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Robertkochia solimangrovi sp. nov., isolated from mangrove soil, and emended description of the genus *Robertkochia*

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Keywords: *Robertkochia solimangrovi*; polyphasic taxonomy; *Flavobacteriaceae*; mangrove

The full length 16S rRNA gene of strain CL23^T has been deposited at EMBL/DDBJ/GenBank with accession number MK258111.

The whole genome shotgun project of strain CL23^T and *R. marina* CC-AMO-30D^T are available at EMBL/DDBJ/GenBank under accession QKWN00000000 and QXMP00000000 respectively.

ABSTRACT

To date, there is sparse information for the genus *Robertkochia* with *Robertkochia marina* CC-AMO-30D^T as the only described member. We report here a new species isolated from mangrove soil of Malaysia Tanjung Piai National Park and perform polyphasic characterization to determine its taxonomy position. Strain CL23^T is a Gram-negative, yellow-pigmented, strictly aerobic, catalase-positive and oxidase-positive bacterium. The optimal growth conditions were determined to be at pH 7.0, 30–37°C and 1–2% (w/v) NaCl. The major respiratory quinone was menaquinone-6 (MK-6) and the highly abundant polar lipids were four unidentified lipids, a phosphatidylethanolamine and two unidentified aminolipids. The 16S rRNA similarity between CL23^T and *R. marina* CC-AMO-30D^T is 96.67%. Strain CL23^T and *R. marina* CC-AMO-30D^T are clustered together and were distinguished from taxa of closely related genera in 16S rRNA phylogenetic analysis. Genome sequencing revealed the strain CL23^T has a genome size of 4.4 Mbp and a G+C content of 40.72 mol%. Overall genome indexes (OGRIs) including digital DNA-DNA hybridization (dDDH) value and average nucleotide identity (ANI) are 17.70% and approximately 70%, below the cut-off 70% and 95% respectively, indicated that strain CL23^T is a distinct species to that of *R. marina* CC-AMO-30D^T. Collectively, based on phenotypic, chemotaxonomic, phylogenetic and genomic evidence presented, strain CL23^T is proposed as a new species with the name *Robertkochia solimangrovi* sp. nov. (=KCTC 72252^T =LMG 31418^T). An emended description of the genus *Robertkochia* is also proposed.

Flavobacteriaceae is one of the widely spread bacterial families composed of 158 genera at the time of writing [1]. The genus *Robertkochia* was introduced by Hameed et al. in 2014 [2] as one of the new genera in the family *Flavobacteriaceae*. Until now, the genus consisted of a single species *Robertkochia marina* CC-AMO-30D^T, which was isolated from surface seawater at Taichung harbour, Taiwan [2]. The species was described as Gram negative, strictly aerobic, orange-pigmented and with iso-$C_{15:0}$, iso-$C_{15:1}$ G and iso-$C_{17:0}$ 3-OH as predominant fatty acids. The report for *Robertkochia* is scarce as the previous study only focused on taxonomic assignment with one species reported so far [2]. Furthermore, the genome of this genus and prospective application have not been studied or reported.

Robertkochia and many other members of the *Flavobacteriaceae* are halophilic or halotolerant bacteria that reside in diverse saline environments such as seawater, mangrove forest and marine sediment [3-5]. Mangroves are inter-tidal wetlands that connect terrestrial and marine ecosystems [6]. Due to periodic tidal flats, drastic changes in salinity and nutrient availability of the mangrove environment make it a unique ecosystem [7]. Free living and symbiotic bacteria in such environment were found to play essential roles in maintaining mangrove ecosystem such as recycling of organic matter and biotransformation of minerals [8-10]. It was estimated that less than 5% of species in mangrove environment have been described so far [11]. Therefore, it could be considered as one of the interesting areas to be explored. In the present study, strain CL23^T was isolated from soil obtained from mangrove forest located at Tanjung Piai National Park, Johor, Malaysia. This strain was characterized using polyphasic approach (phenotypic, chemotaxonomic and genomic aspects) following the recommended guidelines [12, 13] and new criteria for classification [14] to elucidate its taxonomy position. The results indicated that strain CL23^T represents a new species within *Robertkochia* genus, with the name *Robertkochia solimangrovi* sp. nov. is proposed.

ISOLATION AND HOME HABITAT

Soil from the mangrove forest was sampled at Tanjung Piai National Park (GPS location: 1°16'06.0" N, 103°30'31.2" E) in September 2017 with permit (CJB F No. 734342) granted by Johor National Parks Corporation. The soil samples were serially diluted with sterile distilled water (10^{-1} to 10^{-8}). A 0.1 ml of diluted sample was spread onto marine agar 2216 (MA; BD Difco) and incubated at 30–35°C for 1 to 14 days. A yellow-pigmented strain designated as CL23^{T} was isolated from MA and re-streaked twice to obtain a pure culture. The strain was maintained in marine broth 2216 (MB; BD Difco) with 20 % (v/v) glycerol at –80°C. Strain CL23^{T} was deposited at Korean Collection for Type Cultures (KCTC) and Belgian Co-ordinated Collections of Micro-organisms (BCCM) under accession of KCTC 72252^{T} and LMG 31418^{T}, respectively. For comparative polyphasic taxonomy characterization, *R. marina* CC-AMO-30D^{T} (=JCM 18552^{T}) was obtained from Japan Collection of Microorganisms (JCM). Both strains were routinely cultured on MA and in MB at 30°C for 48 h, unless specified otherwise.

16S rRNA PHYLOGENY

Genomic DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) and was purified by DNA Clean and Concentrator™-25 (Zymo Research) following manual instructions. The 16S rRNA gene of strain CL23^{T} was amplified by PCR using universal primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-'3) and 1525R (5'-AAGGAGGTGWTCCARCC-3') [15]. The 16S rRNA gene was sequenced at Apical Scientific Pte. Ltd., Seri Kembangan, Malaysia. After the sequencing, the raw sequences were trimmed, and the sequences were aligned using ClustalW. The nearly full-length 16S rRNA gene was searched against EzBioCloud database for identification. The amplified 16S rRNA gene of strain CL23^{T} was also cross-checked with the genome data to ensure the acquisition of full-length gene (1522 bp). The 16S rRNA gene of strain CL23^{T} (MK258111) shared highest similarity (96.67%) with *R. marina* CC-AMO-30D^{T} (JX235674), which is below the accepted threshold of 98.7% for species delineation [14]. The 16S rRNA gene similarity was less than 94% between strain CL23^{T} and

other members of closely related genera: *Joostella marina* En5^T (93.82%), *Joostella atrarenae* M1-2^T (93.82%), *Zhouia spongiae* HN-Y44^T (93.75%) and *Pustulibacterium marinum* E403^T (93.35%).

Phylogenetic trees of 16S rRNA were built following the Neighbor-joining (NJ) [16] and Maximum Likelihood (ML) [17] algorithms using MEGA 7.0 software [18] based on 1000 bootstrap replications [19] and Kimura-2 parameter. Following the 16S rRNA phylogenetic analysis (Fig. 1), strain CL23^T and *R. marina* CC-AMO-30D^T formed a clade in NJ and ML trees, confirming the placement of strain CL23^T within *Robertkochia* genus. The high bootstrap value at the node separating the branch of strain CL23^T and *R. marina* CC-AMO-30D^T in 16S rRNA phylogenetic tree supported that these two strains are distinct between each other.

PHENOTYPIC AND CHEMOTAXONOMIC CHARACTERIZATION

Colony morphology was observed on MA at 30°C after 48 h of incubation. The Gram staining was performed according to the protocol as described previously [20]. The malachite green staining was used to assess the presence of endospore in 7-day old cultures [21]. The Gram stain reaction and endospore formation were examined under light microscope (Nikon ECLIPSE E200). Cell morphology was examined under scanning electron microscope (SEM; JEOL JSM-IT300LV). The bacterial motility was investigated by hanging-drop approach [22]. The presence of flexirubin-type pigment was determined by flooding the cells with 20 % (w/v) KOH [12].

Catalase activity was detected by effervescence using 3 % (v/v) H_2O_2 while oxidase activity was determined by oxidation of tetramethyl-*p*-phenylenediamine. Hydrolysis of starch, casein, L-tyrosine, hypoxanthine, xanthine, Tween 20, Tween 40, Tween 60, Tween 80, carboxymethyl-cellulose (CMC) and xylan were tested according to Smibert and Krieg [21]. Bile esculin hydrolysis was investigated using the method of Facklam and Moody [23]. Other biochemical characteristics were revealed by API 20 E and API 20 NE kits (BioMérieux, France). Carbohydrate utilization and enzyme activity profile of both strains were

investigated by API 50 CHB and API ZYM kits (BioMérieux, France), respectively. All API kits were carried out by following the manufacturer's instructions with slight modification in which inoculation was supplemented up to 2 % (w/v) NaCl.

Growth under anaerobic condition was tested by incubating the bacteria on MA for 14 days at 30°C using AnaeroGen (Oxoid) in an anaerobic jar (Mitsubishi Gas Chemical). Growth was tested on the following media: Reasoner's 2A agar (R2A; HiMedia), Nutrient agar (NA; Merck), Tryptic soy agar (TSA; Merck), Luria-Bertani agar (LBA; Conda) and Muller Hinton agar (MHA; Sigma) supplemented with 2 % (w/v) NaCl at 30°C for 7 days. The temperature range (4, 9, 15, 20, 25, 30, 37, 40, 42, 45 and 50°C) and the optimum temperature for growth were determined using MB at pH 7. The pH range (in intervals of 1.0 pH unit) and optimum pH for growth were investigated using MB at 30°C. The pH was adjusted with the following buffer systems: 50 mM citrate phosphate (pH 4–5), 50 mM sodium phosphate (pH 6–8) and 50 mM glycine–NaOH (pH 9–10) [24]. The pH was verified after autoclaving. To test NaCl tolerance and optimal concentration, the bacteria were grown in a medium containing yeast extract (1.0 g l^{-1}), peptone (5.0 g l^{-1}), $MgCl_2$ (5.0 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (2.0 g l^{-1}), $CaCl_2$ (0.5 g l^{-1}), KCl (1.0 g l^{-1}) and NaCl (0, 0.5, 1–11 %, w/v) [10].

Antibiotic susceptibility of bacteria against 21 antibiotics was tested using the disk diffusion method on MA at 30°C for 48 h [25]. The antibiotics disks (Oxoid) used were: ampicillin (10 µg), bacitracin (10 IU), carbenicillin (100 µg), chloramphenicol (100 µg), clindamycin (2 µg), doxycycline (30 µg), erythromycin (60 µg), gentamicin (10 µg), kanamycin (50 µg), lincomycin (2 µg), minocycline (30 µg), neomycin (30 µg), novobiocin (5 µg), oleandomycin (15 µg), oxacillin (1 µg), penicillin G (10 IU), piperacillin (100 µg), polymyxin B (300 IU), rifampicin (5 µg), streptomycin (10 µg) and tetracycline (30 µg).

Strain CL23^T was determined as a Gram negative, rod-shaped, non-spore forming, oxidase positive and catalase positive bacterium with motile ability by gliding. The colony was in a circular form with 0.5–1.0 mm diameter, smooth surface, convex elevation, entire margin and has translucent property on MA after 48 h incubation. Under SEM, cells of strain CL23^T were 0.2–0.4 µm in width and 2.3–3.2 µm in length. The notable distinctive features to differentiate strain CL23^T and *R. marina* CC-AMO-30D^T are shown in Table 1. In terms of morphology, strain CL23^T is yellow pigmented while *R. marina* CC-AMO-30D^T was found to be orange pigmented. Strain CL23^T grew well in 15–42°C, pH 5–9 and 0–9 % (w/v) NaCl,

and in general strain CL23^T demonstrated a broader growth range compared to *R. marina* CC-AMO-30D^T (Table 1). The optimal growth conditions of strain CL23^T were observed at 30–37°C, pH 7 and 1–2 % (w/v) NaCl. Strain CL23^T was also able to produce acetoin, β-galactosidase and weakly positive toward amygdaline according to API 20 E but not for *R. marina* CC-AMO-30D^T. Based on API ZYM, strain CL23^T was able to produce α-galactosidase, β-galactosidase and α-mannosidase, which were absent in *R. marina* CC-AMO-30D^T. Both strains were further distinguished by the hydrolysis capability of gelatin, Tween 20, Tween 40, Tween 60, and exhibiting resistance towards ampicillin, penicillin G, piperacillin and bacitracin (Table 1).

For the chemotaxonomic analysis, cellular fatty acids were extracted following the protocol of Microbial Identification System (MIDI, version 6.1) [26]. Biomass of strain CL23^T and its reference strain *R. marina* CC-AMO-30D^T were harvested from MA after 48 h of incubation at 30°C. The cells were saponified with methanolic base, then the resulting sodium salts of fatty acids were methylated. In the final step, methyl esters were transferred to the organic phase and washed. Fatty acid methyl esters were analyzed on an Agilent 6890 equipped with Ultra-2 capillary column and subsequently identified in the RTSBA6 library. As exhibited in Table 2, the predominant cellular fatty acid of strain CL23^T and *R. marina* CC-AMO-30D^T were found to be iso-$C_{15:0}$, iso-$C_{15:1}$ G and iso-$C_{17:0}$ 3-OH (> 10%). Nonetheless, some fatty acid patterns and abundance of strain CL23^T varied when compared to *R. marina* CC-AMO-30D^T, such as summed features 3 (3.64%) and 9 (5.24%) were constituted in strain CL23^T but none for *R. marina* CC-AMO-30D^T. On top of that, the amount of iso-$C_{16:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$ 3-OH of strain CL23^T are remarkably lower than *R. marina* CC-AMO-30D^T (Table 2).

The polar lipids and respiratory quinone analyses of strain CL23^T were performed by Dr. Brian Tindall at the Identification Service, DSMZ, Braunschweig, Germany. In brief, the respiratory quinones were extracted by solvent methanol: hexane (2:1 v/v), separated by TLC and High Performance Liquid Chromatography (HPLC) following the standard method by Tindall [27]. The polar lipids were extracted using chloroform: methanol solvent and separated by two-dimensional silica gel thin layer chromatography (TLC) [28]. Total lipid material was identified using molybdatophosphoric acid and specific functional groups were determined using spray reagents specific for defined functional groups.

The major respiratory quinone of strain CL23^T was identified to be menaquinone-6 (MK-6), which matched to *R. marina* [2] and other members in *Flavobacteriaceae* family [12]. In terms of polar lipids, strain CL23^T has four unidentified lipids (L1, L2, L3 and L4), a phosphatidylethanolamine (PE) and two unidentified aminolipids (AL1 and AL2) as major polar lipids (Fig. S1). Additionally, three unidentified glycolipids (GL1, GL2 and GL3) and an unknown lipid (L5) were observed in minor amounts. The unidentified lipids (L1–L3) and glycolipids (GL1–GL3) were not detected in *R. marina* CC-AMO-30D^T [2]. Moreover, an unidentified phospholipid (PL) was contained in *R. marina* CC-AMO-30D^T in which this lipid was not found in strain CL23^T [2].

GENOMIC CHARACTERIZATION

The genome of reference strain *R. marina* CC-AMO-30D^T was not available at the time of study, therefore, both the genomes of strain CL23^T (NCBI accession: QKWN00000000) and *R. marina* CC-AMO-30D^T (NCBI accession: QXMP00000000) were sequenced in this study. Whole genome sequencing of strain CL23^T was accomplished on an Illumina HiSeq 2500 platform (2 × 150 bp). The raw reads were filtered, and the quality data was *de novo* assembled using SOAPdenovo 2.04 [29]. The resulting genome was annotated using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [30].

The assembled genome of strain CL23^T consists of 23 contigs with 322× depth of sequencing coverage (average), made up the size of genome with 4,407,290 bp in length and a GC content of 40.72 mol%. The genome size of strain CL23^T is significantly larger than *R. marina* CC-AMO-30D^T (3,571,649 bp). The GC content of strain CL23^T is slightly lower than *R. marina* CC-AMO-30D^T (43.67 mol%). Based on PGAP annotation, a total of 3669 protein coding genes was found in genome of strain CL23^T. The genes responsible for phosphatase activity were found in the genome of strain CL23^T and *R. marina* CC-AMO-30D^T with a total of 12 and 7 phosphatases were encoded respectively (Table S1). This correlated to API ZYM results in which both strains were positive to acidic and alkali phosphatases. Notably, the number of phosphatases annotated is higher in strain CL23^T as compared to *R. marina* CC-AMO-30D^T. On the other hand, strain CL23^T consists of a series

of genes for assimilatory sulfate reduction into sulfite (sulfate adenylyltransferase subunit CysN and CysD, adenylylsulfate kinase and phosphoadenylylsulfate reductase) and then sulfite reduction into sulfide (FAD-binding oxidoreductase and LLM class flavin-dependent oxidoreductase) (Table S1). Nevertheless, the genes responsible for reduction of sulfite to sulfide are absent in *R. marina* CC-AMO-30D^T (Table S1). Furthermore, strain CL23^T also encodes a set of genes for reduction of nitrate to ammonia (*NirBD* and *NrfAH*) in which *NirBD* genes were not found in genome of *R. marina* CC-AMO-30D^T (Table S1). These genes suggest that strain CL23^T participates in nutrient recycling in mangrove environments.

Multilocus sequence analysis (MLSA) was conducted on five housekeeping genes of strain CL23^T, *R. marina* CC-AMO-30D^T and related genera, which the sequences were retrieved from genome data. The sequences of housekeeping genes were aligned individually and then concatenated in the following order: *rpoB–gyrB–recA–mutL–atpD*. The phylogenetic tree of concatenated housekeeping genes was constructed using MEGA 7.0 similarly as described in section "16S rRNA phylogeny". In this tree (Fig. 2), strain CL23^T and *R. marina* CC-AMO-30D^T are clustered together but well distinguished from each other with high level of support (>90% bootstrap value). Likewise, the phylogenetic tree based on whole genome sequences that built using REALPHY 1.12 [31] also supported that both strain CL23^T and *R. marina* CC-AMO-30D^T are grouped in the same clade (Fig. S2).

To further underpin the classification of strain CL23^T as a new species, the overall genome related indexes (OGRIs) were determined. Average nucleotide identity based on BLAST (ANIb) was calculated using JSpeciesWS [32]. ANI based on USEARCH (OrthoANIu) was determined by ChunLab's online ANI calculator [33]. The digital DNA-DNA hybridization (dDDH) value was calculated by Genome-to-Genome Distance Calculator [34].

The ANIb and OrthoANIu values between strain CL23^T and *R. marina* CC-AMO-30D^T are 69.35% and 70.47% respectively. These ANI values are below the recommended 95–96% for species delineation [35]. Similarly, the dDDH value between two strains was found to be 17.70%, lower than 70%, the cut off for species boundary [34]. Combining the interpretation of ANI and dDDH values, the result revealed the identity of strain CL23^T as a distinct species within the same genus as *R. marina* CC-AMO-30D^T.

Based on polyphasic taxonomy characterization including phenotypic, chemotaxonomic, phylogenetic and genomic aspects, the results clearly indicated that strain CL23^T (=KCTC 72252^T =LMG 31418^T) represents a new species within the genus *Robertkochia*, for which the name *Robertkochia solimangrovi* sp. nov. is proposed.

DESCRIPTION OF *ROBERTKOCHIA SOLIMANGROVI* SP. NOV.

Robertkochia solimangrovi sp. nov. (so.li.man.gro'vi. L. neut. n. *solum* soil; N.L. neut. n. *mangrovum* a mangrove; N.L. gen. n. *solimangrovi* of soil of a mangrove, pertaining to where the type strain was isolated.)

The cells are Gram-negative, rod shape, approximate 0.2–0.4 µm in width and 2.3–3.2 µm in length with motile ability by gliding. Colony is yellow-pigmented, in circular form with 0.5–1.0 mm diameter, smooth surface, convex elevation, entire margin and has translucent property after 48 hours incubation at 30°C on MA. Flexirubin-type pigment is absent. Cells are positive for oxidase and catalase. Growth occurs at 15–42 °C (optimum, 30–37°C), pH 5–9 (optimum, pH 7) and in the presence of 0–9 % (w/v) NaCl (optimum, 1–2 % (w/v) NaCl). Grows well on MA, however, no growth is observed on R2A, NA, LBA, TSA and MHA media. No growth is observed on MA in anaerobic condition. The predominant fatty acids are iso-$C_{15:0}$, iso-$C_{15:1}$ G and iso-$C_{17:0}$ 3-OH. The major isoprenoid quinone is menaquinone-6 (MK-6). The major polar lipids are four unidentified lipids, a phosphatidylethanolamine and two unidentified aminolipids. Xylan, esculin, Tween 20, 40 and 60 are hydrolyzed. L-Tyrosine is weakly hydrolyzed. Casein, starch, CMC, Tween 80, xanthine and hypoxanthine are not hydrolyzed. In the API 20 E strip, positive for ONP-β-D-galactopyranoside and acetoin production; weakly positive for fermentation/oxidation of amygdaline; negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase, urease and gelatinase, production of H_2S and indole, utilization of citrate, fermentation/oxidation of D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, D-saccharose, D-melibiose and L-arabinose. In the API 20 NE strip, positive for hydrolysis of pNP-β-D-galactopyranoside and esculin ferric citrate; negative for nitrate reduction, indole

production, arginine dihydrolase, gelatinase and urease, fermentation of D-glucose and assimilation of D-glucose, D-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid and phenylacetic acid. In the API 50 CHB strip, acid is produced from D-galactose, D-glucose, D-mannose, esculin ferric citrate, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-melezitose, D-raffinose, amidon, glycogen and gentibiose; acid is weakly produced from methyl-α-D-glucopyranoside, arbutin, salicin and D-turanose; acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, *N*-acetyl-glucosamine, amygdalin, inulin, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. In the API ZYM strip, alkali phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphtol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase and α-mannosidase are present; weak positive reaction for α-fucosidase and negative results for lipase (C14) and α-fucosidase. Cells are susceptible to carbenicillin, clindamycin, doxycycline, lincomycin, minocycline, novobiocin, oleandomycin, rifampicin and tetracycline, but not to ampicillin, bacitracin, chloramphenicol, erythromycin, gentamicin, kanamycin, neomycin, oxacillin, penicillin G, piperacillin, polymyxin B and streptomycin.

The type strain is CL23^T (=KCTC 72252^T =LMG 31418^T), isolated from soil of mangrove collected from Tanjung Piai National Park, Johor, Malaysia.

The EMBL/DDBJ/GenBank accession for 16S rRNA gene of strain CL23^T is MK258111. Genome metrics are as follows: genome size, 4,407,290 bp; number of contigs, 23; G+C content, 40.72 mol%. The Whole Genome Shotgun project of strain CL23^T is available at EMBL/DDBJ/GenBank under accession QKWN00000000. The version described in this paper is QKWN01000000.

EMENDED DESCRIPTION OF GENUS *ROBERTKOCHIA*

The characteristics of the genus *Robertkochia* are described according to Hameed et al. 2014 [2] with following amendments and additional information. Oxidase is either positive or negative and catalase is positive. The DNA G+C content of the type strain of type species is 43.67 mol% based on genome data. The Whole Genome Shotgun project of type strain of type species is available at EMBL/DDBJ/GenBank under accession QXMP00000000. The version described in this paper is QXMP01000000.

AUTHOR STATEMENTS

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Ethical statement

No human and animal experiments are involved.

Conflicts of interest

All authors declared that there are no conflicts of interest.

ABBREVIATIONS

ANI, average nucleotide identity, dDDH, digital DNA-DNA hybridization; MA, marine agar; MB, marine broth; MK, menaquinone; ML, maximum likelihood; NJ, neighbor-joining; OGRI, overall genome related index; PE, phosphatidylethanolamine.

REFERENCES

1. **Parte AC**. LPSN – List of Prokaryotic names with Standing in Nomenclature (bacterio.net), 20 years on. *Int J Syst Evol Microbiol* 2018;68(6):1825-9.
2. **Hameed A, Shahina M, Lin S-Y, Lai W-A, Liu Y-C, *et al.*** *Robertkochia marina* gen. nov., sp. nov., of the family *Flavobacteriaceae*, isolated from surface seawater, and emended descriptions of the genera *Joostella* and *Galbibacter*. *Int J Syst Evol Microbiol* 2014;64(2):533-9.
3. **Thevarajoo S, Selvaratnam C, Goh KM, Hong KW, Chan XY, *et al.*** *Vitellibacter aquimaris* sp. nov., a marine bacterium isolated from seawater. *Int J Syst Evol Microbiol* 2016;66(9):3662-8.
4. **Li Y, Bai S, Yang C, Lai Q, Zhang H, *et al.*** *Mangrovimonas yunxiaonensis* gen. nov., sp. nov., isolated from mangrove sediment. *Int J Syst Evol Microbiol* 2013;63(6):2043-8.
5. **Wang B, Sun F, Du Y, Liu X, Li G, *et al.*** *Meridianimaribacter flavus* gen. nov., sp. nov., a member of the family *Flavobacteriaceae* isolated from marine sediment of the South China Sea. *Int J Syst Evol Microbiol* 2010;60(1):121-7.
6. **Alongi DM.** Mangrove forests. In: *Blue Carbon: Coastal Sequestration for Climate Change Mitigation*. Cham: Springer; 2018. p. 23-36.
7. **Lin X, Hetharua B, Lin L, Xu H, Zheng T, *et al.*** Mangrove sediment microbiome: adaptive microbial assemblages and their routed biogeochemical processes in Yunxiao Mangrove National Nature Reserve, China. *Microb Ecol* 2019;78(1):57-69.

8. **Castro RA, Dourado MN, Almeida JRd, Lacava PT, Nave A, *et al.*** Mangrove endophyte promotes reforestation tree (*Acacia polyphylla*) growth. *Braz J Microbiol* 2018;49(1):59-66.

9. **Kathiresan K.** Salt-tolerant microbes in mangroves: ecological role and bioprospecting potential. In: Dagar JC, Yadav RK, Sharma PC, (editors). *Research Developments in Saline Agriculture*. Singapore: Springer Singapore; 2019. p. 237-55.

10. **Lam MQ, Oates NC, Thevarajoo S, Tokiman L, Goh KM, *et al.*** Genomic analysis of a lignocellulose degrading strain from the underexplored genus *Meridianimaribacter*. *Genomics* 2019. doi: 10.1016/j.ygeno.2019.06.011 (in press)

11. **Thatoi H, Behera BC, Mishra RR, Dutta SK**. Biodiversity and biotechnological potential of microorganisms from mangrove ecosystems: a review. *Ann Microbiol* 2013;63(1):1-19.

12. **Bernardet J-F, Nakagawa Y, Holmes B**. Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol* 2002;52(3):1049-70.

13. **Tindall BJ, Rosselló-Móra R, Busse H-J, Ludwig W, Kämpfer P**. Notes on the characterization of prokaryote strains for taxonomic purposes. *Int J Syst Evol Microbiol* 2010;60(1):249-66.

14. **Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, *et al.*** Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol Microbiol* 2018;68(1):461-6.

15. **Claus D**. A standardized Gram staining procedure. *World J Microbiol Biotechnol* 1992;8(4):451-2.

16. **Smibert RM, Krieg NR**. Phenotypic characterization. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR. *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology; 1994. p. 607-54.

17. **Tittsler RP, Sandholzer LA**. The use of semi-solid agar for the detection of bacterial motility. *J Bacteriol* 1936;31(6):575.

18. **Facklam RR, Moody MD**. Presumptive identification of group D *Streptococci*: the bile-esculin test. *Appl Microbiol* 1970;20(2):245-50.

19. **Lam MQ, Nik Mut NN, Thevarajoo S, Chen SJ, Selvaratnam C, *et al.*** Characterization of detergent compatible protease from halophilic *Virgibacillus* sp. CD6. *3 Biotech* 2018;8(2):104.

20. **Jorgensen JH, Turnidge JD.** Susceptibility test methods: dilution and disk diffusion methods. In: James HJ, Michael AP, Karen CC, Guido F, Marie LL Sandra SR, David WW (editors). *Manual of Clinical Microbiology,* 11th ed. Washington, DC: American Society of Microbiology; 2015. p. 1253-73.

21. **Sasser M.** *Identification of bacteria by gas chromatography of cellular fatty acids*. *USFCC Newsl* 1990;20:16.

22. **Tindall BJ**. Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* 1990;66(1-3):199-202.

23. **Tindall BJ, Sikorski J, Smibert RA, Krieg NR.** Phenotypic characterization and the principles of comparative systematics. In: Reddy CA, Beveridge TJ, Breznak JA, Maxluf G, Schmidt TM, Snyder LR (editors). *Methods for General and Molecular Microbiology,* 3rd ed. Washington, DC: American Society of Microbiology; 2007. p. 330-93.

24. **Lane DJ.** 16S/23S rRNA sequencing. In: Stackebrandt E and Goodfellow M (editors). *Nucleic Acid Techniques in Bacterial Systematics*. Chichester, United Kingdom: Wiley; 1991. p. 125-75.

25. **Saitou N, Nei M**. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4(4):406-25.

26. **Felsenstein J**. Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* 1981;17(6):368-76.

27. **Kumar S, Stecher G, Tamura K**. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;33(7):1870-4.

28. **Felsenstein J**. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985:783-91.

29. **Li R, Li Y, Kristiansen K, Wang J**. SOAP: short oligonucleotide alignment program. *Bioinformatics* 2008;24(5):713-4.

30. **Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, *et al.*** NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res* 2016;44(14):6614-24.

31. **Bertels F, Silander OK, Pachkov M, Rainey PB, van Nimwegen E**. Automated Reconstruction of Whole-Genome Phylogenies from Short-Sequence Reads. *Mol Biol Evol* 2014;31(5):1077-88.

32. **Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J**. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics* 2016;32(6):929-31.

33. **Yoon S-H, Ha S-m, Lim J, Kwon S, Chun J**. A large-scale evaluation of algorithms to calculate average nucleotide identity. *Antonie van Leeuwenhoek* 2017;110(10):1281-6.

34. **Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M**. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013;14(1):60.

35. **Richter M, Rosselló-Móra R**. Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci* 2009;106(45):19126-31.

FIGURES AND TABLES

Table 1. Differential phenotypic characteristics of strain CL23^T and *Robertkochia marina* CC-AMO-30D^T.

Strains: 1, strain CL23^T; 2, *R. marina* CC-AMO-30D^T. All data were obtained from this study. +, Positive reaction; –, negative reaction; w, weakly positive reaction. All strains were positive for catalase; hydrolysis of xylan and aesculin; production of acid from D-glucose, esculin ferric citrate, D-cellobiose, D-maltose, D-saccharose, D-trehalose, D-melezitose, amidon and glycogen in API 50 CHB strips; and activity of alkali phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphtol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase. Both strains were negative for flexirubin-type pigment; growth under anaerobic condition; growth on R2A, NA, LBA, TSA and MHA media; hydrolysis of casein, starch, CMC, Tween 80, xanthine and hypoxanthine; nitrate reduction; indole and H_2S production; urease; acid production from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, *N*-acetyl-glucosamine, amygdalin, inulin, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate and potassium 5-ketogluconate in API 50 CHB strips; and activity of lipase (C14) and β-glucuronidase (API ZYM).

Characteristics	**1**	**2**
Colony pigmentation	Yellow	Orange
Oxidase	+	–
Growth parameters		
pH range	5–9	6–7
Temperature range (°C)	15–42	20–40
Temperature optimum (°C)	30–37	30
NaCl range (%, w/v)	0–9	0.5–4
NaCl optimum (%, w/v)	1–2	2
Hydrolysis of		
Tween 20	+	w
Tween 40	+	–
Tween 60	+	w
Tyrosine	w	–

Gelatin	–	+
Production of		
Acetoin	+	–
Oxidation of		
Amyldaline	w	–
Utilization of		
D-Galactose	+	–
D-Mannose	+	–
Arbutin	w	–
Salicin	w	–
D-Lactose	+	–
D-Melibiose	+	–
D-Raffinose	+	–
Gentiobiose	+	–
D-Turanose	w	–
Enzyme activity (API ZYM)		
α-Galactosidase	+	–
β-Galactosidase	+	–
α-Mannosidase	+	w
α-Fucosidase	w	–
Antibiotic susceptibility (per disc)		
Ampicillin (10 µg)	–	+
Penicillin G (10 IU)	–	+
Piperacillin (100 µg)	–	+
Bacitracin (45 µg)	–	+

Table 2. Cellular fatty acid profiles (%) of strain CL23^T and *Robertkochia marina* CC-AMO-30D^T.

Strains: 1, strain CL23^T; 2, *R. marina* CC-AMO-30D^T. All data presented in the table are from this study. TR, trace (≤ 0.5%); –, not detected. Major components (> 10%) are highlighted in bold.

Fatty acid	**1**	**2**
Branched saturated		
iso-$C_{13:0}$	TR	2.4
iso-$C_{14:0}$	–	2.4
iso-$C_{15:0}$	**21.8**	**19.9**
iso-$C_{16:0}$	3.4	6.1
anteiso-$C_{15:0}$	2.3	5.8
Unsaturated		
$C_{15:1}\omega5c$	0.7	–
$C_{17:1}\omega6c$	1.7	–
$C_{17:1}\omega8c$	0.8	–
Branched unsaturated		
iso-$C_{15:1}$ G	**10.8**	**23.3**
iso-$C_{16:1}$ G	–	1.6
iso-$C_{16:1}$ H	1.0	–
anteiso-$C_{15:1}$ A	TR	2.8
Hydroxy		
$C_{15:0}$ 2-OH	0.9	1.5
$C_{15:0}$ 3-OH	2.0	0.6
$C_{16:0}$ 3-OH	1.4	TR
$C_{17:0}$ 3-OH	1.1	TR
iso-$C_{16:0}$ 3-OH	2.6	6.5
iso-$C_{17:0}$ 3-OH	**29.5**	**15.5**
Summed features *		
3 †	3.6	–
9 #	5.2	–

* Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system.

† Summed feature 3 consisted of iso-$C_{15:0}$ 2-OH, $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$ and annotated here as iso-$C_{15:0}$ 2-OH based on the equivalent chain length (ECL).

Summed feature 9 consisted of iso-$C_{17:1}\omega9c$ and/or $C_{16:0}$ 10-methyl.

Figure legends

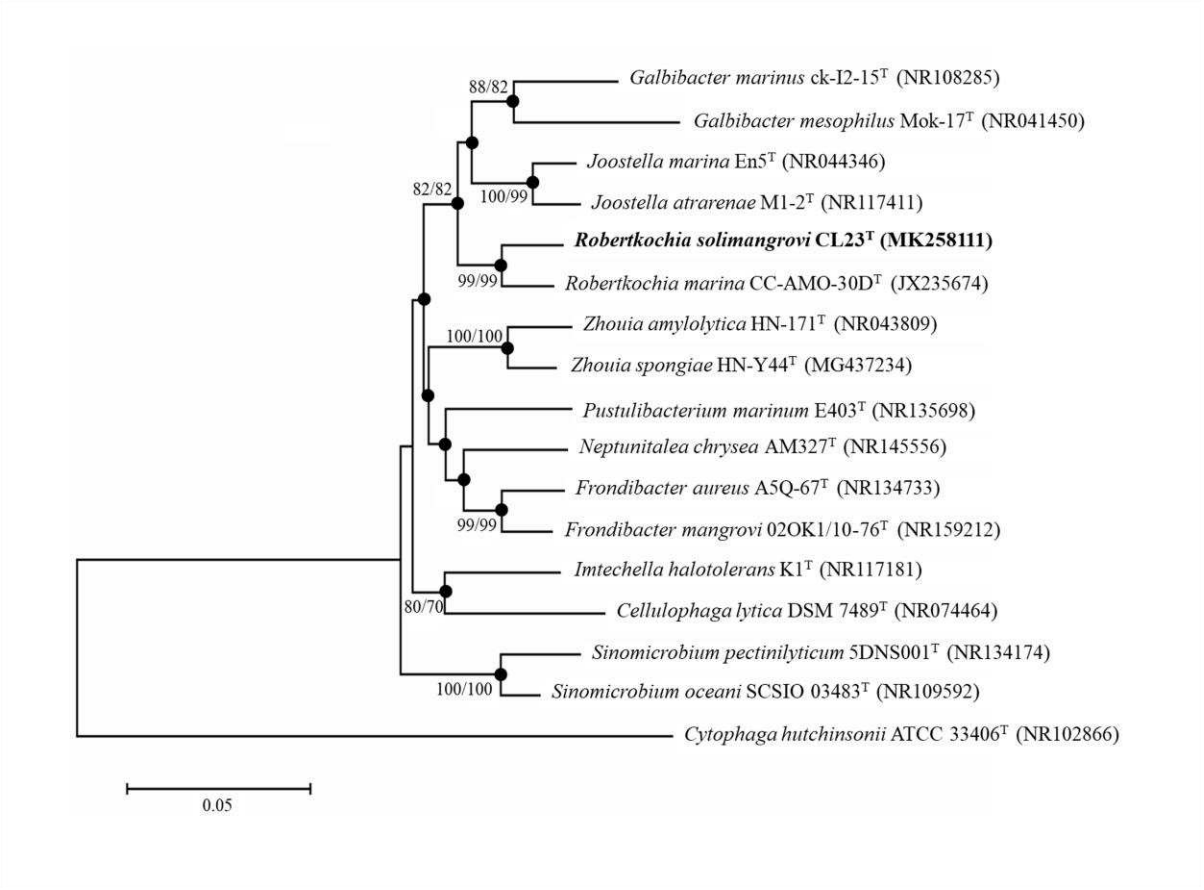

Fig. 1. Neighbor joining 16S rRNA phylogenetic tree manifesting the relationship of strain CL23^T with closely related taxa of family *Flavobacteriaceae*. Corresponding Genbank accession numbers are indicated in parentheses. Bootstrap values ≥70% based on 1000 resampled datasets are depicted as percentages at nodes. Bootstrap value from left to right for NJ and ML calculated with same sequence set. Filled circles indicate that corresponding nodes were also recovered in dendrograms generated using ML algorithm. The sequence of *Cytophaga hutchinsonii* ATCC 33406^T was used as outgroup. Bar, 0.05 substitutions per nucleotide position.

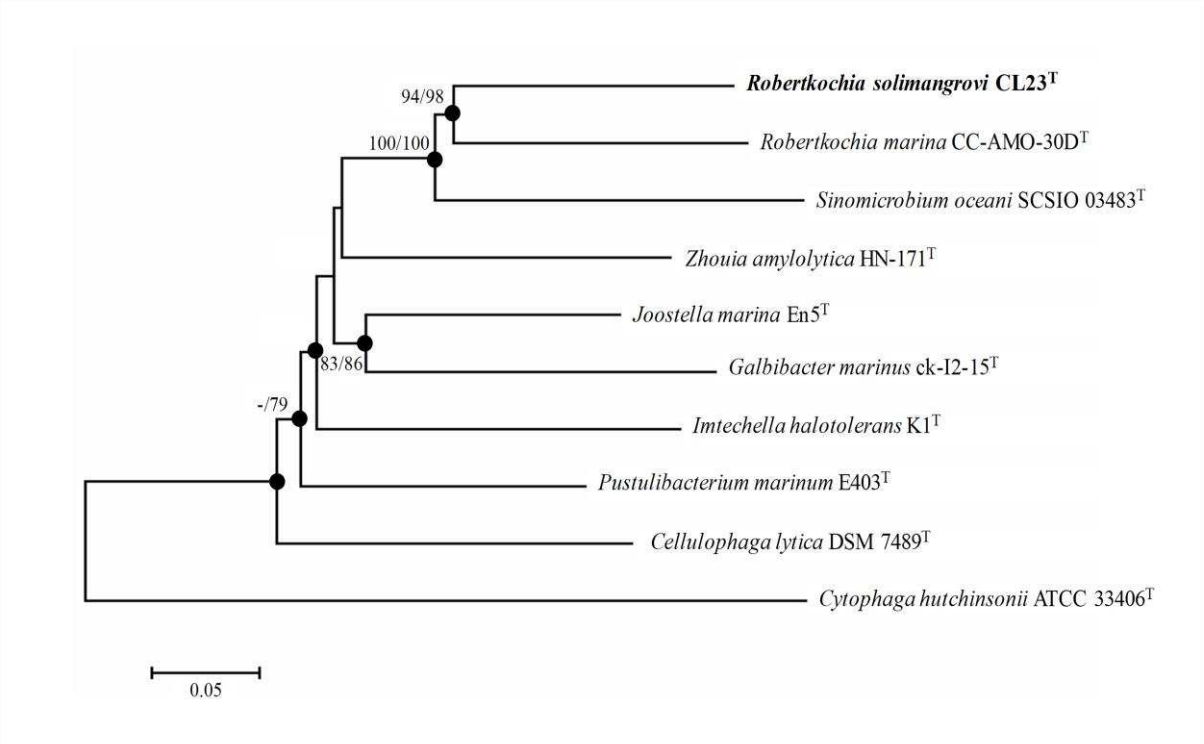

Fig. 2. Neighbor joining phylogenetic tree based on the concatenated sequences of five housekeeping genes: *rpoB–gyrB–recA–mutL–atpD*, indicating the position of strain CL23^T. Bootstrap values ≥70% based on 1000 resampled datasets are depicted as percentages at nodes; value <70% is indicated by a dash. Bootstrap value from left to right for NJ and ML calculated with same sequence set. Filled circles indicate that corresponding nodes were also recovered in dendrograms generated using ML algorithm. The sequence of *Cytophaga hutchinsonii* ATCC 33406^T was used as outgroup. Bar, 0.05 substitutions per nucleotide position.

Supplementary Materials

Robertkochia solimangrovi sp. nov., isolated from mangrove soil, and emended description of the genus _Robertkochia_

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Supplementary figures

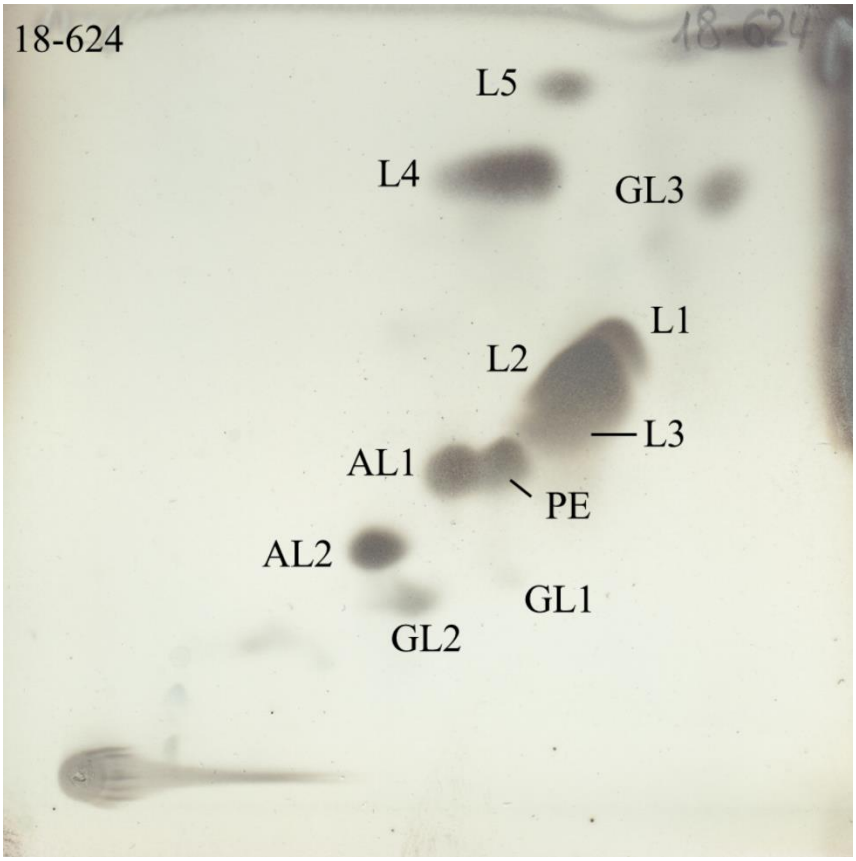

Fig. S1. Polar lipids profile of strain CL23^{T}. Unidentified lipids; L1–L5, phosphatidylethanolamine; PE, unidentified aminolipids; AL1–AL2, unidentified glycolipids; GL1–GL3.

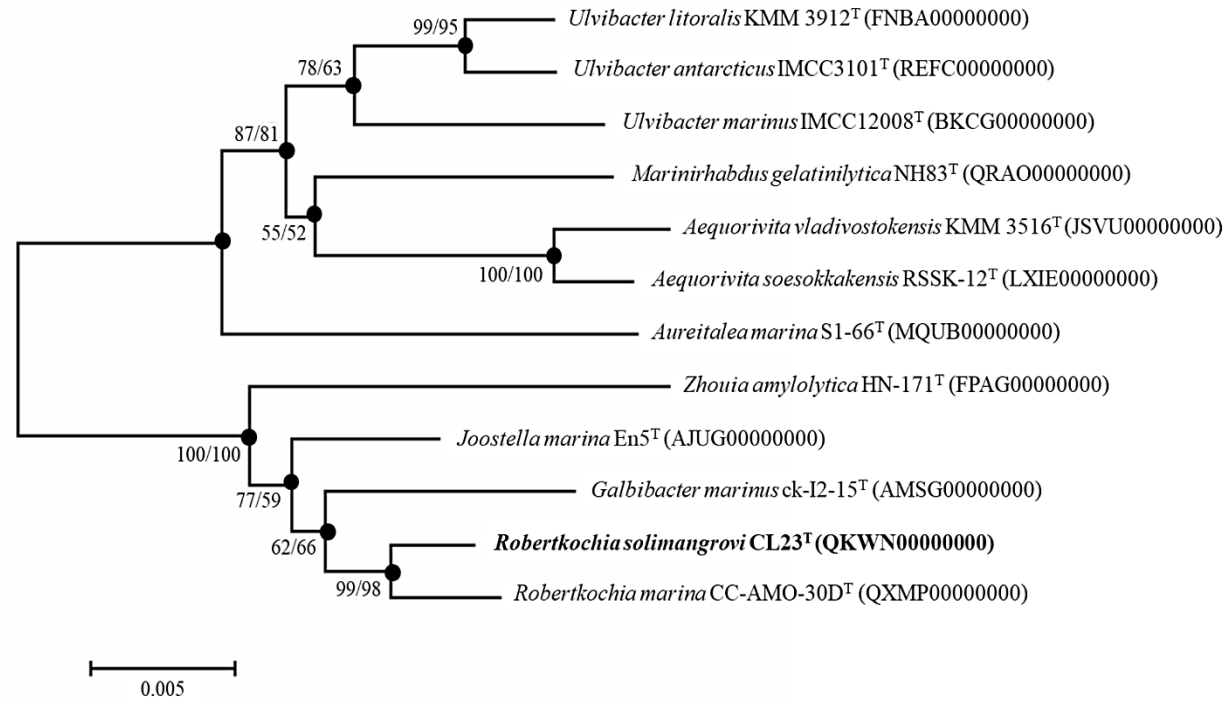

Fig. S2. Neighbor joining phylogenomic tree manifesting the relationship of strain CL23^T with closely related taxa of family *Flavobacteriaceae*. Corresponding Genbank accession numbers are indicated in parentheses. Bootstrap values ≥50% based on 1000 resampled datasets are depicted as percentages at nodes. Bootstrap value from left to right for NJ and ML calculated with same sequence set. Filled circles indicate that corresponding nodes were also recovered in dendrograms generated using ML algorithm. Bar, 0.005 substitutions per nucleotide position.

Supplementary tables

Table S1. List of potential genes for phosphatases, sulfur reduction and nitrate reduction encoded in the genome of strain CL23^T and *R. marina* CC-AMO-30D^T.

Category	Bacterial strain	NCBI Annotation	Accession
Phosphatases	CL23^T	alkaline phosphatase family protein	TRZ44267
		alkaline phosphatase	TRZ44500
		alkaline phosphatase	TRZ44343
		pyrophosphatase	TRZ44378
		sodium-translocating pyrophosphatase	TRZ44400
		alkaline phosphatase family protein	TRZ43533
		alkaline phosphatase family protein	TRZ43596
		alkaline phosphatase	TRZ42861
		HAD family phosphatase	TRZ42760
		alkaline phosphatase family protein	TRZ42969
		alkaline phosphatase family protein	TRZ41972
		HAD family phosphatase	TRZ41063
	R. marina CC-AMO-30D^T	alkaline phosphatase family protein	TRZ46762
		alkaline phosphatase family protein	TRZ45488
		alkaline phosphatase family protein	TRZ45685
		sodium-translocating pyrophosphatase	TRZ44743
		HAD family phosphatase	TRZ42656
		pyrophosphatase	TRZ41149
		HAD family phosphatase	TRZ40862
Sulfur reduction	CL23^T	sulfate adenylyltransferase subunit CysN	TRZ46029
		sulfate adenylyltransferase subunit CysD	TRZ46030
		adenylyl-sulfate kinase	TRZ46031
		phosphoadenylylsulfate reductase	TRZ44200
		phosphoadenylylsulfate reductase	TRZ42776
		FAD-binding oxidoreductase	TRZ41175
		LLM class flavin-dependent oxidoreductase	TRZ41182
	R. marina CC-AMO-30D^T	sulfate adenylyltransferase subunit CysN	TRZ40960
		sulfate adenylyltransferase subunit CysD	TRZ40970
		adenylyl-sulfate kinase	TRZ40959
		phosphoadenylylsulfate reductase	TRZ46694
Nitrate reduction	CL23 ^{T}	nitrite reductase (*NirBD*)	TRZ44395
		nitrite reductase (NAD(P)H) (*NirBD*)	TRZ42280

	nitrite reductase (NAD(P)H) small subunit (*NirBD*)	TRZ42281
	NAD(P)H-nitrite reductase (*NirBD*)	TRZ42287
	ammonia-forming cytochrome c nitrite reductase (*NrfAH*)	TRZ42033
	cytochrome c nitrite reductase small subunit (*NrfAH*)	TRZ42034
R. marina CC-AMO-30D^T	ammonia-forming cytochrome c nitrite reductase (*NrfAH*)	TRZ44150
	cytochrome c nitrite reductase small subunit (*NrfAH*)	TRZ44178