamide, xanthine, hypoxanthine and Tweens 20 and 80 are degraded weakly; urea is not degraded. Lipase, lysine decarboxylase,  $\beta$ -glucosidase,

*N*-acetyl- $\beta$ -glucosaminidase,  $\beta$ -galactosidase,  $\alpha$ -maltosidase and *L*-aspartic arylamidase tests are positive, while arginine dihydrolase, ornithine decarboxylase,  $\alpha$ -galactosidase and  $\beta$ -glucuronidase tests are negative. Resistant to oleandomycin and nalidixic acid but sensitive to penicillin G, vancomycin, polymyxin B, erythromycin, terramycin, aureomycin, tetracycline, streptomycin sulfate, novobiocin, gentamicin and chloramphenicol. The peptidoglycan type is A4 $\alpha$  (L-Lys–L-Ala–L-Glu). The cell-wall sugars are galactose and rhamnose. The polar lipids are phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and an unknown phospholipid. The menaquinones are MK-8(H<sub>4</sub>), MK-7(H<sub>4</sub>) and MK-9(H<sub>4</sub>) (90 : 2 : 1). The cellular fatty acid profile consists mainly of ai-C<sub>15:0</sub> (78.3%) and i-C<sub>15:0</sub> (14.9%), with other minor components, including ai-C<sub>17:0</sub> (1.6%), C<sub>14:0</sub> (1.0%), ai-C<sub>15:1</sub> (1.0%), C<sub>16:0</sub> (1.0%), ai-C<sub>13:0</sub> (0.7%), i-C<sub>16:0</sub> (0.6%), i-C<sub>17:0</sub> (0.5%), i-C<sub>14:0</sub> (0.5%) and i-C<sub>13:0</sub> (0.1%). The DNA G + C content is 72.9 mol%.

The type strain, YIM 004<sup>T</sup> (= CCTCC AB 204065<sup>T</sup> = DSM 17458<sup>T</sup> = KCTC 19029<sup>T</sup>), was isolated from soil in Lijiang, Yunnan Province, China.

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