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ORIGINAL PAPER

Rubellimicrobium roseum sp. nov., a Gram-negative bacterium isolated from the forest soil sample

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Abstract A novel pink-coloured, non-spore-forming, non-motile, Gram-negative bacterium, designated YIM 48858^T, is described by using a polyphasic approach. The strain can grow at pH 6.5–9 (optimum at pH 7) and 25–30°C (optimum at 28°C). NaCl is not required for its growth. Positive for oxidase and catalase. Urease activity, nitrate reduction, starch and Tween 80 tests are negative reaction. 16S rRNA gene sequence similarity studies showed that strain YIM 48858^T is a member of the genus *Rubellimicrobium*, with similarities of 96.3, 95.7 and 95.5% to *Rubellimicrobium mesophilum* MSL-20^T, *Rubellimicrobium aerolatum* 5715S-9^T and *Rubellimicrobium thermophilum* DSM 16684^T, respectively. Q-10 was the predominant respiratory ubiquinone as in the other members of the genus *Rubellimicrobium*. The major polar lipids were diphosphatidylglycerol, phosphatidylcholine, phosphoglycolipid, glycolipid and the major fatty acids were C18:1 ω7c, C16:0 and C10:0 3-OH, which are very different from the valid published species. The DNA G + C content was 67.7 mol%. Both phylogenetic and chemotaxonomic evidence supports that YIM 48858^T is a novel species of the genus *Rubellimicrobium*, for which the name *Rubellimicrobium roseum* sp. nov. is proposed. The type strain is YIM 48858^T (=CCTCC AA 208029^T =KCTC 23202^T).

Keywords *Rubellimicrobium roseum* sp. nov. · Polyphasic taxonomy · 16S rRNA gene

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 48858^T is GU109478.

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Introduction

The genus *Rubellimicrobium* was established by Denner et al. (2006) for a thermophilic bacterium. To this day, the genus comprises three validly published species, *Rubellimicrobium thermophilum* (Denner et al. 2006), *Rubellimicrobium mesophilum* (Dastager et al. 2008) and *Rubellimicrobium aerolatum* (Weon et al. 2009). During our long-term

investigations on the microbial diversity of Southwest of China, a new bacterium YIM 48858^T was isolated. In the present study, the strain was proposed as a novel species of the genus *Rubellimicrobium*.

Materials and methods

Isolation and maintenance of organism

Soil samples were collected from tropical rainforest in Xishuangbanna, Yunnan Province, southwest of China. The soil sample was dried at 28°C for 1 week, then pretreated for 1 h at 80°C. Strain YIM 48858^T was isolated using plate dilution on Gauze 1 agar (Gauze et al. 1983) with nystatin 100 mg and Nalidixic Acid 25 mg (1 l). The incubation for isolation was performed at 28°C for 10 days. The strain was maintained on Medium YIM 38 (Jiang et al. 2007), at 4°C and a glycerol suspension (20%, v/v) at −80°C. Strain YIM 48858^T was deposited in the Collection Center of Typical Cultures, China (CCTCC) with type strain number CCTCC AA 208029^T and in the Korean Collection for Type Cultures (KCTC) as strain KCTC 23202^T.

Morphological, physiological and biochemical characteristics

Morphological, physiological and biochemical studies were performed with cells grown on YIM 38 at 28°C. Morphological characteristics of the strain were observed by light microscopy (model BH 2; Olympus) and by transmission electron microscopy (model H-800; Hitachi). Gram staining was carried out by using the standard Gram reaction (Doetsch 1981) combined with the KOH lysis test method (Gregersen 1978). Cell motility was confirmed by the presence of turbidity throughout the tube including semisolid medium (Leifson 1960). Growth was tested at 0, 4, 10, 15, 20, 25, 28, 30, 37, 45 and 55°C, and at different pH values (pH 4.0–10.0, in increment of 0.5 pH unit) using the buffer systems 0.1 M citric acid/0.1 M sodium citrate (pH 4.0–5.0), 0.1 M KH_2PO_4/0.1 M NaOH (pH 6.0–8.0) and 0.1 M $NaHCO_3$/0.1 M Na_2CO_3 (pH 9.0–10.0). Tolerance against salt were determined at various concentrations of NaCl (0, 1, 3, 5, 7, 10, 15, 20 and 25% w/v). Catalase activity was determined by production of bubbles after the addition of a drop of 3% H_2O_2. Oxidase activity was observed by oxidation of tetramethyl-*p*-phenylenediamine. Urease activity, milk coagulation and peptonization, H_2S production, nitrate reduction and hydrolysis of cellulose were tested as described by Smibert and Krieg (1994). Hydrolysis of Tweens 20, 40, 60 and 80, gelatin and starch were determined as described by Cowan and Steel (1965). Other enzyme activities were tested by using the API ZYM system (bioMérieux) according to the manu manufacturer's instructions. Utilization of sole carbon and sole nitrogen sources and energy were performed using the previous methods (Stevenson 1967; Tsukamura 1966). Three type strains were tested in the identical experiment, too.

Chemotaxonomy

Cell mass of strain YIM 48858^T for chemical and molecular systematic analyses was harvested after incubation at 28°C for 10 days in shaking-flasks (about 150 r.p.m.) of YIM 38 agar. Polar lipids were extracted, examined by two-dimensional TLC, and identified using the procedures described by Minnikin et al. (1984). The lipoquinone was investigated as described by Komagata and Suzuki (1987), using reversed-phase HPLC. For cellular fatty acid analysis, cell mass was harvested from R2A flasks on a rotary shaker at 150 r.p.m. for 3 days at 28°C. The fatty acids were extracted, methylated and analysed using the standard Microbial Identification System (MIDI) (Sasser 1990; Kämpfer and Kroppenstedt 1996). The fatty acids of the three type strains were examined using the same conditions.

Molecular analysis

Genomic DNA of strain YIM 48858^T for the determination of G + C content was extracted as described by Marmur (1961). The G + C content of the DNA was determined by reversed-phase HPLC method (Mesbah et al. 1989).

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were done as described by Li et al. (2007). The resulting 16S rRNA gene sequence was compared to sequences obtained from the EzTaxon server (http://www.eztaxon.org/) (Chun et al. 2007) to find the most closely related species. Multiple alignment with sequences of the most

closely related strains was carried out using CLUSTAL_X (Thompson et al. 1997). Phylogenetic analyses were performed using three tree-making algorithms, the neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Fitch 1971) methods. Distances were calculated by Kimura's two-parameter model (Kimura 1980). A phylogenetic dendrogram was constructed using MEGA version 3.1 (Kumar et al. 2004) with the neighbor-joining method. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by means of 1000 replicates (Felsenstein 1985). *Pleomorphomonas oryzae* F-7^T (AB159680) was used as an outgroup.

Results and discussion

Cells of strain YIM 48858^T were irregular rods, approximately 0.8–1 μm wide and 1.8–2.2 μm long after cultivation for 6 days at 28°C on YIM 38 agar. Cells were Gram-negative, non-motile and non-spore-forming, containing poly-β-hydroxybutyrate. Colonies were rose-coloured, circular and clearly protuberant and hard texture after growth on ISP 2 agar for 6 days at 28°C. Strain YIM 48858^T grew at 25–30°C and pH 6.5–9, but showed no growth when medium was supplemented with 1% NaCl. Physiological and biochemical characteristics of strain YIM 48858^T are given in Table 1 and in the species description.

The diagnostic phospholipids of strain YIM 48858^T were diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphoglycolipid (PGL) and glycolipid (GL). The predominant respiratory lipoquinone was Q-10, and contained a little Q-9. Major cellular fatty acids were C18:1 ω7c, C16:0 and C10:0 3-OH.

The genomic DNA G + C content of strain YIM 48858^T was 67.7 mol%.

An almost-complete 16S rRNA gene sequence (1466 bp) was determined in this study. An association supported by the three tree-making algorithms based on 16S rRNA gene sequence showed that strain YIM 48858^T belongs to the genus *Rubellimicrobium*, and the strain formed a distinct branch in the genus *Rubellimicrobium* supported by a high bootstrap value 94% in the neighbour-joining and 76% in the maximum-parsimony analysis (Fig. 1). This grouping was also supported by the maximum-likelihood analysis (not shown). 16S rRNA gene sequence similarity studies showed that strain YIM 48858^T is a member of the genus *Rubellimicrobium*, with similarities of 96.3, 95.7 and 95.5% to *Rubellimicrobium mesophilum* MSL-20^T, *Rubellimicrobium aerolatum* 5715S-9^T and *Rubellimicrobium thermophilum* DSM 16684^T, respectively.

Taxonomic conclusion

The predominant respiratory lipoquinone Q-10 of the strain YIM 48858^T and some physiological and biochemical characteristics (in the species description) were consistent with the genus *Rubellimicrobium*. However, strain YIM 48858^T could be differentiated obviously from the three related *Rubellimicrobium* species by following feature. Growth temperature range of strain YIM 48858^T is narrow (25–30°C) and living in soil. *Rubellimicrobium aerolatum* 5715S-9^T is from air. *Rubellimicrobium thermophilum* DSM 16684^T is thermophile. The strain can use citrate as sole carbon source, whereas the three species can not. DNA G + C content (67.7 mol%) of strain YIM 48858^T was lower for 1.3–4.7 mol% than other three species. For fatty acid analysis, content of C10:0 3-OH was present in strain YIM 48858^T, but absent in the three validly published species. All these results supported the proposal of strain YIM 48858^T as representing a novel species of the genus *Rubellimicrobium*, *Rubellimicrobium roseum* sp. nov.

Description of *Rubellimicrobium roseum* sp. nov

Rubellimicrobium roseum (ro'se.um. L. neut. adj. roseum rose-coloured, the colour of colony of the type strain).

Aerobic, non-motile, non-spore-forming, irregular rods (0.8–1 × 1.8–2.2 μm), Gram-negative bacterium contains poly-β-hydroxybutyrate. Colonies are pink-coloured, circular and clearly protuberant after growth on ISP 2 agar for 6 days at 28°C. Growth temperature occurs in very narrow range (25–30, optimally around 28°C) and pH range is 6.5–9 (optimum 7). No growth in the presence of NaCl. Oxidase- and catalase-positive. Degrades Tweens 20, 40 and 60. Urease activity, H_2S production, nitrate reduction, milk coagulation and peptonization, hydrolysis of gelatin, cellulose, starch and Tweens 80 tests

Table 1 Characteristics of strain YIM 48858^T and other species of the genus *Rubellimicrobium*

Characteristic	YIM 48858^T	*R. mesophilum* MSL-20^T*	*R. aerolatum* 5715S-9^T*	*R. thermophilum* DSM 16684^T*
Cell width (μm)	0.8–1	0.4–0.7	0.8	0.6–0.8
Cell length (μm)	1.8–2.2	1.6–3.4	1.6–3.6	2.0–4.0
Growth at (°C)	25–30	5–35	20–37	28–56
pH	6.5–9	6.5–11	6–7.5	6.5–8
Motile	Non-motile	Motile	Non-motile	Motile
Flagella	Non	Non	Non	One to three
Tolerance of NaCl (%)	0	<1	≤1	2
Catalase/oxidase	+/+	±	+/+	+/+
Urease	−	−	−	+
Nitrate reduction	−	+	+	+
Hydrolysis				
Gelatin	−	+	−	−
Tween 80	−	+	−	−
Starch	−	+	−	−
Utilization of				
L-histidine	+	+	−	−
L-proline	+	w	−	+
D-mannose	−	−	−	+
Citrate	w	−	−	−
L-fucose	w	w	−	+
Malic acid	+	−	−	+
D-xylose	−	−	−	+
Sucrose	−	+	−	+
Cellobiose	−	−	−	+
D-arabinose	w	w	−	+
D-sorbitol	−	−	−	+
L-malate	−	−	−	+
D-mannitol	−	w	−	−
D-ribose	−	−	−	+
L-rhamnose	w	−	−	+
Gluconate	+	+	−	−
Major fatty acids (>10%)	C10:0 3-OH, C16:0, C18:1 ω7c	C16:0, C18:1ω7c, 11-methyl C18:1 ω7c	C18:1ω7c, C16:0	C19:0 cyclo ω8c, C16:0
G + C content mol%	67.7	72.4	69	70

Note: + Positive, utilized; − negative, not utilized; *w* weak

* Data for three reference strains were gotten from identical experiments with the strain YIM 48858^T

are negative reaction. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase and α-glucosidase are positive. α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-fucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and β-glucosidase in the API ZYM system (bioMérieux) are negative. Utilized D-galactose, D-arabinose, L-rhamnose, fucose, methanol, sodium acetate, gluconate, citrate and malic acid as sole carbon source, and L-histidine, L-proline, L-serine,

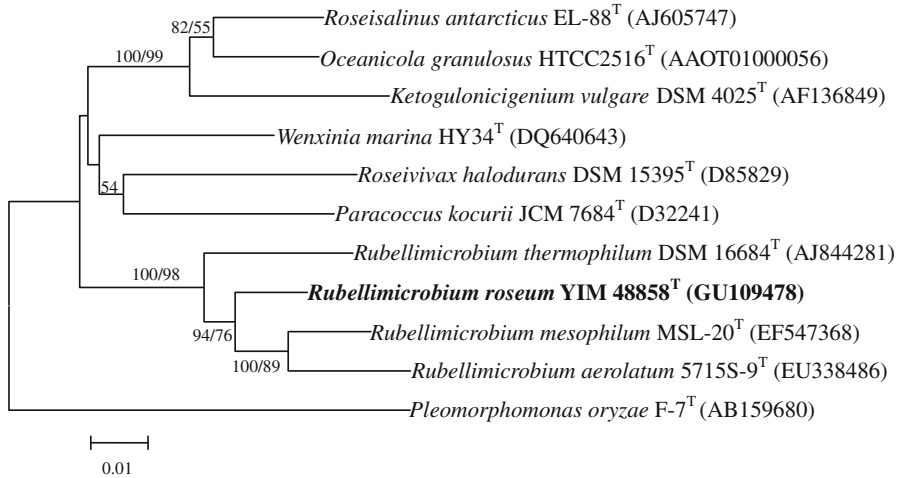

Fig. 1 Phylogenetic tree showing the phylogenetic position of strain YIM 48858^T within the radiation encompassing related genera of the family *Rhodobacteraceae*. The tree of the 16S rRNA gene sequences generated by the two methods (NJ and MP). Numbers (NJ/MP) on branch nodes are bootstrap percentages (1000 resamplings, only values over 50%/500 are given) for NJ and MP analyses. Bar, 1 substitution per 100 nt

tryptophan, phenylalanine, L-arginine, DL-methionine, L-valine and L-leucine as nitrogen source. Not utilized cellobiose, D-glucose, maltose, sucrose, lactose, D-raffinose, fructose, D-mannose, D-ribose, D-xylose, D-sorbitol, *myo*-inositol, D-mannitol, dextrin or glycerol as sole carbon source, and L-ornithine, L-alanine, xanthine, urea or lysine as nitrogen source. The phospholipids include diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphoglycolipid (PGL) and glycolipid (GL). The respiratory quinones comprise Q-10 (97.51%) and a little Q-9 (2.49%). The major cellular fatty acids are C18:1 ω7c, C16:0 and C10:0 3-OH. The DNA G + C content is 67.7 mol%.

The type strain is YIM 48858^T (=CCTCC AA 208029^T =KCTC 23202^T), isolated from a forest soil sample in Yunnan province, Southwest of China.

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