

Hymenobacter latericoloratus sp. nov. and *Hymenobacter luteus* sp. nov., isolated from freshwater sediment

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Abstract Two novel Gram-stain negative, non-motile, rod-shaped and aerobic bacterial strains, designated YIM 77920^T and YIM 77921^T, were isolated from freshwater sediment of Jiuxiang cave, a tourism cave located in Yiliang county, Yunnan province, south-west China. The 16S rRNA gene sequences of strains YIM 77920^T and YIM 77921^T exhibited sequence similarities of 96.59 and 96.66 % to *Hymenobacter xinjiangensis* X2-Y^T, respectively, and indicated that the two isolates belong to the genus *Hymenobacter*. The major fatty acids present in the two strains were identified as C_{16:1}ω5c, iso-C_{15:0} and Summed Feature 4 (C_{17:1} anteiso B/iso I). MK-7 was identified as the respiratory quinone component for both strains. The polar lipids profile of strain YIM 77920^T was

found to consist of phosphatidylethanolamine, four unidentified polar lipids, three unidentified aminophospholipids, two unidentified phospholipids and two unidentified aminolipids, while that of strain YIM 77921^T consisted of phosphatidylethanolamine, four unidentified polar lipids, two unidentified aminolipids, one unidentified phospholipid and four unidentified aminophospholipids. The DNA G+C contents of strains YIM 77920^T and YIM 77921^T were determined to be 57.5 and 59.6 mol%, respectively. DNA–DNA hybridization between them had a low value (56.55 %). Based on the morphological and physiological properties, and phylogenetic analyses, strains YIM 77920^T and YIM 77921^T are considered to represent two novel species of the genus *Hymenobacter*, for which the names *Hymenobacter latericoloratus* sp. nov. (type strain YIM 77920^T = JCM 30327^T = CCTCC AB 2012949^T) and *Hymenobacter luteus* sp. nov. (type strain YIM 77921^T = JCM 30328^T = CCTCC AB 2012947^T) are proposed.

Lan Liu and En-Min Zhou have contributed equally to this work.

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Introduction

The genus *Hymenobacter* is a member of the family *Cytophagaceae* in the phylum *Bacteroidetes*, which was first described by Hirsch et al. (1998) and then emended by Buczolits et al. (2006). The genus is differentiated from other members of the family by having high DNA G+C contents (55–65 mol%). Members of the genus *Hymenobacter* are Gram-negative, pink to red-pigmented and rod shaped. Species of this genus are well known to survive under unfavourable conditions such as desiccation and radiation, and can tolerate high levels of oil and heavy metals. At the time of writing, the genus is comprised of 31 validly named (<http://www.bacterio.net/hymenobacter.html>.2014.; Parte 2014), including the recently described species *Hymenobacter arcticus* (Chang et al. 2014), *Hymenobacter kanuolensis* (Su et al. 2014) and *Hymenobacter qilianensis* (Han et al. 2014). During an investigation into the biodiversity of microorganisms from freshwater sediment collected from a tourism cave (Jiuxiang) located in Yiliang country, Yunnan province, south-west China, two strains designated as YIM 77920^T and YIM 77921^T were isolated. The two strains showed many characters similar to the members of the genus *Hymenobacter*, such as Gram-negative, rod shaped and high DNA G+C contents (55–65 mol%); their 16S rRNA gene sequences similarities were found to below 97 % compared to other validly named species, which inspired us to identify their phenotypic, chemotaxonomic and molecular characters in order to classify them.

Materials and methods

Strains and culture conditions

Strains YIM 77920^T and YIM 77921^T were isolated on Reasoner's 2A agar (R2A) medium from the freshwater

sediment of Jiuxiang tourist cave (E103°22.791', N25°04.270') located in Yiliang county, Yunnan province, south-west China, by serial dilution followed by incubation for 7–10 days at 28 °C. Pure colonies of strains YIM 77920^T and YIM 77921^T were obtained by repeatedly re-streaking on R2A medium at 28 °C and routinely cultivated on the same medium. Both the strains were stored as glycerol suspensions (20 % v/v) in R2A broth at –80 °C for further use. Biomass for chemical and molecular studies was obtained by cultivation on R2A medium or broth at 28 °C. The type strain *Hymenobacter xinjiangensis* JCM 23206^T was obtained from Japan collection of microorganisms (JCM) and cultured under the same conditions as appropriate for specific comparative tests.

Morphological, physiological and biochemical characterization

Cell motility was studied by the development of turbidity throughout a tube of semi-solid medium (Leifson 1960). For morphological studies, strains YIM 77920^T and YIM 77921^T were observed using light microscopy (BH-2; Olympus) and scanning electron microscopy (QUANTA200; FEI). For scanning electron microscopy, harvested cells were suspended with sterilized water and were fixed with 3 % glutaraldehyde for two hours. Subsequently the fixed cells were dehydrated through a gradient series of alcohol (30, 50, 70, 90 and 100 %, respectively). The cell specimens were sputter coated with gold and observed with a scanning microscope. Gram staining was carried out by using the standard Gram reaction and was confirmed by using the KOH lysis test method (Cerny 1978). Strains YIM 77920^T and YIM 77921^T were examined for physiological and biochemical characteristics. Growth at different temperatures (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 °C) and NaCl tolerance at various concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3 and 5.0 % w/v) was determined using R2A medium. The pH range (4.0–10.0, at intervals of 1.0 pH unit) for growth was tested in R2A broth using the buffer system described by Xu et al. (2005). Growth on several media such as nutrient agar (NA), trypticase soy agar (TSA) and Luria–Bertani (LB) at 28 °C were also evaluated. Oxidation of carbon sources was tested using the Biolog GEN III MicroPlate according to the manufacturer's instructions. Oxidase activity was determined by the oxidation of tetramethyl-*p*-phenylenediamine (Kovacs

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1956). Catalase activity was detected by assessing the production of bubbles on addition of a drop of 3 % (v/v) H₂O₂. H₂S production, nitrate reduction and hydrolysis of cellulose, gelatin, starch, urea and Tweens (20, 40, 60 and 80) were performed as described by Gonzalez et al. (1978). Other enzyme activities and biochemical characteristics were additionally determined by using API ZYM and API 20 NE kits according to the manufacturer's instructions (bioMérieux, France). Susceptibility of strains YIM 77920^T, YIM 77921^T and *H. xinjiangensis* JCM 23206^T to antibiotics was investigated by the agar-diffusion method using 0.5 McFarland bacterial suspensions plated onto R2A agar medium for 2 days at 28 °C. The following antibiotics were tested: amikacin (30 µg), ampicillin (30 µg), bacitracin (10 IU), cefeprozone (10 µg), ceftriaxone (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (15 µg), erythromycin (15 µg), gentamicin (10 µg), norfloxacin (10 µg), ofloxacin (10 µg), penicillin (10 IU), piperacillin (100 µg), tetracycline (30 µg) and vancomycin (30 µg). The antimicrobial susceptibility was determined by measuring the zone of inhibition.

Chemotaxonomy

Chemotaxonomic characteristics of strains YIM 77920^T, YIM 77921^T and the reference strain *H. xinjiangensis* JCM 23206^T were observed using several standard methods under the same conditions. The respiratory quinones were extracted and purified as described by Collins et al. (1977) and analysed by HPLC (Kroppenstedt 1982). Polar lipids were extracted as described by Minnikin et al. (1979) and identified by two-dimensional TLC (Collins and Jones 1980). To standardise for physiological age, the biomass for fatty acid analysis was harvested on R2A broth at 28 °C when the population quantity was half of maximum value. Cellular fatty acids were prepared and analysed according to the standard protocol of the microbial identification system (Sherlock Version 6.1; MIDI database: TSBA6). The G+C content of the genomic DNAs were determined by using reversed-phase HPLC (Mesbah et al. 1989) with *Escherichia coli* DH5 α as the reference strain.

Molecular analysis and Molecular analysis and DNA–DNA hybridizations

Extraction of genomic DNAs and PCR amplification of the 16S rRNA genes were performed as described

by Li et al. (2007). The sequences obtained were compared with available 16S rRNA gene sequences of validly named species from the EzTaxon-e server (<http://eztaxon-e.ezbiocloud.net/>; Kim et al. 2012). Multiple alignments with sequences of the most closely related taxa and calculations of levels of sequence similarity were carried out using CLUSTAL_X program (Thompson et al. 1997). Phylogenetic analyses were performed by using three tree-making algorithms, the Neighbour-joining (Saitou and Nei 1987), Maximum-likelihood (Felsenstein 1981) and Maximum-parsimony (Fitch 1971) trees were constructed by using the MEGA version 5.0 software package (Tamura et al. 2011). Kimura's two parameter model was used to calculate evolutionary distance matrices of the phylogenetic trees (Kimura 1980). Bootstrap analysis was performed with 1,000 replications (Felsenstein 1985). *Flavobacterium aquatile* ATCC 11947^T (M62797) was used as an outgroup.

DNA–DNA relatedness between strains YIM 77920^T and YIM 77921^T was studied using the fluorometric micro-well method (Ezaki et al. 1989; Christensen et al. 2000; He et al. 2005), using eight replications for each hybridization reaction.

Results and discussion

Morphological, physiological and biochemical characterization

Cells of strains YIM 77920^T and YIM 77921^T were observed to be Gram-stain negative, aerobic and non-motile. Scanning electron microscopy results showed strain YIM 77920^T is rod-shaped, about 0.6–0.8 µm in width and 1.3–3.5 µm in length, and strain YIM 77921^T is also rod-shaped, about 0.6–0.8 µm in width and 1.4–3.3 µm in length (Fig. S1). Strains YIM 77920^T and YIM 77921^T shared common phenotypic characters but differed in producing brick-red and orange-red coloured colonies, respectively, on R2A medium. Both strains were found to grow on R2A and NA but not on TSA or LB, with a temperature range for growth of 5–35 °C and salt tolerance up to 0.5 % (w/v). The strains were found to differ in their pH ranges for growth. Strain YIM 77920^T was found to grow at a pH range of 5.0–8.0 with an optimum pH 7.0, while strain YIM 77921^T was found to have a pH range of 6.0–8.0 with an optimum pH 7.0. Both strains were found to be

positive for catalase, oxidase and hydrolysis of starch and Tweens (40, 80) but to be negative for H₂S production, nitrate reduction and hydrolysis of cellulose, Tweens (20, 60) and urea. Strain YIM 77920^T was found to be able to hydrolyse gelatine, while strain YIM 77921^T could not. For the antibiotics, strains YIM 77920^T and YIM 77921^T were found to be sensitive to amikacin, ampicillin, cefoperazone, ceftriaxone, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, norfloxacin, ofloxacin, piperacillin, tetracycline and vancomycin, while YIM 77920^T is sensitive to penicillin but strain YIM 77921^T is not. The major differential characteristics between strains YIM 77920^T, YIM 77921^T and the most closely related type strain *H. xinjiangensis* JCM 23206^T are shown in Table 1. The detailed physiological characteristics of strains YIM 77920^T and YIM 77921^T are given in the species description.

Chemotaxonomy

The respiratory quinone identified in strains YIM 77920^T and YIM 77921^T was MK-7. The major fatty acids (>10 %) of strains YIM 77920^T and YIM 77921^T were identified as C_{16:1}ω5c, iso-C_{15:0} and summed feature 4 (C17:1 anteiso B/iso I). The major fatty acid profiles of the two strains were consistent with that of *H. xinjiangensis* JCM 23206^T, but there were also some differences compared with the latter (Table 2). The polar lipids profile of strain YIM 77920^T was found to consist of phosphatidylethanolamine, four unidentified polar lipids, three unidentified aminophospholipids, two unidentified phospholipids and two unidentified aminolipids, while that of strain YIM 77921^T consisted of phosphatidylethanolamine, four unidentified polar lipids, two unidentified aminolipids, one unidentified phospholipid and four unidentified aminophospholipids. The results showed that both strains share similar profiles for quinone and fatty acids but they are different in their polar lipids. The G+C contents of strains YIM 77920^T and YIM 77921^T were determined to be 57.5 and 59.6 mol%, respectively, while that of *H. xinjiangensis* JCM 23206^T was reported to be 54 mol% (Zhang et al. 2007).

Phylogenetic analysis and DNA–DNA relatedness

The almost complete 16S rRNA gene sequences of strains YIM 77920^T (1,540 bp) and YIM 77921^T

Table 1 Different characteristics within strains YIM 77920^T, YIM 77921^T and *H. xinjiangensis* JCM 23206^T

Characteristics	YIM 77920 ^T	YIM 77921 ^T	JCM 23206 ^T
Pigmentation	Brick-red	Orange-red	Pink
Growth			
pH range	5–8	6–8	7–9
Hydrolysis of			
Gelatin	+	–	+
Tween 40	+	+	–
Activity of			
α-Chymotrypsin	–	–	+
Esterase (C4)	+	–	+
β-Fucosidase	+	–	–
α-Galactosidase	+	–	–
β-Galactosidase	+	–	–
β-Glucosidase	+	–	–
α-Mannosidase	+	–	–
Trypsin	–	+	+
Assimilation of			
Adipic acid	–	+	–
D-Glucose	–	–	+
D-Mannose	–	–	+
Malic acid	–	+	–
Maltose	–	–	+
Trisodium citrate	+	–	–
Oxidation of			
L-Alanine	–	+	–
D-Glucuronic acid	+	–	+
Glucuronamide	+	+	–
D-Maltose	–	–	+
1 % Sodium lactate	–	–	+
Tetrazolium Violet	+	+	–
D-Turanose	–	+	–
DNA G+C content (mol%)	57.5	59.6	54*

* Data from Zhang et al. (2007), other data were obtained from this study

+ positive, – negative

(1,545 bp) were obtained; the GenBank accession numbers are AB859260 and AB859261, respectively. Sequence analysis of the almost complete 16S rRNA gene sequences of the two strains using the EzTaxon-e server showed that they both had highest similarities to members of the genus *Hymenobacter* and that both strains are closely related to *H. xinjiangensis* X2-1^T

Table 2 Cellular fatty acid compositions of strains YIM 77920^T, YIM 77921^T and *Hymenobacter xinjiangensis* JCM 23206^T

Fatty acid (%)	YIM 77920 ^T	YIM 77921 ^T	JCM 23206 ^T
Saturated			
C _{12:0}	Tr	ND	ND
C _{14:0}	0.5	0.6	Tr
C _{15:0} 2-OH	ND	Tr	Tr
C _{16:0}	2.1	3.7	2.1
C _{16:0} 3OH	0.6	Tr	Tr
C _{17:0}	Tr	Tr	ND
C _{18:0} 10-methyl	ND	ND	Tr
Unsaturated			
C _{13:1} at 12-13	ND	ND	Tr
C _{16:1} ω5c	16.8	15.7	14.6
C _{17:1} ω6c	Tr	ND	ND
Branched			
Iso-C _{13:0}	Tr	ND	Tr
Iso-C _{15:0}	32.9	32.2	41.4
Iso-C _{15:1} G	Tr	Tr	0.8
Iso-C _{15:0} 3 OH	2.4	2.1	2.3
Anteiso-C _{15:0}	2.4	2.9	2.3
Iso-C _{16:0}	0.6	0.5	0.6
Iso-C _{16:1} H	ND	ND	Tr
Iso-C _{17:0}	6.7	6.9	6.1
Iso-C _{17:0} 3 OH	5.2	3.8	3.9
Iso-C _{18:0}	ND	ND	Tr
Summed feature 1	0.7	0.6	1.0
Summed feature 2	0.5	0.6	Tr
Summed feature 3	6.6	4.8	6.3
Summed feature 4	20.5	23.7	16.5

All data were obtained from this study. Biomass was harvested on R2A broth at 28 °C when the population quantity was half of maximum value. Values are percentage of total fatty acids

Fatty acids that represent <0.5 % in all strains were omitted

Summed feature 1: C_{13:0} 3OH/C_{15:1} i H; Summed feature 2: C_{16:1} iso I/C_{14:0} 3OH; Summed feature 3: C_{16:1} ω6c/C_{16:1} ω7c; Summed feature 4: C_{17:1} anteiso B/iso I

ND not detected, Tr trace, mean values less than 0.5 %

(96.66 and 96.59 % similarity), *Hymenobacter rigui* WPCB131^T (95.54 and 95.54 %) and *Hymenobacter perfusus* A1-12^T (95.42 and 95.28 %), and the two strains are 99.40 % similar to each other. The Neighbour-Joining phylogenetic tree based on 16S rRNA sequences showed the two strains form a clade with *H. xinjiangensis* X2-1^T well separated with other

members of the genus *Hymenobacter* (Fig. 1). The stabilities of trees were further confirmed by Maximum Likelihood and Maximum Parsimony methods (Fig. S3, S4). As the 16S rRNA gene sequence similarities of strains YIM 77920^T and YIM 77921^T with the type strains of members of the genus *Hymenobacter* were below 97 %, DNA–DNA hybridizations between them were not carried. However, the sequence similarity between YIM 77920^T and YIM 77921^T was 99.40 %, so DNA–DNA hybridization between strains YIM 77920^T and YIM 77921^T was carried out and the value was 56.55 ± 2.72 %, which is less than cut-off point (70 %) for the delineation of genomic species (Stackebrandt and Goebel 1994).

Therefore, on the basis of phylogenetic analysis, phenotypic and chemotaxonomic characteristics (Fig. 1, Tables 1 and 2, and Figs S1, S2), strains YIM 77920^T and YIM 77921^T should be affiliated to the genus *Hymenobacter*. However, strain YIM 77920^T and YIM 77921^T could be distinguished from the type strain of *H. xinjiangensis* JCM 23206^T by differences in several properties, such as colony colour, pH range for growth, hydrolysis of gelatin and Tween 40, G+C content, oxidation of carbon sources, as well as the proportions of some fatty acids. In addition, analyses of 16S rRNA gene sequences, DNA–DNA relatedness values and their different properties, notably colony colour, enzyme activities, oxidation of carbon sources, assimilation of adipic acid, malic acid and trisodium citrate, indicates that strains YIM 77920^T and YIM 77921^T represent two novel species within the genus *Hymenobacter*, for which the names *Hymenobacter latericoloratus* sp. nov and *Hymenobacter luteus* sp. nov. are proposed, respectively.

Description of *Hymenobacter latericoloratus* sp. nov

Hymenobacter latericoloratus (la.te.ri.co.lo.ra'tus. L. n. latus brick; L. part. adj. coloratus coloured; N.L. part. adj. latericoloratus brick-coloured).

Cells are Gram-negative, aerobic and non-motile. Colonies are brick-red on R2A medium. Growth occurs on NA but not on TSA or LB. Has a temperature range for growth of 5–35 °C, with optimum at 25–30 °C. Cells can tolerate salt concentrations up to 0.5 % (w/v). pH range for growth is from 5.0 to 8.0, with optimum at pH 7.0. Positive for

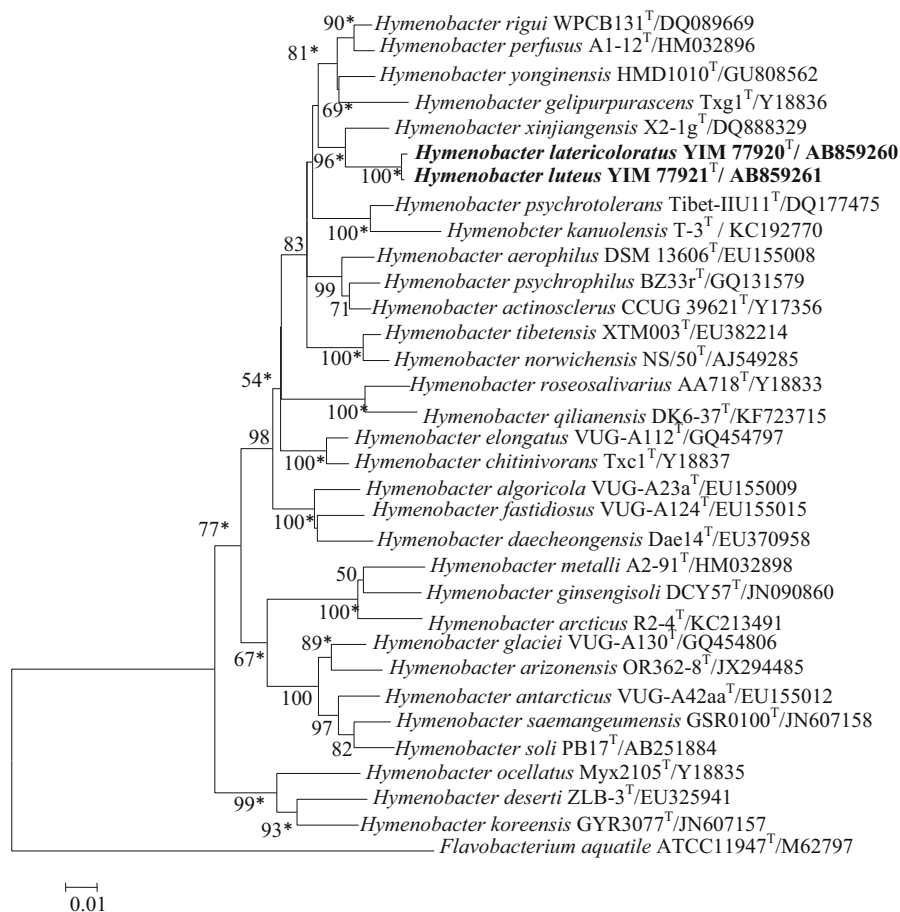


Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strains YIM 77920^T, YIM 77921^T and their closest relatives. Bootstrap values (expressed as percentages of 1,000 replications) of above 50 % are shown at the branch points. Asterisks denote nodes that were also

catalase, oxidase and hydrolysis of gelatine, starch and Tweens (40, 80) whereas negative for H₂S production, nitrate reduction and hydrolysis of cellulose, Tweens (20, 60) and urea. According to the API ZYM system, positive for *N*-acetyl- β -glucosaminidase, acidic phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), β -fucosidase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, leucine arylamidase, lipase (C14), α -mannosidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase; negative for α -chymotrypsin, β -glucuronidase and trypsin. In the API 20NE system, assimilation of *L*-arabinose and trisodium citrate, hydrolysis of aesculin and gelatine are positive, while arginine dihydrolase, galactosidase, glucose fermentation, indole production, nitrate reduction, urease and

recovered using the maximum-parsimony and maximum-likelihood methods. The sequence of *Flavobacterium aquatile* ATCC 11947^T was used as the outgroup. Bar 0.01, represents substitutions per nucleotide position

assimilation of *N*-acetylglucosamine, adipic acid, capric acid, *D*-glucose, malic acid, maltose, *D*-mannose, *D*-mannitol, phenylacetic acid and potassium gluconate are negative. Positive reactions from the GEN III system for oxidation of aztreonam, citric acid, *D*-fucose, *L*-fucose, gelatin, glucuronamide, *D*-glucuronic acid, *L*-glutamic acid, glycyl-*L*-proline, *L*-histidine, lithium chloride, nalidixic acid, tolerance of pH 6 and pH 5, *L*-serine, tetrazolium violet, Tween 40 and vancomycin; negative reactions from the GEN III system for oxidation of others. The major fatty acids are C_{16:1 ω 5c}, iso-C_{15:0}, and Summed Feature 4 (C_{17:1} anteiso B/iso I). The polar lipids consist of phosphatidylethanolamine, four unidentified polar lipids, three unidentified aminophospholipids, two unidentified phospholipids and two unidentified aminolipids. The

respiratory quinone is MK-7. The G+C content of the DNA of the type strain is 57.5 mol%.

The type strain YIM 77920^T (=JCM 30327^T = CCTCC AB 2012949^T) was isolated from the fresh-water sediment of Jiuxiang tourist cave located in Yiliang country, Yunnan province, south-west China. The GenBank accession number for the 16S rRNA gene sequence of strain YIM 77920^T is AB859260.

Description of *Hymenobacter luteus* sp. nov

Hymenobacter luteus (lu.te'us. L. masc. adj. *luteus* orange-yellow).

Cells are Gram-negative, aerobic, non-motile and produce orange-red coloured colonies on R2A medium. Growth occurs on NA but not on TSA and LB. The temperature range for growth is 5–35 °C, with optimum at 25–30 °C. Cells can tolerate salt concentrations up to 0.5 % (w/v). The pH range for growth is pH 6.0–8.0, with optimum at pH 7.0. Positive for catalase, oxidase and hydrolysis of starch and Tweens (40, 80) whereas negative for H₂S production, nitrate reduction and hydrolysis of cellulose, gelatine, Tweens (20, 60) and urea. According to the API ZYM system, positive for *N*-acetyl- β -glucosaminidase, acidic phosphatase, alkaline phosphatase, cystine arylamidase, esterase lipase (C8), α -glucosidase, leucine arylamidase, lipase (C14), naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase; negative for α -chymotrypsin, esterase (C4), β -fucosidase, α -galactosidase, β -galactosidase, β -glucosidase, β -glucuronidase and α -mannosidase. In the API 20NE system, assimilation of adipic acid, L-arabinose and malic acid, hydrolysis of aesculin are positive, while arginine dihydrolase, hydrolysis of gelatine, glucose fermentation, indole production, galactosidase, nitrate reduction, urease and assimilation of *N*-acetylglucosamine, capric acid, D-glucose, maltose, D-mannose, D-mannitol, phenylacetic acid, potassium gluconate and trisodium citrate are negative. Positive reactions from the GEN III system for oxidation of *N*-acetyl- β -dmannosamine, L-alanine, L-aspartic acid, aztreonam, bromo-succinic acid, L-fucose, D-galactose, gentiobiose, D-glucose-6-phosphate, glucuronamide, L-glutamic acid, glycyl-L-proline, L-histidine, D-malic acid, L-malic acid, D-lactic acid methyl ester, methyl pyruvate, tolerance of pH 6, quinic acid, D-saccharic acid, L-serine, tetrazolium violet, Tween 40, D-turanose and vancomycin; negative reactions from the

GEN III system for oxidation of others. The major fatty acids are C_{16:1} ω 5c, iso-C_{15:0}, and summed feature 4 (C_{17:1} anteiso B/iso D). The polar lipids consist of phosphatidylethanolamine, four unidentified polar lipids, two unidentified aminolipids, one unidentified phospholipid and four unidentified aminophospholipids. The respiratory quinone is MK-7. The G+C content of the DNA of the type strain is 59.6 mol%.

The type strain YIM 77921^T = JCM 30328^T = CCTCC AB 2012947^T was isolated from the fresh-water sediment of Jiuxiang tourist cave located in Yiliang country, Yunnan province, south-west China. The GenBank accession number for the 16S rRNA gene sequence of strain YIM 77921^T is AB859261.

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