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ORIGINAL PAPER

Rubellimicrobium rubrum sp. nov., a novel bright reddish bacterium isolated from a lichen sample

Long-Qian Jiang · Kun Zhang · Gui-Ding Li · Xin-Yu Wang · Song-Biao Shi · Qin-Yuan Li · De-Feng An · Lei Lang · Li-Song Wang · Cheng-Lin Jiang · Yi Jiang

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Abstract A novel strain, YIM 131921^T, was isolated from a *Physcia* sp. lichen collected from the South Bank Forest of the Baltic Sea. The strain is Gram-negative, catalase positive and oxidase negative, strictly aerobic, asporogenous, non-motile and reddish brown in colour. The temperature and pH for growth were found to be 20–30 °C (optimum 28 °C) and pH 6.5–12.0 (optimum pH 7.0 ± 0.5). No growth was observed in the presence of NaCl. Based on 16S rRNA gene sequence similarity, strain YIM 131921^T shares high similarities with *Rubellimicrobium roseum* YIM 48858^T (98.3%), followed by *Rubellimicrobium mesophilum* MSL-20^T (96.8%), *Rubellimicrobium aerolatum* 5715S-9^T (96.1%) and *Rubellimicrobium thermophilum* DSM 16684^T (96.0%). Phylogenetic trees showed YIM 131921^T forms a cluster with type strains of the genus *Rubellimicrobium*. The predominant cellular fatty acids (> 20%) were identified as summed feature 8 ($C_{18:1}\omega7c$) and $C_{16:0}$. Q-10 was found to be the predominant respiratory ubiquinone. The polar lipids were identified as diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, glycolipid, phospholipids and an unidentified aminolipid. The DNA G + C content of the draft genome sequence is 66.6 mol%. Strain YIM 131921^T showed an average nucleotide identity value of 80.3% and a digital DNA–DNA hybridizations value of 26.1% with the reference strain *R. roseum* YIM 48858^T based on draft genome sequences. Based on comparative analyses of phenotypic, molecular, chemotaxonomic data and genomic comparisons, strain YIM 131921^T is concluded to represent a novel species of the genus *Rubellimicrobium*, for which the name *Rubellimicrobium rubrum* sp. nov. is proposed. The type strain is YIM 131921^T (= CGMCC 1.13958^T = NBRC 114054^T = KCTC 72461^T).

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L.-Q. Jiang · K. Zhang · G.-D. Li · Q.-Y. Li · D.-F. An · L. Lang · C.-L. Jiang · Y. Jiang (✉)
Yunnan Institute of Microbiology, Key Laboratory for Conservation and Utilization of Bio-Resource, School of Life Sciences, Yunnan University, Kunming 650091, People's Republic of China
e-mail: jiangyi@ynu.edu.cn

G.-D. Li
Institute of Microbial Pharmaceuticals, Northeastern University, Shenyang 110819, People's Republic of China

X.-Y. Wang · L.-S. Wang
Key Lab for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, People's Republic of China

S.-B. Shi
School of Chemistry and Chemical Engineering, Guangxi Key Laboratory of Chemistry and Engineering of Forest Products, Guangxi University for Nationalities, Nanning 530008, People's Republic of China

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Introduction

The genus *Rubellimicrobium*, a member of the *Rhodobacteraceae*, was first proposed by Denner et al. (2006). At the time of writing, the genus *Rubellimicrobium* comprises four validly named species, *Rubellimicrobium thermophilum* (Denner et al. 2006), *Rubellimicrobium mesophilum* (Dastager et al. 2008), *Rubellimicrobium aerolatum* (Weon et al. 2009) and *Rubellimicrobium roseum* (Cao et al. 2010). The members of the genus *Rubellimicrobium* have been isolated from different environments, including coloured slime deposits in paper machines, soil and air. Characteristic features of the members of the genus *Rubellimicrobium* include colonies that are convex and pink to reddish in colour, ubiquinone Q-10 as the major respiratory ubiquinone, and $C_{18:1}\omega7c$ and $C_{16:0}$ as major cellular fatty acids. Their polar lipids include diphosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol and an unidentified aminolipid (Denner et al. 2006).

Lichens are structured associations of a fungus with a cyanobacteria and/or green algae in a symbiotic relationship, which provide specific habitats for diverse bacterial communities, including actinomycetes (Liu et al. 2017). Lichens also provide an extremely rich reservoir for the isolation of novel species. During a study of the diversity of microorganism present in lichen samples, a novel bacterium YIM 131921^T was isolated and its taxonomic status was investigated using a polyphasic taxonomic approach.

Materials and methods

Isolation, maintenance and cultural conditions

A lichen sample was collected from the South Bank Forest of the Baltic Sea (10°12′E, 54°31′N), Germany, then immediately transferred to sterile paper bag and air-dried at 28 °C for 7 days. The lichen sample was pretreated through two steps: a 5-min wash with running water, followed by three washes in sterile water. Finally, the lichen sample was homogenised with 18 ml of sterile 0.1% pyrophosphate using a sterile glass homogeniser. The methods of lichen surface pretreatment were according to Liu et al. (2017). Strain YIM 131921^T was isolated using a standard dilution plate method on humic acid-vitamin agar media (Hayakawa and Nonomura 1987), which was incubated at 28 °C. Individual colonies were further purified onto YIM 38 medium (containing, per litre of distilled water: yeast extract 4.0 g, glucose 4.0 g, malt extract 2.5 g, B-vitamin 1.0 mg, agar 12.0 g, pH 7.2) (Jiang et al. 2007), then maintained on YIM 38 medium slants at 4 °C and glycerol suspensions (20%, v/v) at − 80 °C respectively.

Phenotypic characteristics

Morphological characteristics of strain YIM 131921^T were observed by using a phase contrast microscope (ECLIPSE Ni-U; Nikon), and the flagella of the strain were observed by transmission electron microscopy (JEM-2100; JEOL) after growth on YIM 38 agar at 28 °C for 5 days. Cell motility was confirmed in semi-solid medium tubes containing 0.4% agar by observing the growth spread of cells (Leifson 1960). Gram staining and Gram-reaction was performed by using the standard Gram reaction (Doetsch 1981) combined with the non-staining KOH lysis method (3% KOH; Buck 1982). Anaerobic growth was determined after incubation on YIM 38 for 2 weeks at 28 °C using the GasPak EZ Anerobe Pouch System (BD). Growth at different temperatures (4, 10, 15, 20, 25, 28, 37, 40 and 45 °C), and at various concentrations of NaCl (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%, w/v) were tested on YIM 38 medium at 28 °C. The pH range for growth (pH 4.0–13.0, at intervals of 0.5 pH unit) was tested on YIM 38 at 28 °C using the buffer system from Xu et al. (2005). Oxidase activity was determined by using 1% (w/v) tetramethyl-*p*-phenylenediamine as described by Kovacs (1956). Catalase activity was determined as the production of bubbles after the addition of 3% (v/v) H_2O_2. Hydrolysis of starch and cellulose, Tweens (20, 40, 60 and 80), coagulation and peptonisation of milk, H_2S production and nitrate reduction were observed using the methods described by Smibert and Krieg (1994). Other biochemical properties and enzyme activities were tested using API 20NE, and API ZYM kit (BioMérieux, 80 France) according to

the instructions of the manufacturer. Sole carbon and nitrogen source utilisation were determined using Biolog GEN III MicroPlates according to the manufacturer's instructions. The incubation temperature was 28 °C and results were observed after 48 h. The reference strain *R. roseum* YIM 48858^T for phenotypic comparisons was grown and tested under identical conditions.

16S rRNA gene sequencing and phylogenetic analysis

DNA extraction and amplification of 16S rRNA gene fragments were carried out according to Peng et al. (2006). The 16S rRNA gene sequence was compared to sequences from validly named bacteria using the EzBioCloud server databases (Yoon et al. 2017). Phylogenetic trees were generated by three tree-making algorithms, the neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Tamura et al. 2011) and maximum-parsimony (Fitch 1971) methods using MEGA version 7.0 software (Kumar et al. 2016). The topologies of the phylogenetic trees were assessed using the bootstrap method with 1000 replicates (Felsenstein 1985).

Genomic analysis

The draft genome sequence was determined using the Illumina HiSeq 4000 sequencing platform. The draft genome was assembled using SOAP denovo version 2.04 (Li et al. 2008) and the short oligonucleotide of assembled results was further polished using SOAP aligner 2.21 (Li et al. 2015). Average nucleotide identity (ANI) was calculated using ANI Calculator tool from Ezbiocloud. The estimated genome-sequence based digital DNA–DNA hybridization (dDDH) values were calculated using formula 2 at the Genome-to-Genome Calculator (CGGC) website (http://ggdc.dsmz.de/ggdc.php) as described by Meier-Kolthoff et al. (2013). Gene annotations were conducted through the NCBI prokaryotic genome annotation pipeline. The biosynthetic gene clusters for putative secondary metabolites were identified using the antiSMASH 3.0 program (Vela et al. 2007).

Chemotaxonomic characterisation

Cell biomass for chemotaxonomic characterisation was harvested after cultivation on YIM 38 at 28 °C for 5 days. Polar lipids were extracted and analysed by the method of Denner et al. (2001). Fatty acids were extracted with the method of Kuykendall et al. (1988) and analysed by using the standard protocol of the MIDI System (Sherlock version 6.1; database TSBA6) (Sasser 1990). The ubiquinone was investigated by HPLC according to the method described by Tindall (1990).

Results and discussion

Phenotypic characteristics

Colonies of strain YIM 131921^T were observed to be bright red, irregular, dry and convex at 28 °C on YIM 38 agar for 5 days. Cells of strain YIM 131921^T were observed to be aerobic, Gram-negative, asporogenous, non-motile, smooth and spherical (approximately 1.0 μm in diameter) without flagella after cultivation for 5 days at 28 °C on YIM 38. Strain YIM 131921^T was found to grow well on YIM 38, weakly on R2A medium, but not at all on Tryptic Soy Agar. Optimal growth was found to occur at 28 °C ant pH 7.5 without NaCl. Strain YIM 131921^T is catalase positive and oxidase negative. It can degrade Tweens 20, 40, 60 and 80. Strain YIM 131921^T is positive for urease activity, negative for H_2S production, nitrate reduction, milk coagulation and peptonisation, hydrolysis of gelatin, cellulose and starch tests. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, acid phosphatase, β-galactosidase and α-glucosidase were positive; but lipase (C14), cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase in the API ZYM system (bioMérieux) were found to be negative. In the Biolog GEN III system tests, utilisation of D-glucose, D-mannose, fusidic acid, D-sorbitol, D-arabitol, L-alanine, pectin, D-galacturonic, L-galactonic acid lactone, D-gluconic acid, *p*-hydroxy-phenylacetic acid, methyl pyruvate, D-lactic acid methyl ester, L-lactic acid, citric acid, L-malic acid, Tween 40, γ-amino-butyric acid, α-hydroxy-butyric acid, β-

hydroxy-D, L-butyric acid, α-keto-butyric acid, acetoacetic acid, and propionic acid were positive, while other tests were negative. Detailed phenotypic properties are shown in the species description and Table 1.

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene sequence of strain YIM 131921^T was determined by PCR amplification. The nearly complete 16S rRNA gene sequence (1469 bp; GenBank accession number MK610812) was obtained. Comparative analysis of 16S rRNA gene sequences using the EzBioCloud server indicated that strain YIM 131921^T shows a high level of similarity with *R. roseum* YIM 48858^T (98.3%), less than 97% with other currently described type strains. Phylogenetic trees were constructed by the neighbour-joining, maximum-likelihood algorithms and maximum parsimony. As shown in Fig. 1, strain YIM 131921^T groups within the genus *Rubellimicrobium*. This was also observed in the maximum-likelihood tree (Fig. S1) and maximum-parsimony tree (Fig. S2).

Genomic analysis

The draft genome sequence of strain YIM 131921^T (4615821 bp) contains 104 contigs with N50 contig length of 145678 bp (GenBank accession number VDFU00000000). The G + C content of strain YIM 131921^T was determined to be 66.6 mol% based on the draft genome, which is lower than the G + C contents of other members of the genus *Rubellimicrobium*. The draft genome of the reference strain *R. roseum* YIM 48858^T determined in this study consists of 4851245 bp with an N50 contig length of 86729 bp and a G + C content of 69.1 mol % (GenBank accession number VDFV00000000). dDDH showed that the relatedness between strain YIM 131921^T and *R. roseum* YIM48858^T was 26.1 ± 3.1%, which is much lower than the threshold value (70%) recommended for distinguishing novel prokaryotic species (Wayne et al. 1987). The ANI value between the novel strain and *R. roseum* YIM 48858^T was 80.3% which is clearly lower than the threshold (< 95%) generally accepted for species delineation (Richter and Rossello-Mora 2009). Based on the genomic analysis, the genome of strain YIM 131921^T contains 4284 genes, 4234 coding sequences (CDSs), 3 complete rRNA genes and 43 tRNA genes. In contrast, the draft genome of the reference strain *R. roseum* YIM 48858^T contains 4661 genes, 4610 coding sequences (CDSs), 3 complete rRNA genes and 44 tRNA genes. A phytoene synthase gene was detected in both strains which has something to do with the colony colour. These results clearly indicate that strain YIM 131921^T represents a novel species of the genus *Rubellimicrobium*.

Chemotaxonomic characterisation

The polar lipids of strain YIM 131921^T were found to be diphosphatidylglycerol, phosphatidylcholine, a glycolipid, phosphatidylglycerol, phospholipids and an unidentified aminolipid (Fig. S3). The presence of diphosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol and an aminolipid, and absence of phosphatidylethanolamine, support the conclusion that strain YIM 131921^T belongs to the genus *Rubellimicrobium* (Denner et al. 2006). Phosphatidylethanolamine, commonly present in Gram-negative bacteria, was not detected in lipid extracts of *R. thermophilum*, *R. mesophilum* and strain YIM 131921^T but is present in *R. roseum* and *R. aerolatum*. The phospholipids (PL_{1-2}) distinguish the new isolate from other species of the genus *Rubellimicrobium*. The major cellular fatty acids were identified as $C_{18:1}\omega7c$ (55.7%) and $C_{16:0}$ (22.1%), $C_{10:0}$ 3-OH and $C_{18:1}\omega7c$ 11-methyl were detected in minor amounts. The fatty acid composition was found to be similar to that of the closely related strain *R. roseum* YIM 48858^T, but the proportions of some fatty acids were different (Table S1). The predominant respiratory ubiquinone was found to be Q-10, which is the typical ubiquinone of the genus *Rubellimicrobium*.

In conclusion, based on polyphasic taxonomic approaches including phenotypic, molecular and chemotaxonomic features, strain YIM 131921^T is considered to represent a novel species of genus *Rubellimicrobium*, for which the name *Rubellimicrobium rubrum* sp. nov. is proposed.

Description of *Rubellimicrobium rubrum* sp. nov.

Rubellimicrobium rubrum (ru'brum. L. neut. adj. *rubrum* red, the colour of colonies of the type strain).

Table 1 Comparison of the phenotypic characteristics of strain YIM 131921^T and other related species of the genus *Rubellimicrobium*

Characteristic	1	2	3[b]	4[c]	5[d]
Isolation source	Lichen	Soil	Soil	Air	Biofilms
Colony colour	Bright red	Pink	Pink to light red	Pink	Red
Cell width (μm)	1.0	0.8–1.0[a]	0.4–0.7	0.8	0.6–0.8
Cell length (μm)	1.0	1.8–2.2[a]	1.6–3.4	1.6–3.6	2.0–4.0
Growth at (°C)	20–30	25–30	20–37	5–35	45–52
pH range for growth	6.5–12	6.5–9	7–11	6–7	6.5–8
Tolerance of NaCl (%)	0	0	0	0–1	2
Motile	Non-motile	Non-motile	Motile	Non-motile	Motile
Flagella	None	None[a]	None	None	One to three
Catalase/oxidase	+/−	+/−	w/−	+/+	w/+
Urease	+	−	−	−	+
Hydrolysis of					
Tween 80	+	−	+	−	−
Gelatin	−	−	+	−	−
Starch	−	−	+	−	−
Cellulose	−	−	+	ND	ND
Utilisation of					
D-Glucose	+	+	ND	−	+
Citric acid	+	−	−	−	−
D-Mannose	+	−	−	−	+
L-Histidine	−	W	+	−	−
D-Malic acid	−	+	−	−	+
D-Sorbitol	+	−	−	−	+
L-Fucose	−	W	W	−	+
L-Rhamnose	−	W	−	−	+
D-Cellobiose	−	+	−	ND	+
Predominant fatty acid	$C_{18:1}\omega7c$, $C_{16:0}$	$C_{18:1}\omega7c$, $C_{16:0}$	$C_{16:0}$, $C_{18:1}\omega7c$	$C_{18:1}\omega7c$, $C_{16:0}$	$C_{19:0}$cyclo$\omega8c$
Polar lipids	DPG, PG, PC, GL, AL, PL_{1-2}	DPG, PG, PC, GL, PE, PL, L	DPG, PC, AL	DPG, PG, PC, GL, PE	DPG, PG, PC, AL
G + C content mol%	66.6	69.1	72.3	69	69.4–70.2

Strains: 1, YIM 131921^T; 2, *Rubellimicrobium roseum* YIM 48858^T; 3 *Rubellimicrobium mesophilum* MSL-20^T; 4 *Rubellimicrobium aerolatum* 5715S-9^T; 5 *Rubellimicrobium thermophilum* DSM 16684^T

All strains are negative for nitrate reduction. +, Positive, utilised; –, negative, not utilised; w, weak; ND, not determined

[a]Data from Cao et al. (2010)

[b]Data from Dastager et al. (2008)

[c]Data from Weon et al. (2009)

[d]Data from Denner et al. (2006)

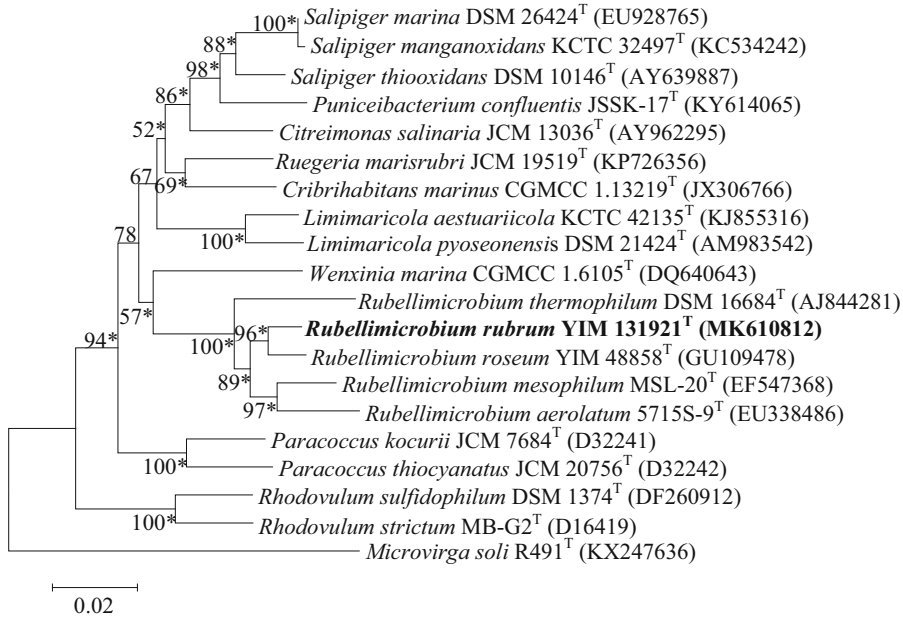

Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain YIM 131921^T and members of the genus *Rubellimicrobium*. Asterisks indicate branches that were also recovered using the maximum-parsimony and maximum-likelihood methods. Bootstrap values (expressed as percentages of 1000 replications) of above 50% are shown at branch points. *Microvirga soli* R491^T (KX247636) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position

Cells are Gram-stain negative, aerobic, non-motile, asporogenous and round (1 μm in diameter). Colonies are red coloured, dry and clearly convex after 5 days of growth on YIM 38 medium at 28 °C. Good growth occurs on YIM 38 medium, weak growth on R2A and does not grow on Tryptic Soy Agar. No growth in the presence of NaCl. Growth occurs at 20–30 °C (optimum 28 °C), pH 6.5–12.0 (optimum pH 7.0 ± 0.5). Degrades Tweens 20, 40, 60 and 80. Catalase positive and oxidase negative. Tests for H_2S production, nitrate reduction, milk coagulation and peptonisation, hydrolysis of gelatin, cellulose and starch are negative. The predominant respiratory ubiquinone is Q-10. The polar lipids of strain YIM 131921^T are diphosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, glycolipid, phospholipids and an unidentified aminolipid. Major cellular fatty acids (> 20%) are summed feature 8 ($C_{18:1}\omega7c$) and $C_{16:0}$. The DNA G + C content of the genomic DNA of the type strain is 66.6 mol%.

The type strain YIM 131921^T (= CGMCC 1.13958^T = NBRC 114054^T = KCTC 72461^T) was isolated from a *Physcia* sp. lichen collected from the South Bank Forest of the Baltic Sea. The GenBank accession numbers of the 16S rRNA gene sequence and draft genome sequence of strain YIM 131921^T are MK610812 and VDFU00000000, respectively.

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Author contributions L-QJ: performed the experiments and wrote the manuscript; X-YW: collected the lichen samples; KZ and G-DL: analysed the data; S-BS and Q-YL: performed the study; D-FA and LL: analysed the data; L-SW: identified the lichen samples; YJ: guided the experiments and revised the manuscript; C-LJ: designed the study.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical standards This article does not contain any studies with human participants and/or animals performed by any of the authors.

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