Crenalkalicoccus roseus gen. nov., sp. nov., a thermophilic bacterium isolated from alkaline hot springs

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Two closely related thermophilic bacterial strains, designated YIM 78023^T and YIM 78058, were isolated from samples collected from two alkaline hot springs in Tengchong county, Yunnan province, south-west China. The novel isolates were Gram-stain-negative, non-motile, aerobic ovoid- to coccoid-shaped and non-spore-forming. Strain YIM 78023^T grew at 20–60 °C and pH 6.0–9.0 with optimal growth observed at 40–50 °C and pH 8.0, while strain YIM 78058 grew at 25–60 °C and pH 6.0–10.0 with optimal growth at 45–50 °C and pH 8.0. Phylogenetic analysis based on 16S rRNA gene sequences affiliated these two isolates within the family *Acetobacteraceae* with high sequence similarities to members of the genera *Roseomonas* and *Belnapia* (all sequence similarities <94.5 %). In addition to the above two genera, these strains also clustered with the genera *Craurococcus* and *Paracraurococcus* (having sequence similarities <93.3 %) in the phylogenetic tree, but with a distinct lineage within the family *Acetobacteraceae*. The major ubiquinone was Q-10 and the major fatty acids observed were $C_{18:1}\omega7c$, summed feature 4 and $C_{16:0}$. The genomic DNA G+C contents observed for strains YIM 78023^T and YIM 78058 were 74.3 and 74.0 mol%, respectively. Morphological, phylogenetic and chemotaxonomic results suggest that strains YIM 78023^T and YIM 78058 are representatives of a novel species of a new genus within the family *Acetobacteraceae*, for which the name *Crenalkalicoccus roseus* gen. nov., sp. nov. is proposed. The type strain of *Crenalkalicoccus roseus* is YIM 78023^T (=JCM 19657^T=KACC 17825^T).

The family *Acetobacteraceae*, proposed by Gillis & De Ley (1980), belongs to the class *Alphaproteobacteria*. This family was separated from the common ancestor of the class *Alphaproteobacteria* for a group of acetic acid bacteria adapted to acidic environments (Kishimoto *et al.*, 1995). Members of the family *Acetobacteraceae* play a role in food industries including production of vinegar (Entani *et al.*, 1985; Nanda *et al.*, 2001), alcohol (Adachi *et al.*, 2001; Takemura *et al.*, 1993), foodstuffs (Du Toit & Lambrechts, 2002; Gosselé *et al.*, 1984) and beverages (Cancalon & Parish, 1995; Moore *et al.*, 2002). It is therefore imperative to look for novel bacteria of the family *Acetobacteraceae* for development of food and related industries. At the time of writing, the family *Acetobacteraceae* comprises 33 genera (http://www.bacterio.cict.fr/) with diverse characteristics, and isolated from different environments including soil (Margesin & Zhang, 2013), plants (Ramírez-Bahena *et al.*, 2013), purified water (Eder *et al.*, 2015), a marine cyanobacterial mat (Yurkov *et al.*, 1994), a soda lake (Boldareva *et al.*, 2009), hot springs (Alarico *et al.*, 2002; Albuquerque *et al.*, 2008; Dong *et al.*, 2014), blood (Han *et al.*, 2003), acidic habitats (Cavalcante & Dobereiner, 1988) and alcohol-containing habitats (Takemura *et al.*, 1993). Members of the family *Acetobacteraceae* are characterized as obligately aerobic with oxygen as the terminal electron acceptor for their

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The GenBank/EMBL/DDJB accession numbers for the 16S rRNA gene sequences of strains YIM 78023^T and YIM 78058 are KJ361470 and KJ361471, respectively.

Four figures are available with the online Supplementary Material.

metabolism. Q-10 is characterized as the predominant ubiquinone for the members of the family, although Q-9 is reported for some members.

During our study on thermophilic bacteria from hot springs in Tengchong county, Yunnan province, south-west China, strains YIM 78023^T and YIM 78058 were isolated from samples of two alkaline hot springs. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strains YIM 78023^T and YIM 78058 clustered with members of the genera *Craurococcus*, *Roseomonas*, *Paracraurococcus*, *Belnapia* and *Humitalea* within the family *Acetobacteraceae*, but were found to have high sequence divergence values with the above related phylogenetic neighbours. The aim of the present study was to determine the taxonomic status of these two isolates by using a polyphasic approach.

Geothermally heated sediments were collected from two hot springs Hehua F (pH 8.5, 57 °C, 24.9636°N, 98.36154°E) and Hehua I (pH 8.0, 60 °C, 24.9758°N, 98.37304°E). Two grams each of the sediment samples was suspended into conical flasks containing 18 ml sterile hot spring water and several glass beads and kept in a orbital shaker (45 °C, 200 r.p.m., 1 h). The suspensions were diluted 10- and 100-fold with sterile water and 0.2 ml aliquots were spread on R2A (Becton, Dickinson and Company) agar plates adjusted to pH 8.0. The isolation plates were incubated at 45, 50 and 55 °C for 7 days. From among the isolates, strain YIM 78023^T from Hehua F hot spring and strain YIM 78058 from Hehua I hot spring samples were selected and purified on modified T5 (Ming *et al.*, 2014) medium, with pH 8.0 and 50 °C as the basal growth conditions unless otherwise stated. The pure cultures were preserved as glycerol suspensions (20 %, v/v) at −80 °C.

For phenotypic characterization, strains YIM 78023^T and YIM 78058 were cultured on R2A agar, modified T5 and ISP 2 (Shirling & Gottlieb, 1966) media. Growth and microscopic morphological characteristics were tested by light microscopy (model BH2; Olympus) and scanning electron microscopy (ESEM-TMP). Samples for scanning electron microscopy were prepared as described by Ming *et al.* (2014). The Gram reaction was tested by the non-staining method using 3 % KOH (Buck, 1982). Cell motility was studied depending on turbidity development in a tube containing semi-solid medium (Leifson, 1960). Growth at different temperatures was tested at 4, 15, 28, 30, 37, 42, 45, 50, 55, 60 and 65 °C. Salt tolerance limit for growth was observed with 0–5 % (w/v) NaCl in ISP2 agar (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 %, w/v, respectively). The pH range for growth was tested from pH 4.0 to 10.0 (at intervals of 1.0 pH units) for 7 days in ISP 2 broth, using the buffer system as described by Nie *et al.* (2012a). The ability to utilize single carbon sources was tested with Pridham and Gottlieb's basal mineral salts medium supplemented with test carbon sources (Pridham & Gottlieb, 1966). Nitrogen source utilization was observed in a basal liquid medium according to Nie *et al.* (2012b). Determination of oxidase activity was carried out using 1 % (w/v) tetramethyl-*p*-phenylenediamine as described by Kovacs (1956). Catalase activity was tested using 3 % (w/v) H_2O_2 by assessing bubble production as the positive result. Tweens (20, 40, 60 and 80) degradation, H_2S production and nitrate reduction, hydrolysis of gelatin, starch and cellulose, activity of urease, and milk peptonization and coagulation were observed as previously described (Gonzalez *et al.*, 1978; Mac Faddin, 1976; Smibert, 1994). API strips (API 20NE, API ZYM and API 50CHB) were used to determine metabolic properties and some enzyme activities according to the instructions of the manufacturer (bioMérieux).

Cells of strains YIM 78023^T and YIM 78058 were Gram-stain-negative, non-motile, aerobic and non-spore-forming. Colonies were irregular, opaque, convex and rose-red. Cells of strains YIM 78023^T and YIM 78058 occurred either singly, in pairs or in clusters. Cells of YIM 78023^T agglutinated with each other on ISP 2, R2A and T5 agar plates and possessed a deeper rose-red colour compared with strain YIM 78058. Cells of strain YIM 78023^T were coccoid in shape while those of strain YIM 78058 were ovoid, with diameter ranging between 0.6 and 0.9 μm (Fig. S1, available in the online Supplementary Material). Strain YIM 78023^T grew at 20–60 °C and pH 6.0–9.0 with optimal growth observed at 40–50 °C and pH 8.0, while strain YIM 78058 grew at 25–60 °C and pH 6.0–10.0 with optimal growth at 45–50 °C and pH 8.0. Both strains could tolerate salt up to a concentration of 2.5 % (w/v) NaCl (optimum growth observed with 0–2.0 %, w/v, NaCl). The strains were positive for oxidase, catalase and urease activities, milk coagulation and peptonization, and nitrate reduction, but were negative for H_2S production. They were able to hydrolyse aesculin, gelatin, and Tweens 20 and 40, but not xylan, cellulose, starch, or Tweens 60 and 80. Phenotypic properties useful for distinguishing strains YIM 78023^T and YIM 78058 from related genera of the family *Acetobacteraceae* are listed in Table 1. The detailed physiological characteristics of strains YIM 78023^T and YIM 78058 are given in the genus and species descriptions below.

Biomass for chemical and molecular studies of strains YIM 78023^T and YIM 78058 was obtained by cultivation on ISP 2 medium for 3 days. Cells were checked for purity by spreading on ISP 2 agar plates, harvested and washed twice with distilled water by centrifugation and freeze-dried.

Respiratory quinones were extracted from lyophilized cells (Collins *et al.*, 1977; Minnikin *et al.*, 1984), purified and analysed by the HPLC method (Hu *et al.*, 2001; Tamaoka *et al.*, 1983). Polar lipids were extracted, separated and examined by two-dimensional TLC on silica gel G 60 plates (Merck); the profile of lipids was identified using previously described procedures (Collins & Jones, 1980; Minnikin *et al.*, 1979). Cells for cellular fatty acids analysis were obtained by culturing strains YIM 78023^T and YIM 78058 in tryptic soy agar (TSA) for 3 days. Cellular fatty acids were extracted, methylated and analysed following the instructions of the Sherlock Microbial Identification System

Table 1. Key taxonomic characteristics of strains YIM 78023^T and YIM 78058 and their closely related genera of the family *Acetobacteraceae*

Taxa: 1, strain YIM 78023^T; 2, strain YIM 78058; 3, *Craurococcus*; 4, *Roseomonas*; 5, *Paracraurococcus*; 6, *Belnapia*; 7, *Humitalea*; 8, *Acetobacter*. +, Positive; −, negative; V, variable; ND, not done; NA, not analysis; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine. Data for strains YIM 78023^T and YIM 78058 were obtained during this study under identical growth conditions; data for related neighbours are from the literature (Albuquerque *et al.*, 2008; Cleenwerck *et al.*, 2002; Han *et al.*, 2003; Jin *et al.*, 2013; Lisdiyanti *et al.*, 2002; Ramírez-Bahena *et al.*, 2013; Reddy *et al.*, 2006; Rihs *et al.*, 1993; Saitoh & Nishimura, 1996; Saitoh *et al.*, 1998; da Costa *et al.*, 2006).

Characteristic	1	2	3	4	5	6	7	8
pH for growth	6.0–9.0	6.0–10.0	7.2–8.0	ND	ND	6.0–8.0	7.0	3.0–9.0
Optimum pH for growth	8.0	8.0	7.5	ND	6.6–6.8	7.0	7.0	4.0–6.0
Growth range (°C)	20–60	25–60	20–37	20–42	20–42	15–30	1–30	≤42
Optimum temperature (°C)	45–50	45–50	28–32	35	30–32	25	25	28–30
Cell shape	Cocci	Ovoid	Cocci	Cocci	Cocci	Cocci	Rod	Rod
Colony colour	Rose-red	Rose-red	Pink	Pink	Red	Red	Pale pink	Pink
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	+	V	+	+	+	−
H_2S production	−	−	+	−	+	−	−	−
Nitrate reduction	+	+	+	−	+	+	−	ND
Gelatin liquefaction	+	+	−	+	−	−	−	−
Starch degradation	−	−	+	−	+	−	ND	−
Utilization of:								
L-Arabinose	−	−	+	+	+	−	+	V
D-Fructose	−	−	+	+	+	−	ND	−
Glycerol	+	−	+	+	+	−	−	+
Inositol	+	+	−	−	−	−	−	ND
Lactose	+	+	−	−	−	+	−	+
D-Mannose	−	−	+	+	+	−	+	+
D-Mannitol	+	+	−	+	−	−	ND	+
D-Sorbitol	+	+	−	−	−	−	−	−
Sucrose	+	+	−	−	−	−	−	−
Sorbose	−	−	ND	−	+	+	−	−
L-Ornithine	+	+	+	+	+	−	−	ND
Cystine	+	+	+	−	+	−	−	ND
Acid production from:								
Arabinose	−	−	+	+	+	+	ND	V
Xylose	−	−	+	−	+	−	ND	V
Glucose	−	−	+	+	+	−	ND	V
Mannose	−	−	+	+	+	−	ND	V
Quinone system	Q-10	Q-10	Q-10	Q-10	Q-10	Q-9	ND	Q-9
Predominant polar lipids	DPG, PG, PE, PC	DPG, PG, PE, PC	NA	DPG, PG, PE, PC	NA	DPG, PG, PC	DPG, PG, PE, PC	
Predominant fatty acids	$C_{18:1}\omega7c$, summed feature 4, $C_{16:0}$	$C_{18:1}\omega7c$, summed feature 4, $C_{16:0}$	$C_{18:1}\omega7c$, $C_{16:1}\omega7c$, $C_{16:0}$, $C_{18:1}$ 2-OH	$C_{18:1}\omega7c$, $C_{16:0}$, $C_{18:1}$ 2-OH	$C_{18:1}\omega7c$	$C_{18:1}\omega7c$, $C_{16:0}$	$C_{18:1}\omega7c$, summed feature 3	$C_{18:1}\omega7c$
G+C content (mol%)	74.3	74.0	70.5	65–71	70.3–71.0	72.1–75	68.2	52–60

(MIDI) version 6.1 and the TSBA6 database (Sasser, 1990). The genomic DNA G+C contents were determined by HPLC after enzymatic degradation (Mesbah *et al.*, 1989) using *E.scherichia coli* strain DH5α as the reference.

The major respiratory quinone observed for both strains was ubiquinone Q-10. The polar lipid profiles of the two strains comprised diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphtidylcholine, one unidentified aminolipid, two unidentified polar lipids and two unidentified lipids (Fig. S2). The major cellular fatty acids detected for strain YIM 78023^T were $C_{18:1}\omega7c$ (49.9 %), summed feature 4 (iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B) (18.9 %), $C_{16:0}$ (10.7 %), $C_{18:0}$ (7.3 %) and $C_{19:0}$ cyclo $\omega8c$ (7.7 %), while for strain YIM 78058 they were $C_{18:1}\omega7c$ (51.0 %), summed feature 4 (iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B) (18.0 %), $C_{16:0}$ (12.1 %), $C_{18:0}$ (7.2 %) and $C_{19:0}$ cyclo $\omega8c$ (6.5 %). Detailed fatty acid profiles of strains YIM 78023^T and YIM 78058 are given in Table S1. The genomic DNA G+C content of strains YIM 78023^T and YIM 78058 were 74.3 and 74.0 mol%, respectively.

Genomic DNA of strains YIM 78023^T and YIM 78058 was extracted, and the 16S rRNA genes were amplified and sequenced by using the universal bacterial primers, as described by Li *et al.* (2007). The amplicons were purified using a PCR purification kit (Sangon Biotech). The almost-complete 16S rRNA genes obtained were assembled via the SeqMan program (DNAStar software). The phylogenetic relationships of strains YIM 78023^T and YIM 78058 were determined after BLAST searches of the 16S rRNA gene sequences in NCBI (Altschul *et al.*, 1990) and the EzTaxon-e server databases (Kim *et al.*, 2012), and the sequences of closedly related strains were retrieved. Multiple alignments were performed with the CLUSTAL X software package (Thompson *et al.*, 1997). Evolutionary distances were calculated by the Kimura two-parameter model (Kimura, 1980, 1984). Phylogenetic trees were reconstructed with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) tree-making algorithms. Phylogenetic and molecular evolutionary analyses were carried out by using the software packages MEGA version 6.0 (Tamura *et al.*, 2013) and PHYML (Guindon & Gascuel, 2003). The topology of each tree with 1000 replications was evaluated by bootstrap analysis (Felsenstein, 1985).

Almost-complete 16S rRNA gene sequences were determined for both strain YIM 78023^T (1514 nt; GenBank accession number KJ361470) and YIM 78058 (1527 nt; GenBank accession number KJ361471). Comparison of the sequences with the corresponding 16S rRNA gene sequences from the GenBank/EMBL/DDBJ and EzTaxon-e server databases clearly indicated that strains YIM 78023^T and YIM 78058 were related to members of the family *Acetobacteraceae*. Therefore, 16S rRNA gene sequences of 33 members of closely related genera of the family *Acetobacteraceae* were retrieved from the NCBI database for phylogenetic analysis. Phylogenetic trees based on the 16S rRNA gene sequence with *Methylobacterium organophilum* JCM 2833^T (D32226) as outgroup showed that strains YIM 78023^T and YIM 78058 formed a monophyletic lineage (99.9 % similarity) by themselves, but a distinct lineage in the family *Acetobacteraceae* (Fig. 1). The two strains shared high similarities to members of the genera *Roseomonas*, *Belnapia* (sequence similarities below 94.5 %), *Craurococcus* and *Paracraurococcus* (sequence similarities below 93.3 %), and were found to cluster with the above genera. A similar distinct lineage was also found in the maximum-parsimony and maximum-likelihood phylogenetic trees (Figs. S3 and S4).

DNA–DNA hybridizations between strains YIM 78023^T and YIM 78058 were performed at the optimal hybridization temperature (48 °C) using the fluorometric micro-well method (Christensen *et al.*, 2000; Ezaki *et al.*, 1989). The DNA probes labelled with photobiotin (A1935; Sigma) and 96-well microdilution plates (Greiner BioOne) were prepared, and each reaction was set with eight replications. Mean DNA–DNA relatedness between strains YIM 78023^T and YIM 78058 was 88.2 ± 2.3 %, indicating that the two strains belong to the same species, which is consistent with their homogeneous phylogenetic, physiological and biochemical characteristics.

In addition to the observations based on phylogenetic analyses with respect to high sequence divergence values from members of the family *Acetobacteraceae*, the novel strains could be easily distinguished based on several phenotypic characteristics as indicated in Table 1. For example, the presence of summed feature 4 (iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B) as a major fatty acid has not been reported in other genera of the family *Acetobacteraceae*. The genomic DNA G+C contents of strains YIM 78023^T and YIM 78058 are higher than for most members of the family *Acetobacteraceae* (55–70 mol%), but similar to the genera *Belnapia* (72.1–75 mol%) and *Sediminicoccus* (73.9 mol%). Growth conditions for strains YIM 78023^T and YIM 78058 (optimum 45–50 °C and pH 8.0) were different from other members of the family, except for the genera *Elioraea* (Albuquerque *et al.*, 2008) and *Rubritepida*, which were isolated from a similar geographical environment. Based on the results from phylogenetic analysis, and morphological and chemotaxonomic characterization, we suggest that strains YIM 78023^T and YIM 78058 should be considered to represent a novel species of a new genus within the family *Acetobacteraceae*, for which the name *Crenalkalicoccus roseus* gen. nov., sp. nov. is proposed.

Description of *Crenalkalicoccus* gen. nov.

Crenalkalicoccus (Cren.al.ka.li.coc'cus. Gr. n. *krene* a fountain, spring; Arab. n. *alqali* potash, used to refer to alkaline compounds; N.L. masc. n. *coccus* from Gr. masc. n. *kokkos* grain or berry; N.L. masc. n. *Crenalkalicoccus* an ovoid- or coccoid-shaped bacterium isolated from alkaline hot spring).

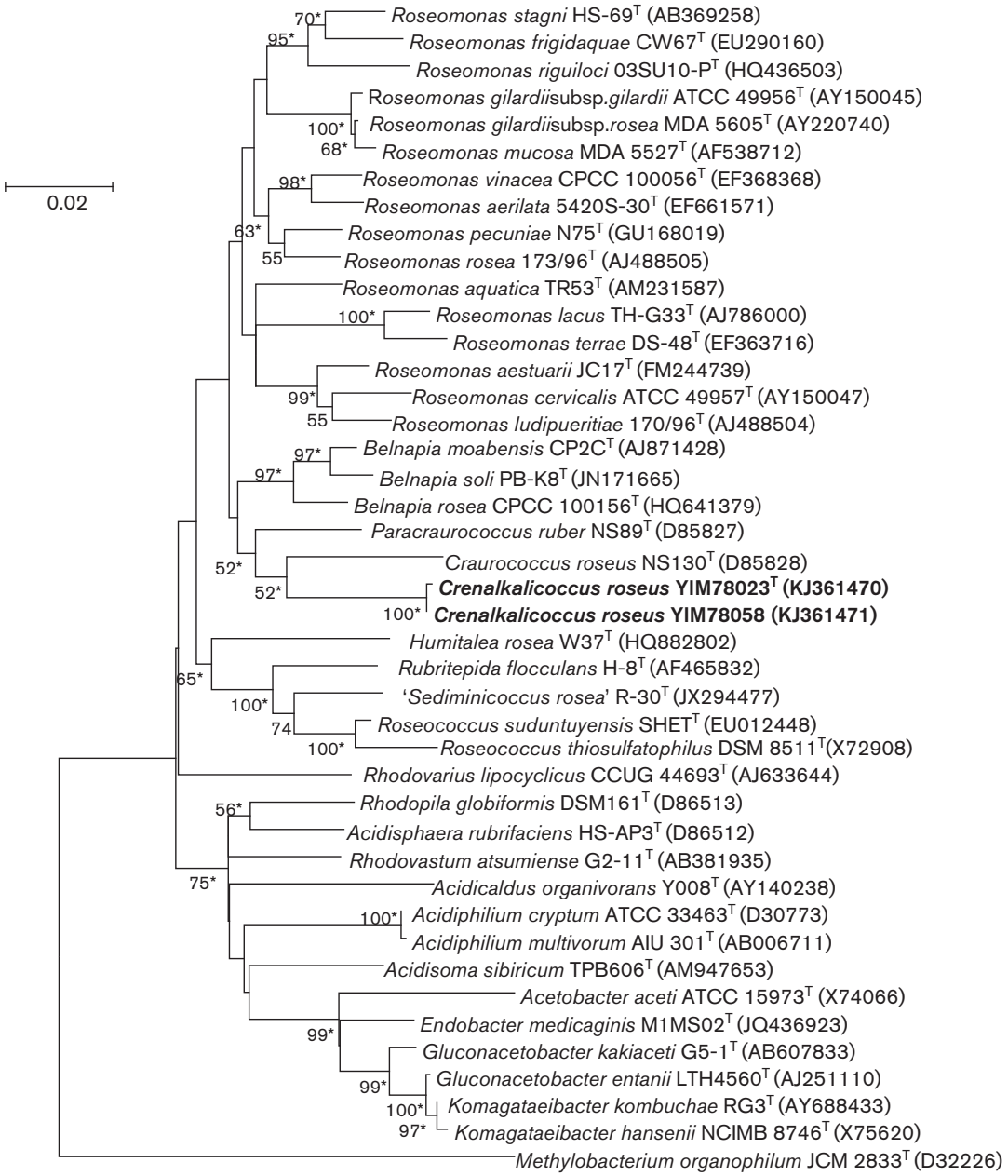

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strains YIM 78023^T and YIM 78058 and members of the family *Acetobacteraceae.* Bootstrap percentages ($\geq$ 50 %) based on 1000 resamplings are given at the nodes. Asterisks indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony and maximum-likelihood methods. Bar, 0.02 substitutions per nucleotide position. The 16S rRNA gene sequence of *Methylobacterium organophilum* JCM 2833^T (D32226) was used as outgroup.

Cells are Gram-stain-negative, non-motile, aerobic, ovoid- or coccoid- shaped and non-spore-forming. Colonies are irregular, opaque, convex and rose-red. Cells occur either in singly, in pairs or in cluster on ISP 2, R2A and T5 agar media. No pigments are produced. Positive for oxidase, catalase and urease activities. The predominant ubiquinone is Q-10. Main cellular fatty acids are $C_{18:1}\omega7c$, summed feature 4 and $C_{16:0}$. The polar lipid profile comprises of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphtidylcholine, one unidentified aminolipid, two unidentified polar lipids and two unidentified lipids.The genomic DNA G+C contents are about 74.0–74.3 mol%. The type species is *Crenalkalicoccus roseus.*

Description of *Crenalkalicoccus roseus* sp. nov.

Crenalkalicoccus roseus (ro'se.us. L. masc. adj. *roseus* rose coloured, rosy, the colour of colonies grown on ISP 2 medium).

Displays the following properties in addition to those described for the genus. Cells measure 0.6–0.9 μm in diameter and agglutinate within themselves after 5 days at 50 °C on ISP 2, R2A and T5 agar plates. Growth occurs at 20–60 °C, at pH 6.0–9.0 and with 0–2.5 % (w/v) NaCl with optimum growth at 40–50 °C, at pH 8.0 and with 0–2.0 % (w/v) NaCl. Utilizes dulcitol, fumaric acid, D-galactose, D-glucose, glycerol, inositol, lactose, D-mannitol, raffinose, sodium acetate, sodium pyruvate, D-sorbitol, sucrose, trehalose, trisodium citrate, D-xylitol and D-ylose as the sole carbon source, but not L-arabinose, cellobiose, D-fructose, maltose, D-mannose or sodium succinate. Utilizes L-alanine, L-arginine, L-asparagine, L-glutamic acid, hypoxanthine, L-lysine, L-serine, L-threonine, L-tyrosine and L-valine as nitrogen and energy sources, but not cystine, glycine or L-phenylalanine. Activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and β-glucosidase are positive, but activities of lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and β-fucosidase are negative. In the API 20 NE test, positive for assimilation of D-glucose, D-mannitol and potassium gluconate, but negative for indole production, glucose acidification, activities of arginine dihydroalase and galactosidase, assimilation of L-arabinose, D-mannose, *N*-acetylglucosamine, maltose, caprate, adipate, trisodium citrate and phenylacetate. Positive for nitrate reduction, and milk coagulation and peptonization tests, but negative for H_2S production. Hydrolyses aesculin, gelatin, Tweens 20 and 40 but not xylan, cellulose or starch.

The type strain, YIM 78023^T (=JCM 19657^T=KACC 17825^T), was isolated from a sediment sample collected from an alkaline hot spring in Tengchong county, Yunnan province, south-west China. The genomic DNA G+C content of the type strain is 74.3 mol%.

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